Part 4
Methods

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Methods for Profiling Drug-like Properties: General Concepts

Overview

- *Property profiling should be rapid and use relevant assay conditions.*
- *Use a diverse set of assays that have high impact for the organization.*
- *Property assays consume resources and influence projects, so assays should be carefully implemented.*
- *Evaluate the cost versus benefit of assays.*

The assessment of compound properties has become an integral activity in drug discovery. It provides data for the selection of leads, the development of structure–property relationships (SPR), and the judging of whether project advancement criteria have been met. The data also guide property optimization by structural modification. Several aspects of the property-profiling program that should be carefully considered are discussed here.

22.1 Property Data Should be Rapidly Available

In order for property data to be relevant for discovery, they need to be reported to project teams rapidly.[1] This allows decisions to be made rapidly so that aggressive discovery time lines can be met. Faster data allow more iterations of trying new ideas to optimize the compounds, thus increasing the success rate. A general guide is to provide data in a few days to 2 weeks, the same time frame as biological data.

22.2 Use Relevant Assay Conditions

The conditions of property assays need to be relevant to the environment faced by the compound.[2] Variables, such as concentration, pH, matrix components in solution, and biological tissue extracts, must be controlled and designed to be reflective of the barriers faced by the compound.

22.3 Evaluate the Cost-to-Benefit Ratio for Assays

In any discovery organization there are always tradeoffs on the allocation of resources. Thus, it is important to decide which properties are of greatest importance to the organization.[3] The properties that have the greatest impact on the projects and goals of the organization should prevail. Assays should not be put in place because other organizations have them but should be decided based on what the organization considers to be the critical issues.

One approach to resource allocation is to use the "appropriate assay" for the particular stage of discovery. Higher-throughput and simpler methods can be used in earlier discovery stages, when the purpose is to select compounds that are in the right general portion of property space. In later discovery stages the purpose is to carefully optimize lead series; thus, more detailed methods are appropriate. This approach mirrors the strategy in discovery biology, where in vitro enzyme or receptor assays are used early, while functional cell-based assays and in vivo models are used in later stages. This approach balances data needs and resources so that there is not an "overkill" of detailed data from resource-intensive assays early and not overinterpretation of, or reliance on, undetailed data later in the process. Resources should be used wisely and evaluation of the cost-to-benefit ratio of assays should be constant.

An example of overkill is the use of Caco-2 for hits from high-throughput screening (HTS) or virtual screening, when structural rules on lipophilicity or polar surface area or high-throughput parallel artificial membrane permeability assay (PAMPA) methods would provide sufficient information for selecting the most favorable from among the fatally flawed compounds. An example of overinterpretation is the use of data for solubility in an aqueous buffer at pH 7.4 as the primary solubility value in late discovery, when solubility can be very different with the different pHs and concentrations of bile salts and lipids present throughout the length of the intestine.

Rules and in silico tools are useful for rapid and inexpensive screening of compounds to check if they meet certain criteria or to form a rapid opinion about whether the properties will be drug-like. *High-throughput in vitro assays* (HT) can process hundreds of compounds per day but tend to use generic conditions. HT assays are most appropriate for earlier drug discovery studies where a large number of compounds are studied in a short period of time and the data need not be highly detailed to be adequate for the decisions made in this stage. *Customized assays* usually are developed and used to answer specific questions for project teams. They can have quite an impact on team success. *In-depth assays* provide detailed information that is necessary for advancement to clinical development. They test specific issues and help to predict and plan for development studies that will be necessary to develop the candidate.

22.4 Choose an Ensemble of Key Properties to Evaluate

Each organization must select the properties they are most interested in monitoring. For example, the assays shown in Figure 22.1 are from one company's property profiling

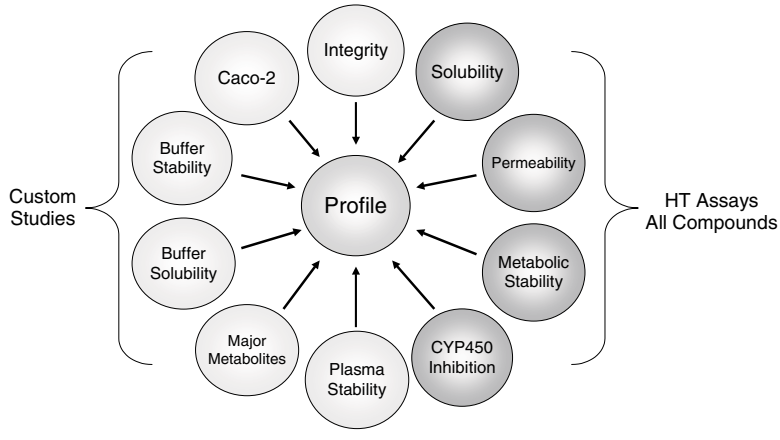

Figure 22.1 ► An ensemble of property assays can be available to support discovery, some as HT assays for all compounds and others as custom assays for selected compounds. (Reprinted with permission from [3].)

program.[4] Some assays can be run as standard assays for all compounds, while others are best used as custom assays to answer specific project team questions.

22.5 Use Well-Developed Assays

Data used for decision-making should be generated using assays that have been well developed. The assay conditions can greatly affect the results. For example, if the compound is not soluble in the assay, if a high compound concentration saturates the enzyme, if substrate turnover is too high, or if a co-solvent inhibits the enzyme, the data will be misleading. It is important that scientists generating the data fully validate the method against the known properties of well-characterized compounds, and that scientists using the data know how it was generated and discuss with the data generator how particular characteristics of the compounds in their series might affect the assay.

Problems

(Answers can be found in Appendix I at the end of the book.)

1. Is it useful to use data from a generic pH 7.4 solubility assay to predict solubility in the GI tract?
2. Is it useful to obtain Caco-2 A > B and B > A permeability data for 200 HTS hits for lead selection?
3. Is PAMPA data at pH 7.4 sufficient for inclusion in the data package for clinical candidate nomination?
4. Is microsomal stability data sufficient for assessing the potential of hydrolysis?
5. Is microsomal stability in multiple species necessary during lead optimization?

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Lipophilicity Methods

Overview

- *In silico lipophilicity predictions are reliable and inexpensive.*
- *High-throughput methods include partitioning in a microtiter plate, high-performance liquid chromatography, and capillary electrophoresis.*
- *In-depth methods utilize equilibrium shake flask partitioning and titration.*

Lipophilicity is one of the most studied physicochemical properties. It also has one of the most developed sets of methods for its prediction and measurement. Lipophilicity values usually are measured and published for new drugs as part of the development process. Many laboratories have measured Log P and Log D values as part of their research. An extensive set of published lipophilicity values for drugs and other compounds has been compiled by Hansch and Leo.[1]

Although a lipophilicity experiment at first may seem simple and easy, it is important to remember the effect of conditions on lipophilicity (see Chapter 5). The following conditions should be carefully controlled and listed when reporting lipophilicity data: partitioning solvents, pH, ionic strength, temperature, buffer composition, co-solutes, co-solvents, and equilibration time. The data can vary significantly with the different conditions.

23.1 In Silico Lipophilicity Methods

Databases provide a rich source of lipophilicity data for historic compounds. The MedChem Database from BioByte Corp. is available through Daylight Chemical Information Systems (*www.daylight.com/products/Medchem.html*). It contains Log P values for 61,000 compounds.

Software tools for the prediction of properties typically are developed using a set of compounds for which the property has been measured. The quality of this "training" dataset is higher with greater structural diversity and the reliable measurement of the property data. Following development of the algorithm, the software should be tested using a separate "validation" set of compounds. The widespread and long-term use of octanol/water partitioning has produced a large published set of compounds that have been carefully measured. Thus, Log P and Log D prediction software can be some of the most reliable in silico tools.

Many commercial software packages for lipophilicity are available. A partial list is given in Table 23.1. Web sites are available for free calculations of Log P by the entry of a SMILES string (e.g., *www.vcclab.org*). Even structure drawing programs, such as ChemDraw, include Log P calculation software.

Hansch and Leo[1] developed the fragment method for calculating Log P. The Log P contributions of a large number of substructures have been determined. The Log P of a new

TABLE 23.1 ► Partial List of Commercial Software for Log P and Log D Calculation

Name	Company	Web site
CLog P	Daylight Chemical Information	*www.daylight.com*
PrologP, PrologD	CompuDrug	*www.compudrug.com*
Bio-Loom	BioByte	*www.biobyte.com*
KowWin	Syracuse Research Corporation	*www.syrres.com/esc/kowwin.htm*
Log*D and* Log*P* DB	Advanced Chemistry Development	*www.acdlabs.com/products/*
QikProp	Schrodinger	*www.schrodinger.com*
ADMET Predictor	Simulations Plus	*www.simulations-plus.com*
ADME Boxes	Pharma Algorithms	*www.ap-algorithms.com*
KnowItAll	Biorad	*www.biorad.com*
ALOGPS	Virtual Computational Chemistry Lab	*www.vcclab.org*
Molinspiration Prop. Calculator	Molinspiration	*www.molinspiration.com*
CSLogP, CSLogD	ChemSilico	*www.chemsilico.com*
SLIPPER-2001	ChemDB	*http://software.timtec.net*
DSMedChem Explorer	Accelrys	*www.accelrys.com*
SciLogP	Scivision	*www.amazon.com*
ChemDraw	CambridgeSoft	*www.cambridgesoft.com*

structure is predicted by breaking it up into these substructures and calculating the sum of the individual contributions. Other Log P calculation approaches use neural net algorithms to construct a model using structural descriptors that correlate to Log P.

The user should not assume that all software produces the same Log P or Log D values, or that the values will be accurate when compared to laboratory measurements. Data for comparison of data obtained from the software package Prolog D with literature values[1,2] are listed in Table 23.2. The average difference between the predicted and measured values is about 1.05 log units. This correlation is represented in the graph in Figure 23.1. $R^2 = 0.72$ indicates a typical correlation between experimental and calculated values of Log D. The same type of comparison for structurally diverse discovery compounds, which typically have less drug-like properties than commercial drugs, are shown in Figure 23.2. The experimental determinations of Log D were performed at Wyeth Research using the gold standard pH-metric assay (see Section 23.3.2). Figure 23.2 shows how discovery compounds follow the same trend as commercial compounds but often have a lower correlation between calculated and measured properties. In addition to having generally poorer drug-like properties, discovery compounds may contain substructures that are not covered by the software development training set. Therefore, the full contribution of each of the discovery compound's substructures or descriptors to Log D may not be fully calculated.

Another aspect of predictive software is its use within a series of analogs around the same core scaffold or template. Software usually is reliable when comparing the structure–lipophilicity relationships of compounds in a series. Thus, although the predicted Log D may differ from the experimental Log D by an average of 1.05 log units (see above), the comparison of compounds within a series involves small substructural differences, and the software should be more predictive for indicating the increasing or decreasing lipophilicity trends resulting from substructural modification.

TABLE 23.2 ► Comparison of Literature Values and Calculated Values of Log D (Using Prolog D) for 70 Commercial Drugs

Compound	Literature Value	pLogD 7.4	Difference	Compound	Literature Value	pLogD 7.4	Difference
Acetylsalicylic Acid	−1.14	−1.58	0.44	Oxprenolol	0.32	1.16	−0.84
Salicylic Acid	−2.11	−2.99	0.88	Labetalol	1.07	−1.2	2.27
Acetaminophen	0.51	0.61	−0.1	Flurbiprofen	0.91	0.33	0.58
Amoxicillin	−1.35	0.08	−1.43	Ibuprofen	1.37	0.85	0.52
Theophylline	−0.02	−2.03	2.01	Propranolol	1.26	1.69	−0.43
Ceftriaxone	−1.23	−5.61	4.38	Alprenolol	0.97	2.14	−1.17
Terbutaline	−1.35	−1.39	0.04	Dexamethasone	1.83	1.49	0.34
Metoprolol	−0.16	0.4	−0.56	Oxazepam	2.13	0.36	1.77
Atenolol	−1.38	−1.21	−0.17	Corticosterone	1.82	2.83	−1.01
Sulpiride	−1.15	−1.08	−0.07	Chloramphenicol	1.14	0.69	0.45
Cephalexin	−1.45	1.17	−2.62	Lorazepam	2.51	1.03	1.48
Pindolol	−0.21	0.58	−0.79	Desipramine	1.28	1.3	−0.02
Nadolol	−1.21	−0.62	−0.59	Promazine	2.52	2.16	0.36
Timolol	−0.047	−0.92	0.873	Imipramine	2.4	2.72	−0.32
Acebutolol	−0.29	0.18	−0.47	Diltiazem	2.06	2.93	−0.87
Warfarin	1.12	−0.42	1.54	Verapamil	1.99	4.98	−2.99
Ketoprofen	−0.13	−0.18	0.05	Triphenylene	5.49	6.71	−1.22
Sulfasalazine	0.08	0.91	−0.83	L-Dopa	−2.57	−2.13	−0.44

3,4,5 - Trihydroxybenzoic Acid	−0.40	−2.22	1.82	Bifonazole	4.77	5.29	−0.52
3,5 - Dinitrobenzoic Acid	0.91	−1.9	2.81	Diethystilbestrol	5.07	6.25	−1.18
Pipemidic Acid	−1.52	−1.78	0.26	Clotrimazole	5.20	5.4	−0.2
2,4 - Dihydroxybenzoic Acid	2.06	−3.2	5.26	Ephedrine	−1.48	−0.82	−0.66
Furosemide	−1.02	−1.44	0.42	Sotalol	−1.35	−1.83	0.48
Sulfamerazin	−0.12	−0.73	0.61	Sumatriptaon	−1	−0.39	−0.61
Sulfathiazole	−0.43	0.55	−0.98	Disopyramide	−0.66	0.51	−1.17
Naproxen	0.3	0.18	0.12	Atropine	−0.25	0.57	−0.82
Allopurinol	−0.44	−3.74	3.3	Ranitidine	−0.29	1.14	−1.43
Thiamphenicol	−0.27	−0.4	0.13	Procaine	0.33	0.75	−0.42
Caffeine	−0.07	−1.8	1.73	Triflupromazine	3.61	3.31	0.3
Metronidazole	−0.02	−0.72	0.7	Clozapine	3.13	3.55	−0.42
Nitrofurazone	0.23	−0.98	1.21	Thioridazine	3.34	3.59	−0.25
Prednisone	1.41	0.96	0.45	Bupivacaine	2.65	4.37	−1.72
Carbamazepine	2.19	2.2	−0.01	Chlorpromazine	3.38	2.9	0.48
Testosterone	3.29	4.81	−1.52	Loratadine	4.4	5.61	−1.21
Estradiol	4.01	4.96	−0.95	Amiodarone	6.1	8.12	−2.02
						Average Difference:	**1.05**

The average difference is 10.5 log units

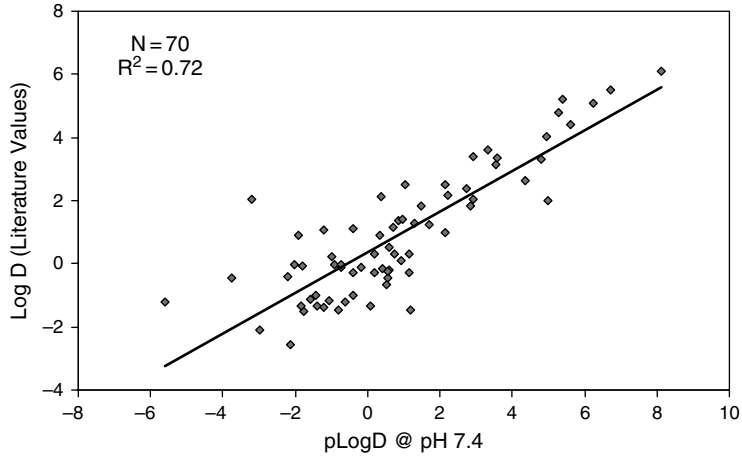

Figure 23.1 ► Correlation of literature values and calculated values of Log D (using Prolog D) for 70 commercial drugs, as listed in Table 23.2. $R^2 = 0.72$ indicates a typical correlation between experimental and calculated Log D.

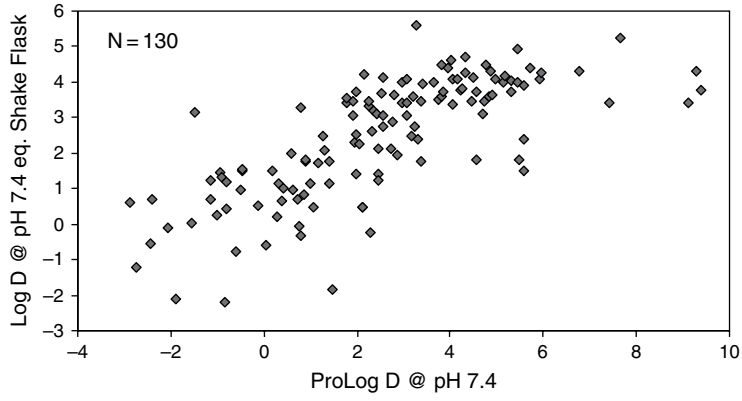

Figure 23.2 ► Correlation of experimental shake flask and calculated values of Log D for 130 discovery compounds at Wyeth Research. The general trends are comparable to the correlation for the commercial compounds.

Log P and Log D prediction from software has many advantages. Predictions are reasonably reliable. Software is fast and highly accessible, often via the company's computer network. The medicinal chemist does not need to have compound material to assay. There is no interference to the measurement by impurities, low solubility, or the need for co-solvents. Software covers a wide dynamic range. In silico predictions are much less expensive than experimental measurements, thus saving company resources for the measurement of other important properties.

23.2 Experimental Lipophilicity Methods

The three main in vitro high-throughput methods used for lipophilicity measurement are scaled-down shake flask, reversed-phase high-performance liquid chromatography (HPLC), and capillary electrophoresis (CE). Each method is meant to model the partitioning

environment and can use internal standards or known compounds for calibration of the analytical response to Log P or Log D. Table 23.3 tabulates the methods and indicates characteristics such as throughput.

TABLE 23.3 ► Methods for Determination of Lipophilicity

Method	Type assay	Speed (min/cpd)[a]	Throughput (cpd/day/instrument)[b]
Scaled-down shake flask	High throughput	10	100
Reversed-phase HPLC	High throughput	5	200
Capillary electrophoresis	High throughput	10	100
Shake flask	In depth	60	20
pH-metric	In depth	60	20

[a] min/cpd: minutes per analysis of one compound
[b] cpd/day/instrument: number of compounds that can be assayed in one day using one instrument

23.2.1 Scaled-Down Shake Flask Method for Lipophilicity

The octanol/water partitioning experiment was traditionally performed in larger vials and flasks (see Section 23.3.1), but the procedure can be scaled down to the titer plate level for higher throughput.[3–6] As shown in Figure 23.3, a 1-mL deep, 96-well plate can be filled with 0.5 mL of aqueous buffer and 0.5 mL of octanol to form the shake flask setup. The test compound is dissolved in dimethylsulfoxide (DMSO), and a small volume is added to the well. DMSO can alter the experiment via interaction with the analyte and solvents, so the volume should be kept as low as possible (<1% of the aqueous volume). It is important to control the ionic strength (e.g., 0.15 M NaCl) and the buffer pH and molarity in order to achieve comparable results between compounds and on different days. The plate is sealed and then agitated to produce good mixing between the phases. An aliquot is obtained from each of the phases and is analyzed, typically by HPLC, to determine the concentration of compound in each phase. A complication of the method is that the concentration of compound in each phase can be quite different, depending on the difference of Log D from 0 (equal concentration in each phase). Also, injection of octanol onto the HPLC can disrupt the chromatography of the analyte, compared to injection in aqueous phase. Carryover from one HPLC injection to the next should be checked and reduced to a minimum. Analiza has commercialized the scaled-down shake flask method into an integrated instrument. Commercial instruments for various lipophilicity methods are listed in Table 23.4.

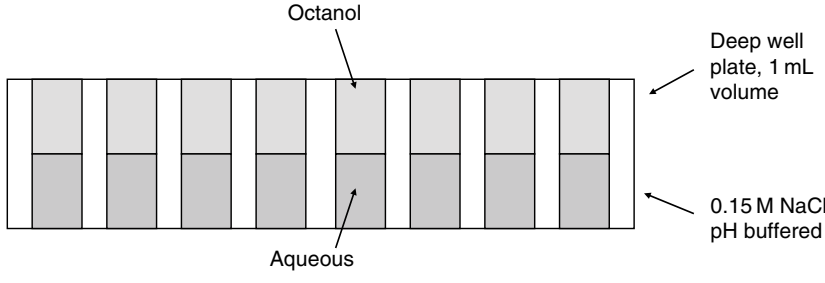

Figure 23.3 ► Scaled-down shake flask method for lipophilicity.

TABLE 23.4 ► Commercial Instruments for Measurement of Log D and Log P

Method	Product name	Company	Web site
Scaled-down shake flask	AlogPW	Analiza	*www.analiza.com*
Reversed-phase HPLC	PCA200	Sirius Analytical Instruments	*www.sirius-analytical.com*
Reversed-phase HPLC	Veloce	Nanostream	*www.nanostream.com*
Capillary electrophoresis	cePRO 9600	CombiSep	*www.combisep.com*
pH-metric	GLpKa	Sirius Analytical Instruments	*www.sirius-analytical.com*

23.2.2 Reversed-Phase HPLC Method for Lipophilicity

Reversed phase HPLC is fundamentally a separation scheme involving two immiscible media. It is technically related to a multistage partitioning experiment. Successive partitioning of the solute between the aqueous HPLC mobile phase and the stationary organic phase performs multiple partitions along the length of the HPLC column. The HPLC retention time is affected by these partitions. The retention time increases as the compound has higher affinity for the organic stationary phase than the aqueous phase. A common reversed-phase HPLC separation uses octadecane groups attached to the stationary support particles.

The lipophilicity of compounds is assessed by first injecting a series of standards for which Log D or Log P has already been determined using definitive analytical methods. The retention times of the standards are plotted against their respective previously measured Log D values (Figure 23.4). When the test compound is run, its retention time (t_R) is

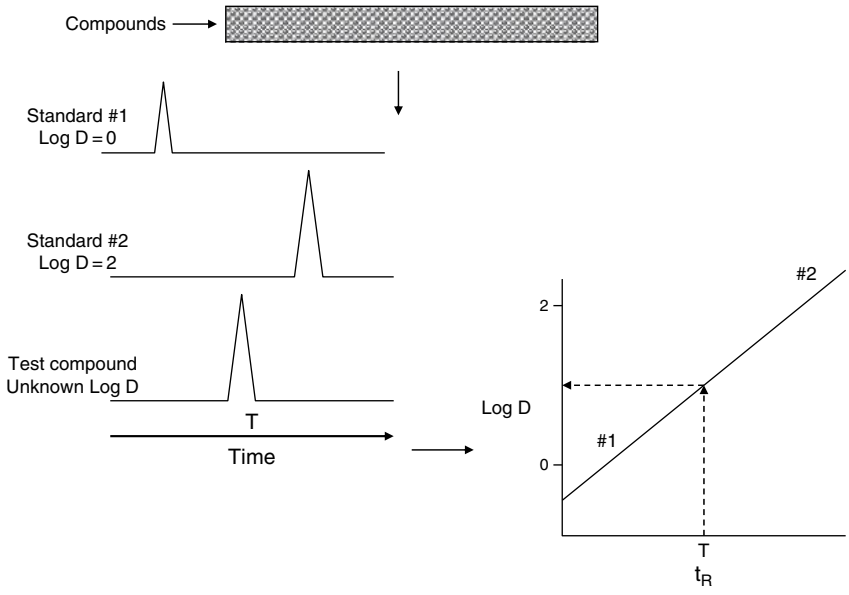

Figure 23.4 ► Reversed-phase HPLC method for lipophilicity. The stationary C18 column packing simulates the nonpolar phase and the mobile phase simulates water. t_R of the standards are plotted against their previously measured Log D. t_R of the test compound is compared to this calibration to determine the compound's Log D.

compared to the calibration curve to determine its Log D. Several articles have reported this type of method.[2,7–22] Any of the commercial HPLC instruments can be used. Nanostream has developed a 24-parallel HPLC system in one instrument for high-throughput analysis that accelerates lipophilicity determination. Sirius Analytical Instruments has developed an integrated instrument for Log D based on the HPLC approach. For HPLC-based methods, it is necessary to ensure that the same mobile phase and column product are always used because t_R varies with conditions.

23.2.3 Capillary Electrophoresis Method for Lipophilicity

As with the HPLC method, CE is another chromatographic method that, utilizing the microemulsion electrokinetic chromatographic (MEEKC) technique, has been used to determine lipophilicity.[21] The test compound partitions between the organic nonpolar microemulsion phase and the aqueous phase. When the compound is in the microemulsion phase, it moves more slowly through the column. Thus, the greater affinity it has for the lipophilic microemulsion phase, the longer will be t_R, which is calibrated to Log D in the same manner as in the HPLC method. CombiSep has developed an integrated 96-channel CE system for determination of lipophilicity.

23.3 In-Depth Lipophilicity Methods

The two primary in-depth methods for lipophilicity measurement are shake flask and pH-metric. Both methods provide reliable data for definitive lipophilicity determination of compounds. Both require significantly greater resources than the high-throughput methods, so they are applied to relatively few compounds. These methods are primarily used as gold standards for compounds entering development toward the end of discovery.

23.3.1 Shake Flask Method for Lipophilicity

The traditional shake flask method for lipophilicity is diagrammed in Figure 23.5. Solid test compound is placed into a flask or vial, and measured volumes of octanol and water are added. The flask is agitated for 24 to 72 hours, and the test compound in each phase is

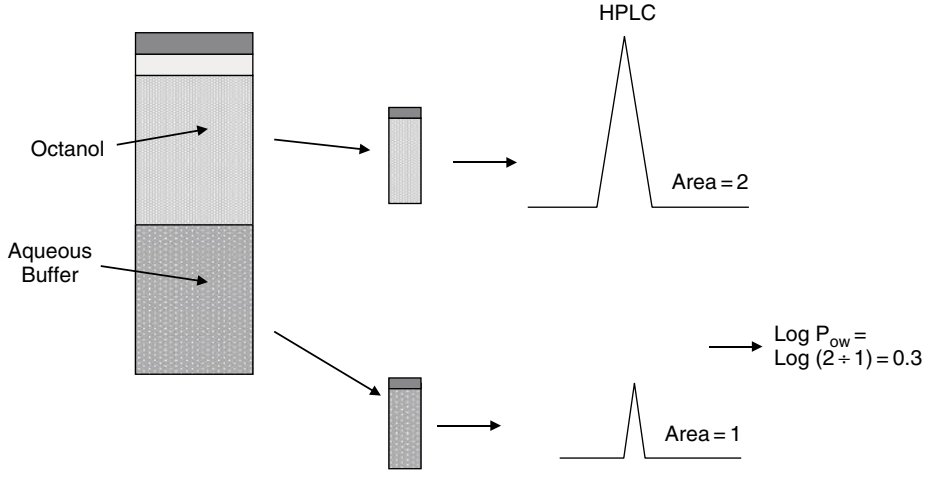

Figure 23.5 ► Traditional shake flask method for lipophilicity.

sampled and quantitated using HPLC. The areas under the peaks, or concentrations calculated from a standard curve, are divided and the log taken. The shake flask method is considered a gold standard for Log P (Log D) determination. It is necessary to control all of the conditions listed in the introduction to this chapter to obtain the highest quality data.

23.3.2 pH-Metric Method for Lipophilicity

Titration is a common method for determining pK_a. However, it also can be used to determine lipophilicity.[22] The test compound is first titrated by the addition of known equivalents of acid or base to produce a titration curve (Figure 23.6). Then the titration is repeated in the presence of octanol. A shift of the titration curve is obtained, owing to partitioning of the test compound into octanol. From the curve shift the lipophilicity is calculated. Sirius Analytical Instruments has developed an instrument that is in widespread use in the industry for pK_a and Log P.

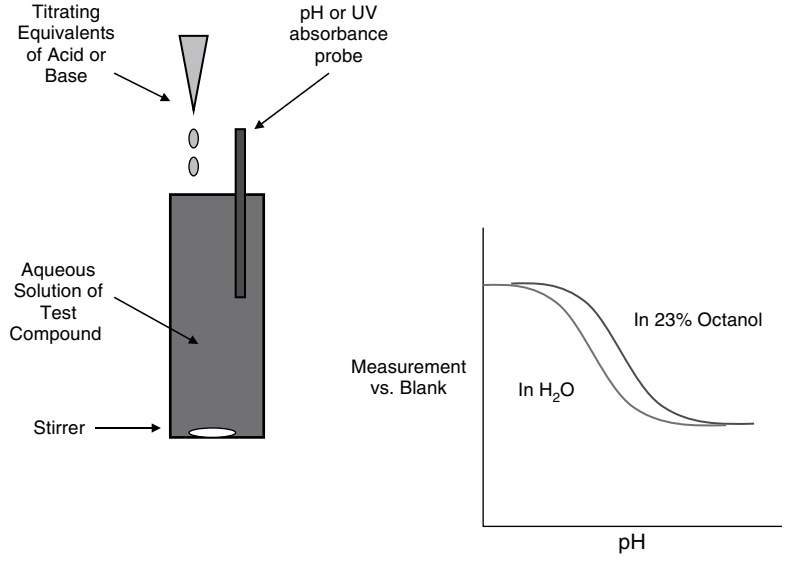

Figure 23.6 ► pH-metric method for lipophilicity.

Problems

(Answers can be found in Appendix I at the end of the book.)

1. Why are in silico predictions often poorer for drug discovery compounds than for commercial drug compounds?
2. Are in silico predictions for lipophilicity any better than other property predictions?
3. Which of the following can affect measurement of Log D data?: (a) % DMSO, (b) the buffer components, (c) humidity, (d) temperature, (e) ionic strength, (f) pH, (g) time.
4. With chromatographic HPLC and CE methods for lipophilicity, how does the analyst correlate retention time to Log D?

5. In general, how close should one expect in silico Log D predictions to be to actual values?

6. Which of the following are common methods of measuring Log P?: (a) partition between octanol and aqueous buffer, (b) infrared, (c) NMR in the presence of octanol, (d) HPLC, (e) titration in the presence of octanol.

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pK_a Methods

Overview

- *In silico* pK_a *predictions can be reliable, calculate tautomers, and indicate which ionizable center has which* pK_a.
- pK_a *can be predicted in higher throughput using spectral gradient analysis and capillary electrophoresis techniques.*
- *The in-depth method for* pK_a *is titration.*

Knowledge of the pK_a of substructures in the lead structure allows the "tuning" of ionizability in order to modify its permeability and solubility. Like lipophilicity, pK_a has been studied for many years, providing a solid body of research and quality measurements upon which reliable tools for pK_a prediction for compounds can be based for application in medicinal chemistry.

24.1 In Silico pK_a Methods

Databases can provide a useful source of comparison pK_a data for compounds that have been reported (Table 24.1). For example, the Daylight/BioByte product contains pK_a values

TABLE 24.1 ► Partial List of Commercial Software and Instruments for Determination of pK_a

Name	Company	Web site
Software		
MedChem Database	Daylight Chemical Information	*www.daylight.com*
Masterfile/CQSAR	BioByte	*www.biobyte.com*
pK_a DB	Advanced Chemistry Development	*www.acdlabs.com/products/*
pKalc (formerly PALLAS)	CompuDrug	*www.compudrug.com*
ADMET Predictor	Simulations Plus	*www.simulations-plus.com*
ADME Boxes	Pharma Algorithms	*www.ap-algorithms.com*
KnowItAll	Biorad	*www.biorad.com*
CSpKa	ChemSilico	*www.chemsilico.com*
SPARC	University of Georgia	*http://ibmlc2.chem.uga.edu/sparc*[a]
Instruments		
SGA	Sirius Analytical Instruments	*www.sirius-analytical.com*
GLpKa	Sirius Analytical Instruments	*www.sirius-analytical.com*
cePRO 9600	CombiSep	*www.combisep.com/pKa.html*

[a] Online calculator.

for 13,900 compounds, and ACD pKa/DB has 16,000 structures with over 31,000 experimental pK_a.

Software for the prediction of pK_a from structure has greatly progressed. A partial listing of available software is provided in Table 24.1. As with any software, the predictability should be validated by comparison to known compounds that have been measured using high-quality "gold standard" methods.

An example of the correlation of predictive software with measurements is shown in Figure 24.1. The compound set has 98 diverse real-world drug discovery compounds that were synthesized, measured, and predicted using ACD/pKa DB software at Wyeth Research. The R^2 for the correlation is 0.90, which is very good for in silico predictions.

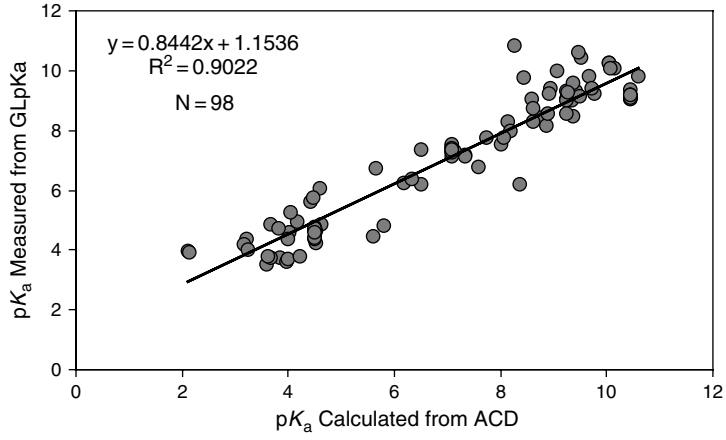

Figure 24.1 ► Correlation of pK_a measured using the GLpKa technique and calculated using software from ACDLabs.

pK_a software can provide more advanced information for chemists. Figure 24.2 shows how ACDLabs software provides structural assignment of which ionization center in the molecule has which pK_a. In addition, the different pK_a values of tautomers can be predicted.

Software can be applied when planning the synthesis of structural series analogs, when the goal is to modify permeability or solubility by the substitution of substructures with

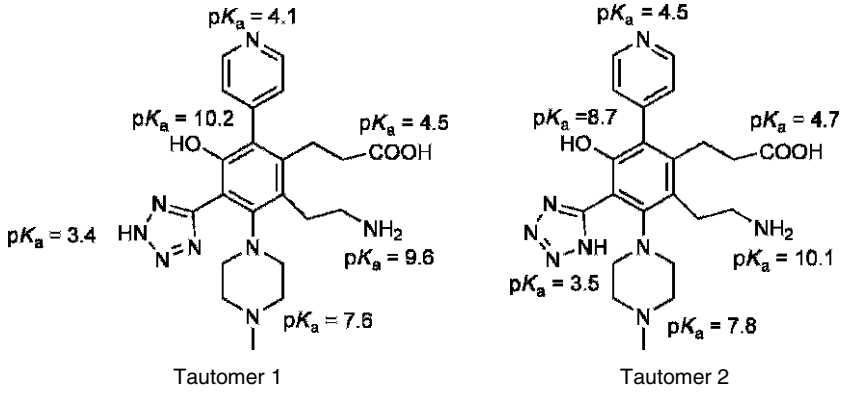

Figure 24.2 ► In silico products often indicate which ionization centers have which pK_a, as well as predicting pK_a for tautomers. These results are from ACDLabs software.

varying pK_a values in order to optimize absorption. The most promising of all the possible modifications then can be synthesized, or several analogs distributed over a pK_a range can be selected for synthesis.

pK_a has been measured accurately for many diverse compounds. Its relationship to structure has been studied in detail. Thus, in many cases, pK_a software is very predictive and is sufficient for many purposes of medicinal chemists.

24.2 Experimental pK_a Methods

Two higher-throughput methods have been introduced. Each can be implemented in drug discovery laboratories with the proper equipment, and each has been offered as an integrated commercial instrument. For both of these methods, only a small quantity of compound is needed (<0.1 mg), and throughput is about 5 minutes per compound.

24.2.1 Spectral Gradient Analysis Method for pK_a

Spectral gradient analysis (SGA) was invented at GSK by Bevan et al.[1,2] It is based on the concept of a gradient high-performance liquid chromatography pump, with the substitution of aqueous acidic and basic buffers for the two mixed liquid phases. The instrument is diagrammed in Figure 24.3. The test compound is dissolved in dimethylsulfoxide (DMSO) at 10 mM and placed in a 96-well plate. Each solution in the plate is diluted with aqueous buffer. The aqueous solution then is continuously mixed into the aqueous pH buffer. Throughout the 2-minute experiment, a gradient program is run, which starts with a high percentage of one buffer and progresses to a low percentage of that buffer over 2 minutes. Thus, the test compound is mixed with a continuously changing pH. As the pH changes, the fraction of the compound that is ionized changes. The absorption of an ultraviolet or visible chromophore, which is near the ionization center (within three to four bonds), changes with ionization. As the mixture flows into the diode array UV detector/spectrometer, the ultraviolet/visual (UV/VIS) absorption changes with pH (Figure 24.4). pK_a is the inflection point of this absorption curve. The instrument can produce a pK_a screen for test compounds about every 3 to 4 minutes for high throughput. A commercial instrument is available (Table 24.1).

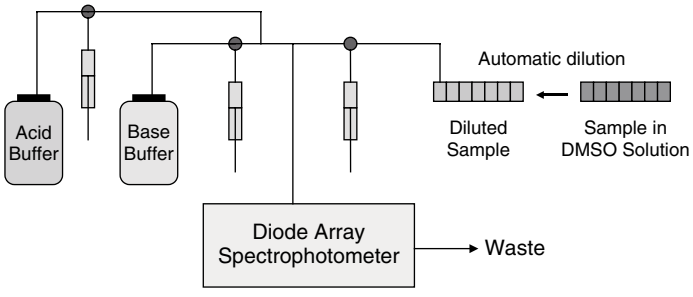

Figure 24.3 ► Spectral gradient analysis pK_a instrument diagram.

24.2.2 Capillary Electrophoresis Method for pK_a

Higher-throughput pK_a measurement is available using capillary electrophoresis (CE).[3–9] This is based on the different electrophoretic mobility of a compound in the neutral and ionized form. The test compound is diluted into aqueous buffer and injected into a CE column. The compound is run several times using CE mobile phase buffers at different

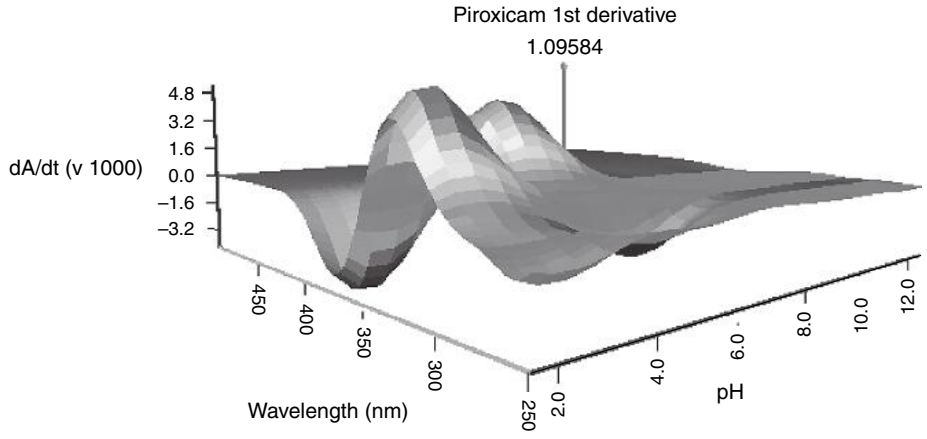

Figure 24.4 ► Spectral gradient analysis (SGA) pK_a system UV/VIS absorbance data output for piroxicam and its use in pK_a screening determination. (Reprinted with permission of John Comer.)

pHs. Ionized molecules move through the mobile phase faster because CE mobility is proportional to charge. Thus, as the fraction of ionized molecules increases, the effective mobility (retention time) is progressively shorter. By plotting the effective mobility versus mobile phase pH, pK_a is calculated as the inflection point.

The CE pK_a method can be automated using commercial CE instruments. A commercial instrument that runs 96 experiments in parallel for high throughput and has pK_a processing software is available (Table 24.1). The CE method separates components and, therefore, has low potential for interference from impurities in the sample.

24.3 In-Depth pK_a Method: pH-Metric

The definitive method for pK_a determination is potentiometric titration. A diagram of this approach is shown in Figure 24.5. The compound is dissolved in water and titrated with an acidic or basic buffer of known molarity. In the classic method, the pH of the test compound solution changes as the titrant is added. This change is monitored using a pH electrode. This change in pH with titrating equivalents is plotted versus solution pH. pK_a is the pH of the inflection point of the curve. A variation of this method is to measure the UV absorbance

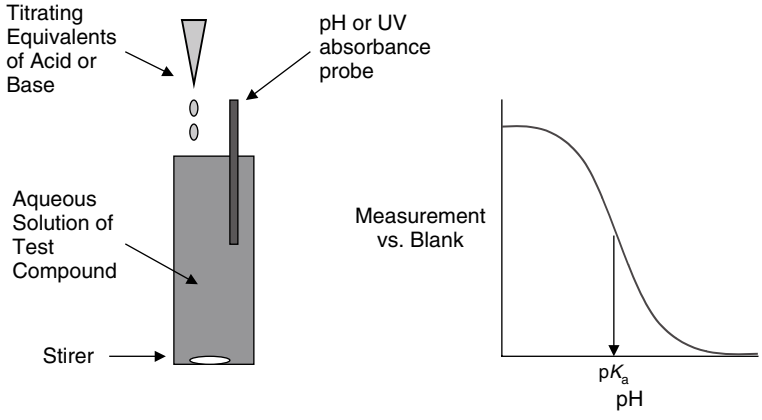

Figure 24.5 ► In-depth titration method for pK_a determination is the pH-metric method.

using a UV spectrophotometric probe in the solution of the test compound. As in the case of the SGA method, the absorbance of a chromophore near the ionization center changes with ionization and can be monitored to determine the extent of ionization.

This method has been studied in detail by Avdeef et al.[10] and termed the *pH-metric method*. It has been integrated as the GLpK_a instrument that is commercially available (Table 24.1). This method is considered the "gold standard" for pK_a and Log P analysis in drug development laboratories. If the compound has low solubility, a co-solvent is added to the test compound solution. Titrations at three co-solvent concentrations allow extrapolation to zero co-solvent aqueous concentration. The throughput for pK_a measurements is about 0.5 to 1 hour per soluble compound and up to 2 hours per compound requiring needs the co-solvent method.[11]

Problems

(Answers can be found in Appendix I at the end of the book.)

1. Detection methods for pK_a use the change in what three measurable parameters?
2. Rank the following pK_a methods in terms of throughput: CE, pH-metric, SGA, in silico.
3. What useful features can advanced software for pK_a provide?
4. Is low solubility a potential issue for pK_a measurement?

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Chapter 25

Solubility Methods

Overview

- *Solubility can be estimated using* pK_a, pH, Log P, *and melting point.*
- *Commercial software calculates equilibrium solubility for comparing series analogs.*
- *High-throughput (HT) kinetic solubility is most relevant in discovery because it mimics discovery conditions.*
- *HT kinetic methods include direct UV, nephelometry, and turbidimetry.*
- *Custom solubility methods are used to mimic a specific project issue.*
- *Equilibrium solubility is relevant for animal dosing and development studies.*

Earlier chapters have emphasized the importance of solubility in drug discovery. Solubility plays a crucial role in drug absorption in the gastrointestinal tract, accurate biological assays in vitro, and dosage form selection for in vivo administration. With this central role, measurement of solubility is a frequent activity of discovery scientists, and attention should be paid to accurate and relevant analyses. This chapter provides an overview of in silico and in vitro solubility methods. The fundamentals of kinetic and thermodynamic solubility are discussed in Chapter 7.

25.1 Literature Solubility Calculation Methods

The total solubility of an ionizable compound at a particular pH can be estimated using its intrinsic solubility (solubility of the neutral form) and pK_a, according to the Henderson-Hasselbalch equation.[1] The following equation is used for acids. Concentration is in molarity:

$$S_{tot} = S_{HA}(1 + 10^{(pH - pK_a)}),$$

where S_{tot} = total solubility, and S_{HA} = intrinsic solubility of the neutral acid. Solubility is in g/mL.

The intrinsic solubility can be estimated using the Yalkowsky equation.[2] It uses lipophilicity and melting point, as follows:

$$\text{Log S} = 0.8 - \text{Log P} - 0.01(\text{MP} - 25),$$

where Log S = log of the solubility, Log P = log of the octanol/water partition coefficient, and MP = melting point in degrees Centigrade. Solubility is in mol/L.

25.2 Commercial Software for Solubility

Several software products for solubility are commercially available (Table 25.1). No study has compared results from these products against each other. Results for one solubility software package have been compared to experimental measurements.[3] The training and validation sets for these algorithms use equilibrium (thermodynamic) solubility values from the literature. Therefore, they may not be predictive of kinetic solubility values.

TABLE 25.1 ► Commercial Software for Solubility

Name	Company	Web site
Aqueous Solubility		
QikProp	Schrodinger	*www.schrodinger.com*
Solubility DB, Solubility Batch	Advanced Chemistry Development	*www.acdlabs.com*
ADMET Predictor	Simulations Plus	*www.simulations-plus.com*
Volsurf	Tripos	*www.tripos.com*
ADME Boxes	Pharma Algorithms	*www.ap-algorithms.com*
DSMedChem Explorer	Accelrys	*www.accelrys.com*
WSKow	Syracuse Research Corporation	*www.syrres.com*
KnowItAll	Bio-Rad Laboratories	*www.biorad.com*
SLIPPER	ChemDB	*www.chemdbsoft.com/SLIPPER*
CSLogWS	ChemSilico	*www.chemsilico.com*
Quantum	Quantum Pharmaceuticals	*www.q-pharm.com*
DMSO Solubility		
Volsurf	Tripos	*www.tripos.com*
DMSO Box	Pharma Algorithms	*www.ap-algorithms.com*

One widely distributed software, QikProp, predicts several absorption, distribution, metabolism, excretion, and toxicity (ADME/Tox) properties based on regression models using molecular descriptors. William L. Jorgensen, the developer of QikProp, suggests three rules for compounds, based on 90% of 1,700 oral drugs. The three rules are as follows:

- QikProp Log S > –5.7 (M)
- QikProp Caco-2 Permeability >22 nm/s
- Number of primary metabolites predicted by QikProp <7

Thus, in addition to providing predictions of specific properties, software can rapidly provide a profile of the drug-like properties compared to a benchmark of success.

Dimethylsulfoxide (DMSO) solubility has recently become an interest of many organizations because of its effects on biological assays (see Chapter 40). Many discovery scientists have considered DMSO a universal solvent. However, investigations have shown that salts and polar compounds may not be fully soluble in DMSO. Two software products for DMSO solubility are listed in Table 25.1.

25.3 Kinetic Solubility Methods

Kinetic solubility methods first dissolve the solid compound in DMSO and then add an aliquot of DMSO solution to the aqueous buffer. Kinetic solubility is most appropriate for drug discovery for the following reasons:

- It mimics the conditions of discovery experiments (initial dissolution in DMSO followed by addition to aqueous buffer, incubation time of hours)
- Initially compounds are dissolved in DMSO, thus negating differences in the solid form among compounds (even batches of the same compound can vary in solid form: metastable crystal form, amorphous material)
- Only 1 to 2 mg of compound is consumed (consistent with the small amount of each compound initially synthesized in discovery)
- Only 1 day is necessary for the assay (consistent with the fast decision-making in discovery)
- In many companies, 10 to 20 mM DMSO solutions of compounds are already available in a company's screening laboratory or compound repository, which minimizes sample preparation work.

As more stable crystal forms are prepared in discovery and development, the equilibrium (thermodynamic) solubility drops. The solubility will be lower than the less stable crystal form that precipitates in kinetic solubility measurements. The focus on kinetic solubility in discovery is to provide an optimistic estimate of solubility values when only limited material with unknown crystal forms is available. The purpose of kinetic solubility measurements is to identify compounds that do not have good kinetic solubility even in aqueous buffer containing DMSO, to guide modification of structures to improve solubility, and to guide formulation selection for animal dosing. As compounds progress to development, typically the most stable crystal form is selected, and more detailed thermodynamic solubility studies are conducted in various solvents. Kinetic solubility is used during lead selection and optimization to:

- Rank order compounds
- Guide modification of structures to optimize solubility.

The final DMSO concentration should be kept as low as practical (typically 1%–5%) in solubility methods. This is because DMSO increases the aqueous solubility of lower solubility compounds, especially lipophilic compounds.

It is important to remember that kinetic solubility data vary with the conditions of the solution. Small changes in pH, organic solvent, ionic strength, ions in solution, co-solutes, incubation time, and temperature can result in large changes in the solubility of a compound. Methods for kinetic and thermodynamic solubility are listed in Table 25.2.

25.3.1 Direct UV Kinetic Solubility Method

The direct UV method, developed by Avdeef,[4] measures the concentration of a compound that is dissolved in solution. A diagram of the method is shown in Figure 25.1. A small volume of compound in DMSO solution is added to the well of a 96-well plate containing

TABLE 25.2 ► Methods for Determination of Solubility

Method	Type assay	Speed (min/cpd)	Throughput (cpd/day/instrument)
Kinetic Solubility			
Nephelometry	High throughput	4	300
Direct UV	High throughput	4	300
Turbidimetry	Moderate throughput	15	50
Thermodynamic Solubility			
Equilibrium shake flask	Low throughput	60	10
Potentiometric	Low throughput	60	10

Note: cpd = compound

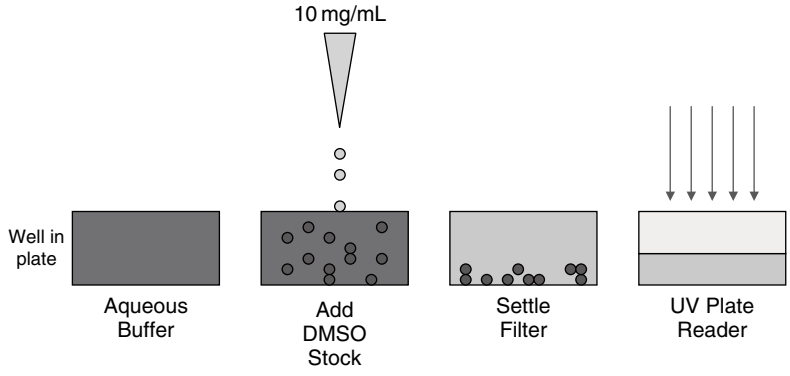

Figure 25.1 ► Direct UV kinetic solubility method diagram.

aqueous buffer and mixed. Typically the solution is covered and held at ambient temperature for a designated time (e.g., 1–18 hours). The maximum target concentration is set at about 100 μg/mL to minimize material consumption and volume of added DMSO. If a compound is not completely soluble, the extra portion will precipitate from solution. The precipitate is removed by filtration, and the UV absorbance of the supernatant is measured using a UV plate reader. The concentration of the compound is proportional to the UV absorbance, according to Beer's law. Solubility is determined against a single point standard, owing to the linearity of concentration versus UV absorbance. pION Inc. has implemented this method as a commercial instrument (Table 25.3). The pION software can compensate for the contribution of DMSO in enhancing compound aqueous solubility. Filters for the method can be obtained from Millipore, Corning, and other manufacturers. The filters must have a minimum of nonspecific absorption so that all of the dissolved compound stays in solution and is quantitated. Analiza has implemented this method in a commercial instrument using a nitrogen-specific high-performance liquid chromatography (HPLC) detector (Table 25.3).

The direct UV method is amenable to automation using laboratory robots. In addition to higher sample throughput, automation offers the opportunity of rapidly measuring solubility at multiple pHs. For example, it is useful to measure solubility at various pHs to simulate physiological and assay conditions: pH 1 (stomach), 6.5 (small intestine), 7.4 (neutral pH in blood, tissues, and bioassay buffers), and 8 (large intestine). One efficient strategy is to measure all compounds using generic conditions (e.g., pH 7.4, ambient temperature, 16 hours) for screening purposes and then to measure solubility using customized conditions for selected compounds of interest, as needed.

TABLE 25.3 ► Commercial Instruments and Products for Measurement of Solubility

Method	Product name	Company	Web site
Kinetic Solubility			
Direct UV	μSol Evolution, Explorer	pION Inc.	*www.pion-inc.com*
Direct UV	Multiscreen Solubility	Millipore	*www.millipore.com*
Direct UV	ASolW	Analiza Inc.	*www.analiza.com*
Nephelometry	NEPHELOstar	BMG LABTECH	*www.bmglabtech.com*
Nephelometry	Solubility Scanner	BG Gentest	*www.bdbiosciences.com*
Nephelometry	Nepheloskan Accent	Thermo Electron	*www.thermo.com*
Thermodynamic Solubility			
Potentiometric	pSOL	pION Inc.	*www.pion-inc.com*

25.3.2 Nephelometric Kinetic Solubility Method

The nephelometric method, developed by Bevan and Lloyd,[5] measures the precipitation of a compound after it reaches its maximum concentration in solution. A diagram of the method is shown in Figure 25.2. A small volume of compound in DMSO solution is added to the first well of a row in a 96-well plate containing aqueous buffer and mixed using the pipetter. If the compound is not fully soluble, it will precipitate from solution and form particles. A small aliquot is removed from this solution, added to the next well in the row, and mixed. Subsequent dilutions result in lower concentrations of compound in subsequent wells. Serial dilutions are performed into 5% DMSO/95% phosphate-buffered saline (pH 7.4). Following a brief incubation, the plate is placed in a plate reader that sequentially impinges laser light on each well. Precipitate in the well from undissolved compound scatters the light. The "counts" of scattered light are plotted versus the well concentration, and the turning point is the maximum concentration, or solubility, of the compound. Bevan and Lloyd suggested the

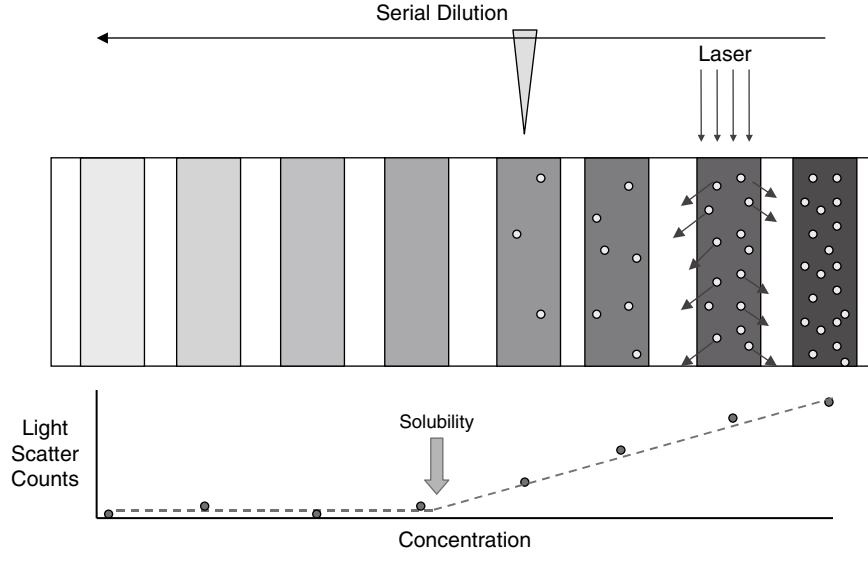

Figure 25.2 ► Nephelometric kinetic solubility method.

following solubility ranges: sparingly soluble (<10 μg/mL), partially soluble (10–100 μg/mL), and soluble (>100 μg/mL); these ranges are similar to those used by Lipinski et al.[6,7]

BMG LABTECH has implemented this method using its NEPHELOstar plate reader with a laser light source at 633 nm. A 96-well plate is scanned in 60 seconds. Thermo Electron has the Nepheloskan Accent reader (Table 25.3), which dispenses DMSO solutions into plate wells, shakes, scans the plate, and calculates the results. BD Gentest has implemented a variation of the method, which uses a flow cytometry detection system to detect the precipitate (Table 25.3).[8,9] The nephelometric method is the most commonly used method in industry[10,11]; however, users should be aware that the method measures precipitate, not actual compound concentration in solution, as in the direct UV method.

25.3.3 Turbidimetric In Vitro Solubility Method

The turbidimetric method also measures the precipitation of a compound from solution when it exceeds its solubility. The method is diagrammed in Figure 25.3. Small volumes (0.5 μL) of compound DMSO solution (20 mg/mL) are added stepwise at 1 min intervals to a cuvette containing stirred 2.5 mL of pH 7 phosphate buffer. When the concentration exceeds the compound solubility, precipitate forms and the turbidity scatters the light and reduces transmission (620–820 nm) through the cuvette. Subsequent additions of DMSO solution increase the precipitate and further decrease the light transmission. Fourteen additions of DMSO solution cover the solubility range of 5 to 65 μg/mL, with increasing DMSO concentration up to 0.375%. Light transmission is plotted versus compound, concentration and the turning point is the solubility of the compound. This method was developed by Lipinski et al.[6,7] and has not been commercialized.

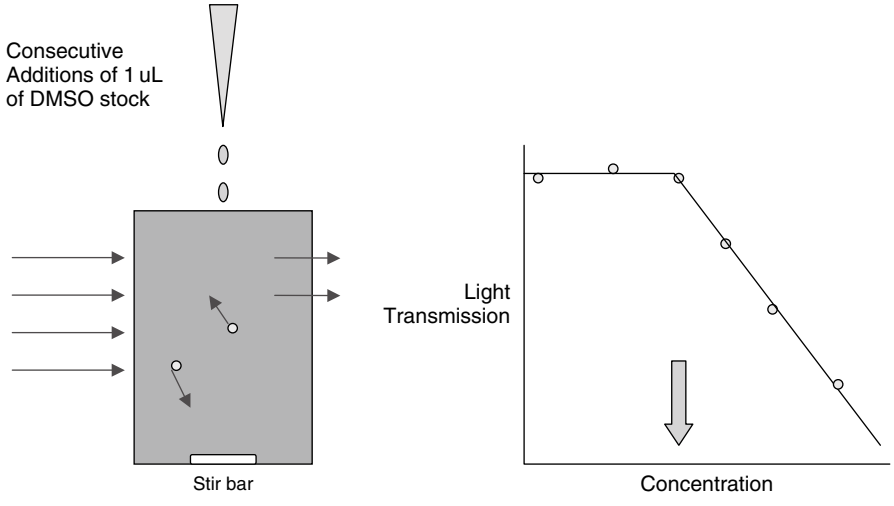

Figure 25.3 ► Turbidimetric kinetic solubility method.

Lipinski et al.[6] suggested that compound solubility <20 μg/mL is problematic. More than 85% of commercial drugs have solubilities >65 μg/mL by the turbidimetric method and have good bioavailability. Those commercial drugs having lower solubilities usually overcome this deficiency by having high potency, high permeability, or active transport across the intestinal epithelial cells.

25.3.4 Customized Kinetic Solubility Method

Often there are cases where generic solubility method conditions are not adequate to accurately assess the solubility of compounds under specific conditions. For example, the solubility of a compound can differ greatly among the following:

- Generic solubility assay buffer
- Enzyme or receptor assay buffer
- Cell-based assay buffer
- In vivo dosing vehicle
- Buffer with surfactant, protein, or DMSO
- pH 1–8

Solubility also varies with temperature, crystal form, and incubation time. In recent years, biological scientists have recognized that solubility differences between bioassay media can greatly affect IC_{50}. If the compound is not fully dissolved, IC_{50} is shifted to a higher concentration. This occurs to a greater extent for lower solubility and insoluble compounds in discovery.

When the solubilities of compounds are in question, a customized solubility experiment can be developed for a set of compounds. Such a "customized" solubility experiment can be performed using the same buffer composition, dilution procedure, incubation time, and temperature as the biological assay. Useful methodology for customized solubility assays was discussed by Di.[12,13] Compounds are added to the bioassay buffer under the conditions of the biological assay and then incubated according to bioassay time schedules. Precipitate is removed by filtration, and the supernatant is diluted accordingly and analyzed by LC/UV/mass spectrometry (MS) techniques. Results from such an assay are shown in Figure 25.4. In this example, the concentration of test compounds 1 to 3 were close to the target concentration of 10 μM in the receptor assay buffer, but in the cell-based assay buffer the concentrations were in the range of 1.4 to 4.8 μM. This likely is due to the high amounts of bovine serum albumin (BSA) and DMSO in the receptor assay buffer compared to the cell-based assay buffer. BSA and DMSO enhance the solubility of lower-solubility compounds. It is advisable to perform such an analysis during the development of each biological assay so that the buffer and dilution protocols can be optimized to maximize

Compounds	Solubility in Receptor Binding Assay Buffer (uM)	Solubility in Cell-based Assay Buffer (uM)
1	11	2.4
2	10	4.8
3	10	1.4
Buffer	5% BSA, 2.5% DMSO	0.1% DMSO

* Target assay concentration 10 μM

Figure 25.4 ► Customized solubility assays using different biological assay buffers indicate that the cell-based assay buffer does not keep the compounds fully solubilized at the target concentration of 10 μM.

solubility of test compounds and produce reliable biological data. (See Chapter 40 for further discussion of solubility issues in biological assays.)

25.4 Thermodynamic Solubility Methods

Thermodynamic solubility methods add aqueous buffer directly to compound solid and mix the solution for an extended time until it reaches or approaches equilibrium. Thermodynamic solubility data are most appropriate for crystalline material that is studied in detail during late drug discovery and development.

Thermodynamic solubility is most often determined using the equilibrium shake flask method or the pH-metric method. A clinical candidate often will undergo thermodynamic solubility studies in a wide set of conditions, as discussed below.

25.4.1 Equilibrium Shake Flask Thermodynamic Solubility Method

Thermodynamic solubility is often measured by placing solid crystalline compound in a vial and adding the solvent (Figure 25.5). The vial then is shaken for 24 to 72 hours at a controlled temperature (25°C–37°C), and undissolved material is separated by filtration. The compound dissolved in the supernatant is diluted accordingly and measured using LC/UV/MS. This method is often considered the "gold standard" solubility method.

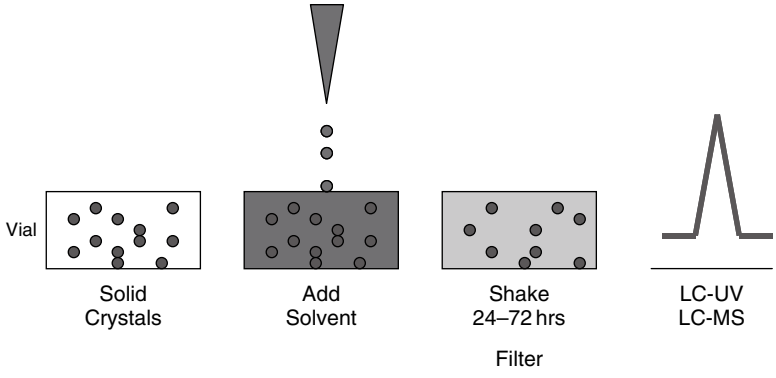

Figure 25.5 ► Equilibrium shake flask thermodynamic solubility method.

In a modification of this method,[14,15] 1 mg of solid compound is placed in a Whatman Mini-UniPrep filter chamber and 0.45 mL of aqueous buffer is added. After the filter cap is attached, the vials are placed in a HPLC autosampler vial block and shaken for 24 hours. The block is transferred to a Whatman filter processor device, and all the vials are simultaneously filtered. HPLC is used to measure the filtrate concentration. This approach automates portions of the thermodynamic solubility method.

25.4.2 Potentiometric In Vitro Thermodynamic Solubility Method

If the actual pK_a of a compound has been determined, potentiometric titration can be used to determine its intrinsic solubility (S_0; Figure 25.6). Known volumes of acid or base are added to a solution of the compound in the assay tube. The pH change during titration produces a titration curve. The apparent pK_a (pK_a^{app}) is shifted from the actual pK_a due to precipitation

of the compound, and the S_0 is calculated from the equation shown in Figure 25.6. This method is implemented on the GLpK_a instrument from Sirius Analytical, which is often used in development laboratories. The potentiometric solubility method is suitable only for compounds with ionization centers.

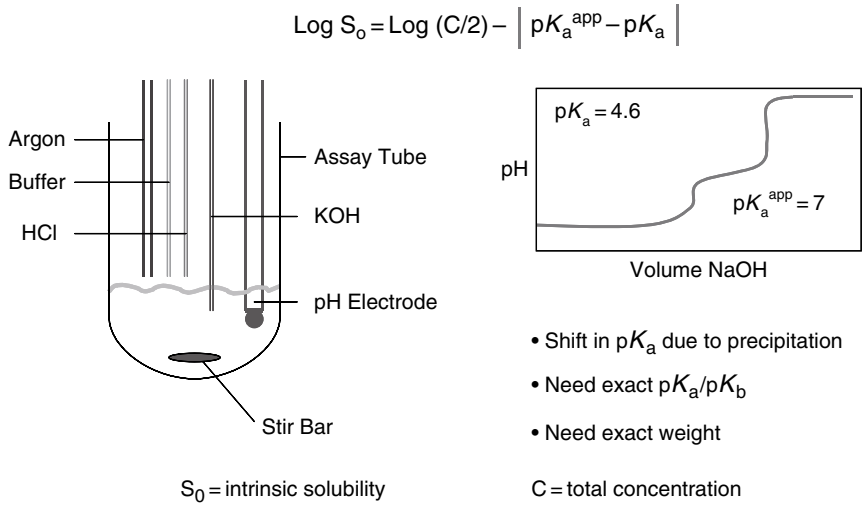

Figure 25.6 ► Potentiometric thermodynamic solubility method.

25.4.3 Thermodynamic Solubility in Various Solvents

Thermodynamic solubility studies are often performed to assist discovery and development formulation and in preparation for clinical development. Examples of solvents commonly tested are listed in Table 25.4. Such studies provide perspectives on compound solubility in various physiological fluids to aid absorption studies, in various formulary solvents to aid formulation, and in solvents that are used to measure lipophilicity.

TABLE 25.4 ► Various Physiological and Formulary Solvents Used to Test Thermodynamic Solubility of Development Candidates

Physiological buffer	Formulary solvent	Lipophilicity
pH 1	Tween 80	Octanol
pH 4.5	PEG 200	Cyclohexane
pH 6.6	PEG 400	
pH 7.4	Phosal 53 MCT	
pH 9	Phosal PG	
SGF	Benzyl Alcohol	
SIF	EtOH	
SIBLM	Corn Oil	
Plasma	2% Tween/0.5%MC	

Reprinted with permission from [16].
MC, Methylcellulose; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; SIBLM, simulated intestinal bile lecithin media.

Problems

(Answers can be found in Appendix I at the end of the book.)

1. For the following compounds, estimate their equilibrium solubilities:

Compound	Melting point (°C)	Log P	Estimated solubility (mol/L)
1	125	0.8	
2	125	1.8	
3	225	1.8	
4	225	3.8	

2. For the following acids, estimate their equilibrium solubilities:

Compound	Intrinsic solubility	pK_a	pH	Total solubility (g/mL)
1	0.001	4.4	7.4	
2	0.001	4.4	4.4	
3	0.001	4.4	8.4	
4	0.00001	4.4	7.4	

3. What is/are measured in the nephelometric and direct UV methods?
4. Why is it useful to use customized solubility methods as opposed to generic high-throughput assays?
5. List some differences between kinetic and thermodynamic (equilibrium) solubility methods.
6. Which of the following are *true* about *thermodynamic* solubility measurements?: (a) aqueous buffer is added to solid compound and agitated for 2–3 days, (b) are unaffected by the form of the material (i.e., amorphous, crystalline, polymorphic), which varies with batch, (c) are used for discovery lead optimization, (d) are used for development and clinical batches, (e) assist with product development.

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Permeability Methods

Overview

- *In silico methods are available for calculating in vivo and in vitro permeability, such as intestinal absorption, Caco-2, and parallel artificial membrane permeability assay (PAMPA).*
- *High-throughput permeability methods utilize high-performance liquid chromatography (immobilized artificial membrane), artificial membranes (PAMPA), and cell layers (Caco-2).*
- *In-depth study of permeability uses portal vein cannulation and in situ perfusion.*

26.1 In Silico Permeability Methods

Computational models for intestinal drug absorption have been reported.[1] Several commercial software packages are available for the prediction of gastrointestinal (GI) absorption. A partial list of software is given in Table 26.1. As with all software, the user should evaluate the software using the compounds with which they are familiar and for which internal data are available before purchasing and implementation. Permeability is a more complex process than lipophilicity, and only a limited number of measurements[1–3] of intestinal absorption are available upon which to develop algorithms. Thus, the predictions should be taken as a guide but not overinterpreted. As with other software, predictions likely are best for comparing compounds in a series on a relative scale, as opposed to setting expectations for an exact value in vivo. One application of predictive permeability tools is for medicinal chemists to study the potential GI absorption effects of various substituents on a core scaffold when planning compounds to synthesize.

TABLE 26.1 ► Partial List of Commercial Software for Permeability

Name (prediction)	Company	Web site
GastroPlus (human intestinal)	Simulations Plus	*www.simulations-plus.com*
ADME Boxes (passive intestinal)	Pharma Algorithms	*www.ap-algorithms.com*
ADME Index (database of marketed drugs)	Lighthouse Data Sol.	*www.lighthousedatasolutions.com*
KnowItAll (human intestinal)	Biorad	*www.biorad.com*
CSHIA (human intestinal)	ChemSilico	*www.chemsilico.com*
Admensa (human intestinal)	Inpharmatica	*www.inpharmatica.com*
QikProp (Caco-2, MDCK)	Schrodinger	*www.schrodinger.com*
ADMET Predictor (MDCK, human jejunal)	Simulations Plus	*www.simulations-plus.com*

Egan et al.[4] demonstrated that Log P and polar surface area were good descriptors for a model of well-absorbed (>90%), moderately absorbed (30%–90%), and poorly absorbed compounds. Well-absorbed compounds group in an ellipse in the region of AlogP98 –1 to 5.9 and polar surface area of 0 to 132 $Å^2$.

Software for predicting permeability in cell layer methods is available, as for Caco-2 and the Madin Darby Canine Kidney cell line (MDCK), and are listed in Table 26.1. Quantitative structure–activity relationship methods for Caco-2, parallel artificial membrane permeability assay (PAMPA), PAMPA-BBB, and human intestinal absorption have been published.[5,6]

26.2 In Vitro Permeability Methods

Three types of permeability assays are most commonly used: (a) "immobilized artificial membrane" (IAM) high-performance liquid chromatography (HPLC), (b) cell layer, and (c) PAMPA. Each of these methods involves partitioning the test compound between aqueous and lipophilic phases. The IAM and PAMPA methods model only the passive diffusion mechanism. The cell layer method models passive diffusion, active uptake transport, efflux, and paracellular permeability mechanisms.

26.2.1 IAM HPLC

IAM is a convenient method because it uses the common HPLC format. Instead of octadecyl groups covalently bonded to the solid support, as in reversed-phase HPLC, the IAM technique uses phospholipids bonded to the solid support. These contain the polar head groups and aliphatic side chains of the lipids. Pidgeon developed this new HPLC phase concept.[7–16] Test compounds partition between the aqueous mobile phase and the phospholipid phase. The chromatographic capacity factor (k) increases with increasing affinity for the phospholipid phase. Compounds are rank ordered by k, which indicates a higher lipophilicity or phospholipids affinity. The parameters of this affinity correlate with permeation. Retention time is calibrated against permeability or absorption using standard compounds for which permeability or absorption has been measured using another technique.

The HPLC format is commonly available in discovery laboratories, is convenient to use, and is readily automated using autosamplers to save scientists' time. The correlation to permeability may be better than Log D because the lipids are close in structure to biological membrane lipids. IAM columns are commercially available (Table 26.2). Because IAM involves only physicochemical interaction with the phospholipids and not the polarity transitions and molecular volume space constraints of the lipid bilayer of a biological membrane, it is less predictive of permeability than some other methods. Traditionally, IAM uses an isocratic mobile phase and, therefore, has long retention times for highly lipophilic compounds. Valko et al.[16] developed a gradient IAM method that has higher throughput. Advantages of IAM are that the method requires very little material, and impurities do not interfere with the permeability prediction.

26.2.2 Cell Layer Method for Permeability

The first practical method for in vitro permeability assessment in discovery was the cell layer method. This assay models the epithelial cell layer permeability barrier that compounds encounter in the small intestine. Caco-2 is the best known cell line for this assay.[17–23] It is an immortal human colon carcinoma cell line that is readily available from the American Type Culture Collection (ATCC; Table 26.2). Desirable aspects of this cell line are its morphology

TABLE 26.2 ► Commercial Instruments and Products for the Measurement of Permeability

Method	Product Name	Company	Website
Caco-2	Transwell™	Corning	*www.corning.com/lifesciences/*
Caco-2	MultiScreen Caco-2™	Millipore	*www.millipore.com*
Caco-2	BioCoat™	BD Biosciences	*www.bdbiosciences.com*
Caco-2	BIOCOAT® HTS	BD Biosciences	*www.bdbiosciences.com*
Caco-2	Caco-2 Assay Kits	In Vitro Technologies	*www.invitrotech.com*
Caco-2	CAC-BD	Nichiryo	*www.nichiryo.co.jp/e/pdf/cac-bd.pdf*
Caco-2	EVOM, REMS TEER	World Precision Instr.	*www.wpiinc.com*
Caco-2	Biomek	Beckman Coulter	*www.beckman.com*
Caco-2	Caco-2 cells	ATCC	*www.atcc.org*
MDCK	MDCK cells	ATCC	*www.atcc.org*
MDCK	MDR1-MDCKII cells	Netherlands Cancer Ins.	*p.borst@nki.nl*
PAMPA	PAMPA Evolution™	pION Inc.	*www.pion-inc.com*
PAMPA	PAMPA Explorer™	pION Inc.	*www.pion-inc.com*
PAMPA	Gut-Box™	pION Inc.	*www.pion-inc.com*
PAMPA	AperW™	Analiza Inc.	*www.analiza.com*
PAMPA	MultiScreen PAMPA™	Millipore	*www.millipore.com*
IAM	IAM Columns	Regis Technologies	*www.registech.com*

and multiple permeability mechanisms. Caco-2 develops microvilli on its apical surface that resemble the morphology of GI epithelial cells that line the intestinal villi. Caco-2 cells also express cell membrane transporters on the apical surface, such as P-glycoprotein (Pgp), breast cancer resistance protein (BCRP), and multidrug resistance protein 2 (MRP2). This provides the opportunity to investigate various permeability mechanisms.

A variation of the cell layer method uses the MDCK (Madin Darby Canine Kidney) cell line or other cell lines such as LLC or A1/2/4. MDCK has been actively used in drug discovery for passive diffusion permeability prediction.[22]

A diagram of the cell layer experiment is shown in Figure 26.1. Cells are plated in the insert of a device that is commonly called the *cell culture insert*. The cells settle onto a porous filter support. Over approximately 21 days, the cells grow to confluence and cover the surface of the support. It is preferable to form a cell monolayer, but a mix of monolayers and multilayers might form. It is important that there be no gaps, otherwise the test compound will rapidly pass unimpeded through the porous filter. With time, the cells develop the microvilli morphology on the apical (upper) surface. They also increasingly express transporter proteins. The cell maintenance for media changes over this 21-day period makes Caco-2 a relatively expensive cell assay. A shorter 5-day culture technique[24] is commercially available (Table 26.2), but care must be taken to ensure full transporter functionality. By contrast, MDCK cells grow to confluence in 3 to 4 days and are ready

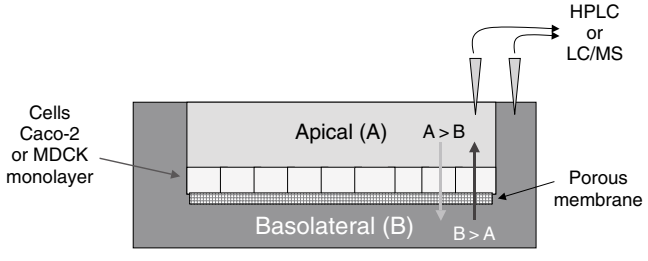

Figure 26.1 ► Cell layer permeability cell culture insert experiment setup.

for a permeability experiment. During the permeability experiment, the growth medium is replaced with buffered saline that contains glucose and the test compound.

Several different permeability experiments can be performed with this technique. The simplest experiment is to place the test compound in the buffer on the apical (A) side of the cell layer. Buffer with no test compound is placed on the basolateral (B) side. The test compound diffuses from the apical compartment through the cells and into the basolateral compartment. Aliquots from the two compartments are removed at specific time points over a 1- to 2-hour period. The concentration in each compartment is measured using HPLC or liquid chromatography/mass spectrometry (LC/MS) techniques, and the rate of permeation is calculated. This apical to basolateral (A > B) experiment provides a value for permeability in the absorptive direction, which models absorption in the GI. This permeability value (P) has been correlated by several research groups to absorption in the small intestine or to fraction of dose absorbed (FA). When such a correlation is established, it can assist medicinal chemists with predicting in vivo absorption of their compounds.

It is important to remember that Caco-2 data are not likely to completely agree between laboratories, even within the same company. This is due to differences caused by divergence of the characteristics of the cell lines, culturing conditions (e.g., serum source, frequency of media changes), and practice (e.g., experimental apparatus, percent dimethylsulfoxide [DMSO], media components). Thus, although trends are likely to agree, the actual permeability values can vary among laboratories.

Another experiment that can be performed is to place the test compound in the buffer on the basolateral side of the cell layer and buffer without compound on the apical side. This experiment is performed to study the permeability of test compounds by cell membrane transporters. If the permeability is the same in the A > B direction ($P_{A>B}$) and the B > A direction ($P_{B>A}$), then the compound primarily permeates by passive diffusion permeation. If the rates are significantly different, then a transporter may be involved. These experiments are discussed in greater detail in Section 27.2.1.

The cell layer permeability assay has been run under various conditions. Some of the important variables to consider are test compound concentration, pH of the aqueous buffers in the apical and basolateral compartments, use of materials that sequester the test compound in the basolateral compartment (sometimes called *sink compounds*), and use of solubilizers in the basolateral compartment. These are diagrammed in Figure 26.2.

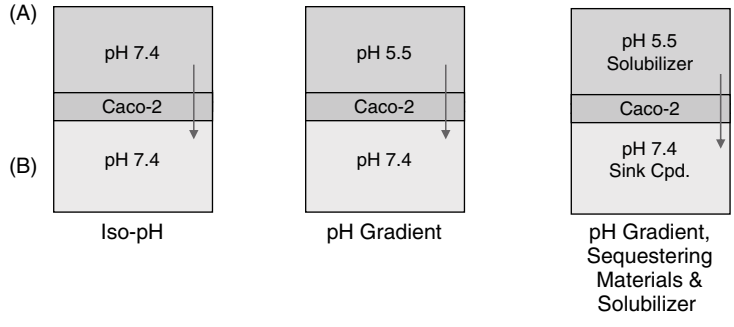

Figure 26.2 ► The cell layer assay has been run under various conditions, including the pH of the aqueous buffer, solubilizers, and sequestering materials. Shown are some of the variations in conditions in the apical **(A)** and basolateral **(B)** compartments.

Test compound concentration selection can affect the results. Many laboratories have used the concentration range of 5 to 10 μM. Alternatively, it has been argued that the concentration of compound in the GI lumen following an oral dose is closer to 50 to 100 μM, and some

laboratories use this concentration range. At this concentration range, transporters are likely to be saturated. Thus, there may be differences in the measured permeability between low and high concentrations. If the test compound is an efflux transport substrate, the ratio of permeability by transporters relative to passive diffusion is lower as the concentration is increased, and transporters are saturated. Therefore, it is important to note the concentration of the experiment. Higher concentrations do not properly model other permeability barriers (e.g., blood–brain barrier [BBB]), where the concentration is much lower ($\sim$1 μM level) and the effects of transporters are much greater (e.g., Pgp efflux at the BBB).

It is important in cell layer assays to first test for the transepithelial electrical resistance (TEER). The TEER value indicates if the cell layer is forming a continuous network of tight junctions. Devices for measuring the TEER of individual wells in manual or automated format are commercially available (Table 26.2). If the TEER value is low, there will be much higher levels of paracellular permeation. Lucifer yellow can be added to each well to detect high paracellular permeability. Lucifer yellow is detected in the receiver well using a plate reader.

Two types of pH conditions are used in cell layer assays. The first is to use an acidic pH (e.g., pH 5–6.5) in the apical compartment and neutral pH in the basolateral compartment. This models the upper small intestine, where the intestinal lumen is at an acidic pH. The problem that occurs with using a pH gradient (as discussed in Section 8.1.1) is that passive diffusion of acids is enhanced in the direction of higher pH and of bases is enhanced in the direction of lower pH. Therefore, it may appear that acids are undergoing active uptake transport and bases are undergoing efflux transport. For data generated using a pH gradient, this effect should be kept in mind if the discovery project team is trying to elucidate the permeability mechanisms of a compound. For this reason, some laboratories use pH 7.4 in both chambers.

Another experimental variation is the use of solubilizers in the apical compartment. These help to solubilize low-solubility compounds during the experiment in order to produce a measurable and accurate value for the permeability. Materials used for this purpose include serum albumin. Sequestering materials can be used in the basolateral chamber. These compounds sequester the compound once it has passed through the permeability barrier and are meant to model the conditions of the intestine. Materials used for this purpose include bile salts. Such conditions are colloquially termed *sink conditions*. Alternatively, conditions could be chosen such that the concentration in the receiver compartment never exceeds 10% of the starting concentration.

Cell layer experiments are performed in several different cell culture insert plate formats. The number of wells varies from 12 to 96 per plate. The 96-well plates allow more compounds to be assayed per plate. The tradeoff is that as the number of wells per plate increase, the volume and surface area decrease, resulting in lower method sensitivity. If highly sensitive LC/MS/MS systems are available, the sensitivity issue may not be a problem, and higher-density plate formats may enable higher throughput. Most laboratories run the Caco-2 permeability assay in 24-well plates, although 96-well applications are becoming more common.

Caco-2 permeability values differ among laboratories; however, sometimes it is useful to have a benchmark for comparison. Here is one set of permeability ranges used for Caco-2:

- $P_{app} < 2 \times 10^{-6}$ cm/s Low permeability
- $2 \times 10^{-6} < P_{app} < 20 \times 10^{-6}$ cm/s Moderate permeability
- $P_{app} > 20 \times 10^{-6}$ cm/s High permeability

26.2.3 Artificial Membrane Permeability Assay

PAMPA was invented by Kansy et al.[25] It reduces the cost and increases the throughput of permeability assays. Instead of a barrier made of living cells, the PAMPA barrier is made of phospholipids (e.g., phosphatidyl choline, egg lecithin) solubilized in a long-chain hydrocarbon (e.g., dodecane). A diagram of the PAMPA permeability experiment is shown in Figure 26.3. Other groups have also reported variations of the PAMPA method.[24,26–39]

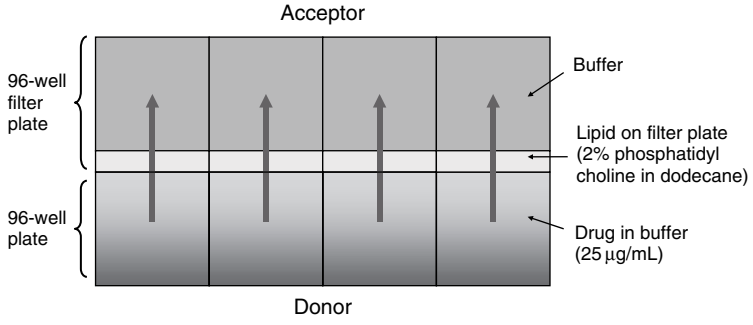

Figure 26.3 ► Parallel artificial membrane permeability assay (PAMPA) experiment.

The test compound is diluted in aqueous buffer ("donor solution") and is placed in the well of a 96-well plate. Each of these wells is termed a *donor*. A concentration of 25 μg/mL is commonly used. The liquid completely fills the wells. A 96-well filter plate, which has a porous filter on the bottom, is placed on top of this plate so that it is in contact with the aqueous buffer. A few microliters of a solution of the phospholipid is placed onto the top of the porous filter and soaks down into the holes of the filter to form the artificial barrier. Blank buffer is placed in the wells of the filter plate, on top of the artificial barrier. Each of these wells is termed an *acceptor*. This "sandwich" of 96-well plate and filter plate is maintained at a constant temperature and humidity for between 1 and 18 hours, depending on the laboratory's protocol and the permeability of the compounds. Samples are taken from the acceptor wells, the filter plate is removed, and samples are taken from the donor wells. The concentration of compound in the wells is quantitated using an LC/MS, LC/ultraviolet (UV), or a UV plate reader instrument. The unused "donor solution" that was not placed in the donor wells is used as a standard for quantitating the concentration of compound in the donor and acceptor wells, to calculate the permeability. The permeability often is termed *effective permeability* (P_e).

An advantage of PAMPA is that no cell culture maintenance is required. The artificial barrier is created at the time of the experiment. The capability of using the UV plate reader allows the method to be high throughput. The method measures only passive diffusion, but this provides a way to evaluate this important property independent of other permeability mechanisms.

PAMPA has been shown to correlate to human jejunal permeability with approximately the same reliability as Caco-2. An example of this correlation is shown in Figure 26.4.[28,40–42] Thus, if the project team wants to project far ahead to in vivo absorption, PAMPA provides a high-throughput approach with adequate predictability.

As with Caco-2, PAMPA has been run in many different ways in different laboratories. Therefore, it is not wise to compare data among laboratories unless the methods and quality control values are identical. Variations on PAMPA are similar to Caco-2. The pH of the buffers is sometimes run with the same pH on each side of the barrier and sometimes with

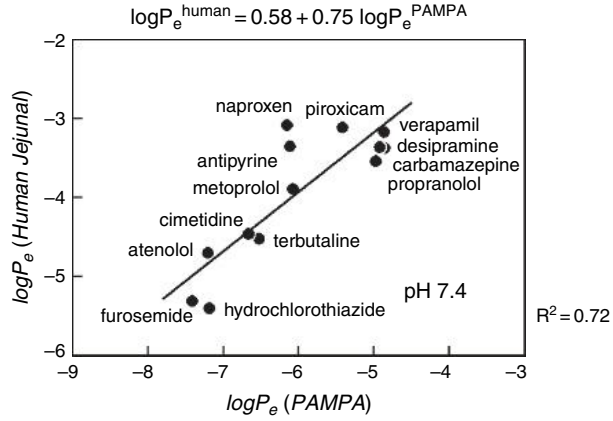

Figure 26.4 ► Prediction of human jejunal permeability using PAMPA.[28,40–42] Used with permission of A. Avdeef.

neutral pH on the acceptor side and lower pH on the donor side, to simulate the GI tract. The gradient pH condition has the same effect on acids. Compound flux (rate of flow through barrier) is increased in the direction of the higher pH. For bases, the flux is increased in the direction of the lower pH. Solubilizing components have been used on the donor side to enhance solubility, and sequestering materials have been used on the acceptor side to simulate conditions in the GI. The components in the artificial barrier also have been varied. Examples include 2% phosphatidylcholine in dodecane,[28] 20% egg lecithin in dodecane,[25] and hexadecane alone.[26] Stirring of the donor may be performed to reduce the gradient that forms due to diffusion rates and depletion at the barrier surface ("unstirred water layer"). Stirring can reduce the experiment time from 18 hours to 1 hour or less. Laboratories also have used various thicknesses of barriers by using different filter supports. All of these conditions will affect the data but allow modification of the experiment to model a specific set of conditions of interest to the project team.

26.2.4 Comparison of Caco-2 and PAMPA Methods

The widespread implementation of PAMPA in the pharmaceutical industry has prompted many questions regarding how the methods compare and which method produces "more reliable data." One way to compare the two methods is with relative characteristics, which reveal advantages and limitations.

Table 26.3 compares aspects of the two methods. PAMPA can be used over a wider pH range than Caco-2. This allows permeability at wider pHs to be studied. At the more

TABLE 26.3 ► Comparison of PAMPA and Caco-2 Permeability assays.

Comparison	PAMPA	Caco-2
Barrier	Phospholipids in Solvent	Cell Layer
pH	4–8	5.5–7.4
Mechanisms	Passive	Passive, Active, Efflux, Metabolism
Throughput	500/week	30/week
Cost	<$1/sample	~$30/sample
Manpower	0.35 FTE[1]	2 FTE[1]

*[1] FTE = full time equivalent (i.e., one scientist working full time)

extreme pH ranges, the pores between the cells in the Caco-2 cell layer increase in size and, thus, overpredict the paracellular mechanism. PAMPA correlates only with passive diffusion, whereas Caco-2 provides additional permeability mechanisms. The simplest Caco-2 permeability values ($P_{A>B}$) contain permeability components from each of these mechanisms. If the discovery project team is interested only in getting an estimate of permeability for absorption, then this may be sufficient. However, if a medicinal chemist wants to know the relative contributions of each mechanism, multiple Caco-2 experiments are necessary ($A > B$, $B > A$). PAMPA provides passive diffusion insights in only one experiment.

Throughput and cost are two clear advantages of PAMPA. In about one third to one half of a scientist's time and with one instrument, at least 500 compounds can be run by PAMPA in 1 week. Experimental supplies for PAMPA are less expensive than the cell culture materials used for Caco-2. For Caco-2, one to two scientists run 30 compounds in 1 week. This results in an approximately 30-fold lower throughput and 30-fold greater cost per analysis for Caco-2.

If data from Caco-2 and PAMPA on the same compounds are compared, the pattern in Figure 26.5 is obtained.[43] For compounds that are primarily permeable by passive diffusion, the data fall along the central correlation line. Compounds having strong Pgp efflux tend to plot in the section (lower right) of relatively higher PAMPA permeability than Caco-2 permeability. Compounds having strong paracellular or active uptake transport tend to plot in the section (upper left) of relatively higher Caco-2 than PAMPA permeability. This type of comparison can help to elucidate the predominating permeability mechanisms, if data are available from both methods.

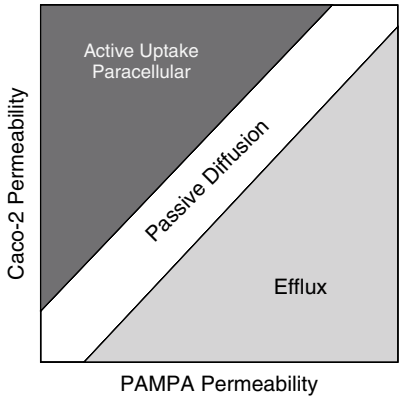

Figure 26.5 ► Comparison of Caco-2 and PAMPA permeability values from the same compound can provide insight on permeability mechanisms. (Reprinted with permission from [37].)

These comparisons suggest how Caco-2 and PAMPA can be used together synergistically (Figure 26.6). In early discovery, when large numbers of compounds are being considered by a project team, PAMPA is a rapid, low-cost, fast means for obtaining permeability insights. In mid-discovery, the two techniques can be used together to provide enhanced permeability mechanisms information. In late discovery, Caco-2 can be used for in-depth permeability mechanism studies.

26.3 In Depth Permeability Methods

The most detailed permeability studies are performed with living systems or organs from living systems. Investigators have cannulated the hepatic portal vein to measure the concentration of drug absorbed in the intestine, prior to first-pass liver metabolism. This provides a

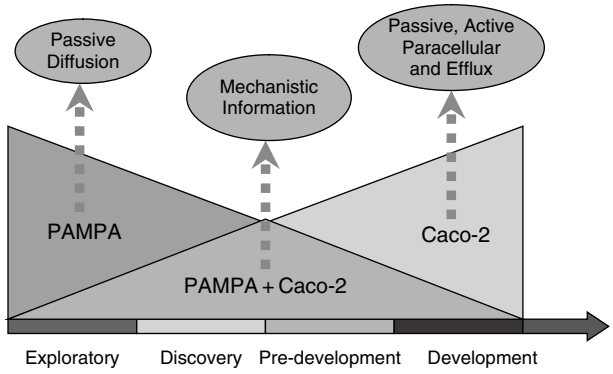

Figure 26.6 ► Strategy for combined use of Caco-2 and PAMPA in drug discovery. (Reprinted with permission from [37].)

concentration versus time profile of compound absorption by the intestine. Another in-depth approach, termed *in situ perfusion*, passes a drug solution through a section of the small intestine in a living animal and observes the rate of absorption.. This provides a profile of compound absorption in a specific portion of the intestine (e.g., jejunum). Such studies are performed rarely in discovery but are more common in development, when they provide data for biopharmatics classification system (BCS) studies that are submitted to the Food and Drug Administration (see Section 7.2.3.1).[44] A comparison of the throughput of these in depth methods and in vitro methods is provided in Table 26.4.

TABLE 26.4 ► Methods for the Determination of Permeability

Method	Type Assay	(min/cpd)	(cpd/day/instrument)
IAM	High throughput	10	120
PAMPA	High throughput	0.5	200
Cell layer-MDCK	Moderate throughput	40	12
Cell layer-Caco-2	Moderate throughput	80	6
In situ intestinal perfusion	Low throughput	250	2
Hepatic portal vein cannulation	Low throughput	250	2

Problems

(Answers can be found in Appendix I at the end of the book.)

1. For the following permeability methods, which permeation mechanisms contribute to the measured permeability value?

Method	Passive diffusion	Active Uptake	Efflux	Paracellular
IAM				
PAMPA				
Caco-2				

2. Why is IAM convenient for permeability estimation?

3. What factors add to the expense of Caco-2 compared to PAMPA?
4. What additional information can be obtained from Caco-2 compared to PAMPA?
5. How do IAM HPLC columns differ from other reversed-phase HPLC columns?
6. When the apical compartment in Caco-2 (donor in PAMPA) is more acidic than the basolateral compartment (acceptor in PAMPA), what artificial effects can occur for basic and acidic test compounds?
7. Compare Caco-2 and PAMPA permeability data for the following compounds:

Compound's el Permeability Mechanism(s)	PAMPA relatively higher than Caco-2	Caco-2 relatively higher than PAMPA	PAMPA and Caco-2 relatively the same
Passive diffusion only			
Passive diffusion, active uptake			
Passive diffusion, efflux			
Passive diffusion, paracellular			

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Transporter Methods

Overview

- *In silico predictions are increasingly available for transporters.*
- *In vitro cell layer assays use inhibitors and cell lines with transfected transporters.*
- *High-throughput ATPase and calcein acetoxymethyl ester assays are used, but cell layer transport is most relevant.*
- *Transporters are knocked out genetically or chemically in vivo for in-depth study.*

Transporters are an emerging area in drug discovery. The extent of their impact on absorption, distribution, metabolism, excretion, and toxicity (ADME/Tox) is still being investigated. Most pharmaceutical companies have heeded the evidence that P-glycoprotein (Pgp) causes significant drug resistance and ADME/Tox effects. In response, methods have been implemented, and synthetic strategies that counteract Pgp efflux have gained wider understanding and application. The intensity of the effects of other transporters and their relative impact on the success of discovery projects in finding a quality clinical candidate remain under investigation. As with all properties, resources for transporters must be carefully allocated. If transporters are found to have minimal effects, in general, assaying all compounds for several transporters is unwise. Currently, assays for transporters are generally implemented to study selected compounds and diagnose poor or nonlinear pharmacokinetic performance or an unexplained observation. Various in vitro and in vivo assays for efflux transporters have been implemented.[1]

27.1 In Silico Transporter Methods

Initial approaches for developing in silico tools for Pgp transporter substrates have been reported. Crivori et al.[2] developed a model using VolSurf descriptors and partial least squares discriminant (PLSD) analysis. The model had 72% predictability for classifying Pgp substrates and inhibitors (not quantitative prediction of efflux ratio). Pharmacophore structural descriptors for substrates were determined using GRIND software.

Characterization of transporters currently is based on a multidisciplinary approach combining insights from chemistry, function, quantitative structure–activity relationships (QSAR), homology modeling, comparative modeling, and structural studies.[3,4] Comparative modeling using a low-resolution x-ray crystal structure of *Escherichia coli* lipid A transporter (MsbA) provided a model for Pgp, but this has been criticized.[5–7] If high-quality Pgp structural data become available, they will be very useful, however, at this time a multidisciplinary approach currently appears to be the strategy by which improved in silico predictions of transporter substrates will be achievable in the near term. These models will guide structural modifications to reduce efflux, such that it reduces drug exposure to the

target, and enhance transport, when it is desirable to use transporters to enhance absorption, improve distribution, or reduce elimination.

Commercial software for transporters is limited to the classification of Pgp substrates (Table 27.1).

TABLE 27.1 ► Commercial Software for Transporters

Name	Company	Web site
ADME Boxes (Pgp)	Pharma Algorithms	*www.ap-algorithms.com*

27.2 In Vitro Transporter Methods

Several in vitro methods are available for assessing the susceptibility of a compound to transport. The indispensable method component is the presence of the transporter in a living cell or membrane system. Transporters have been expressed in various formats: cell lines transfected with a specific transporter gene, isolated primary cell cultures, immortalized cell cultures, microinjected oocytes, isolated membranes, and inverted vesicles. Each of these formats has its particular characteristics and applications. Commercial products for transporter studies are listed in Table 27.2.

TABLE 27.2 ► Commercial Suppliers of Products for Transporter Assays

Product name	Company	Web site
Transwell	Corning	*www.corning.com/lifesciences/*
MultiScreen Caco-2	Millipore	*www.millipore.com*
BioCoat	BD Biosciences	*www.bdbiosciences.com*
Caco-2 Assay Kits	In Vitro Technologies	*www.invitrotech.com*
EVOM, REMS TEER	World Precision Instr.	*www.wpiinc.com*
Biomek	Beckman Coulter	*www.beckman.com*
Transporter Vesicles	Solvo Biotechnology	*www.solvo.hu*
MultiDrugQuant Kits	Solvo Biotechnology	*www.solvo.hu*
PREDEASY ATPase Assay Kits	Solvo Biotechnology	*www.solvo.hu*
Transportocytes	BD Gentest	*www.gentest.com*
Human Vesicles	BD Gentest	*www.gentest.com*
Human Membranes	BD Gentest	*www.gentest.com*
Calcein AM Fluorescent Dye	BD Gentest	*www.gentest.com*
Caco-2 and MDCK Cell Lines	ATCC	*www.atcc.org*
Mdr1a/b Transgenic Mice	Taconic Laboratories	*www.taconic.com*

27.2.1 Cell Layer Permeability Methods for Transporters

The cell layer experiment for transporters is performed in the same manner as shown in Figure 26.1 and in 27.1 for Caco-2. In addition to the A>B experiment, a B>A experiment is performed for transporter permeability studies. This experiment is performed by placing buffer, which contains the test compound, in the basolateral compartment of the transwell apparatus and buffer without test compound in the apical compartment. The test compound passes through the porous membrane to reach the basolateral cell membrane and then permeates through the cells to reach the apical compartment. This basolateral to apical (B>A) experiment provides a value for permeation in the "secretory" direction. If the

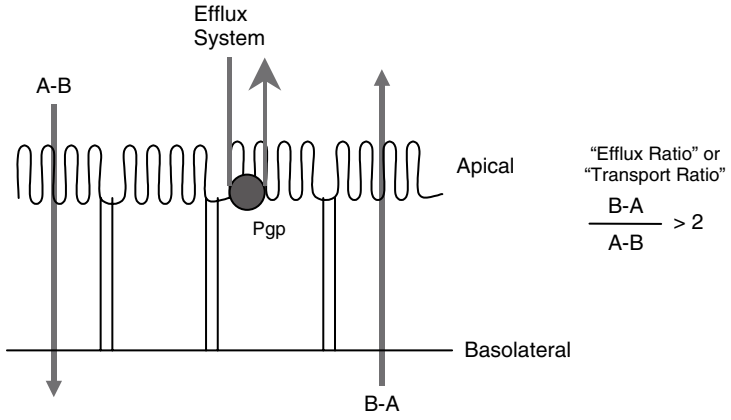

Figure 27.1 ► Cell layer efflux assays measure the permeability both the apical to basolateral (A-B) and basolateral to apical (B-A) directions and calculate an efflux or transport ratio. A ratio >2 indicates efflux.

compound permeates only by passive diffusion or paracellular permeation, then the $P_{A>B}$ and $P_{B>A}$ permeability values are approximately the same. However, if the compound is transported actively, then the values will differ. If $P_{A>B}$ is greater than $P_{B>A}$ and the *uptake ratio* ($P_{A>B}/P_{B>A}$) is ≥ 2, the compound likely will be actively transported for uptake. If $P_{B>A}$ is greater than $P_{A>B}$ and the *efflux ratio* ($P_{B>A}/P_{A>B}$) is ≥ 2, the compound likely will be actively transported for efflux. The actual permeability values from cell layer permeability experiments are known to vary among laboratories. Therefore, each laboratory should establish the values of these ratios that indicate convincing evidence for uptake or efflux transport. This validation is performed by assaying known transporter substrates and evaluating the resulting ratio values.

A complementary approach for assaying or confirming that a test compound is a transporter substrate is the use of an inhibitor. Certain compounds have been recognized as specific inhibitors for transporters (e.g., cyclosporin A [CsA] for Pgp). If the transport ratio (uptake or efflux) changes when the inhibitor is coincubated with the test compound, the test compound likely is a substrate for the transporter.

27.2.1.1 Caco-2 Permeability Method for Transporters

The Caco-2 method for permeability is discussed in Chapter 26. The most common application of Caco-2 is to estimate intestinal absorption. In this application, the A>B permeability has contributions from passive diffusion, paracellular permeation, and active transport. Caco-2 also is used to study some transporters because it expresses several transporters of pharmaceutical interest.

When Caco-2 is used for transporter studies, two points are important to understand and control. First, expression levels of transporters can vary. Caco-2 cells are not genetically identical, and, over time, the relative populations of different cell strains can vary. Therefore, the expression levels of different transporters can vary. In most companies, it is common to run quality control (QC) compounds with Caco-2 assays to monitor for Pgp efflux (e.g., digoxin), paracellular permeation (e.g., atenolol), and passive transcellular permeation (e.g., propranolol). However, it is uncommon to measure the permeability of control compounds for other transporters because the process is time consuming. If a specific transporter is being studied using Caco-2, one or more specific transporter QC compounds should be run to verify the expression level and activity of the transporter of interest.

A second point with Caco-2 use is that a test compound may be a substrate for more than one transporter. The multiple transporter expression of Caco-2 could confuse the results unless good control is built into the experiment with specific inhibitors.

Caco-2 has been reported to express several transporters of interest in drug discovery. These include Pgp (MDR1), breast cancer resistance protein (BCRP; ABCG2), PepT1, PepT2, multidrug resistance protein 2 (MRP2), and others. Culture conditions can affect the expression level of transporters. The functional activity of the transporters should be verified before a cell line is used for screening.

27.2.1.2 Transfected Cell Line Permeability Method for Transporters

Transfected cell lines are more commonly used for cell layer transporter assays. MDCK (a canine kidney line), LLC-PK1 (a porcine kidney epithelial line), 2008 (human ovarian carcinoma), and HEK 293 (human embryonic kidney line) cells have been transfected with transporter genes to produce stable immortal cell lines that express individual transporters. These cell lines have advantages over Caco-2 for the study of specific transporters:

- These cell lines naturally express only low levels of membrane transporters, so background signal is low.
- Transporter expression levels are high, so high signal-to-noise ratios are obtained. Larger transport ratios are produced. The assay can differentiate among project compounds for rank ordering.
- They require fewer resources than Caco-2 to maintain. For example, MDCK cells are ready for use in 3 days after plating, whereas Caco-2 requires 21 days of culture maintenance prior to use.

Various transfected cell lines have been prepared and discussed in the literature; examples are listed in Table 27.3.

TABLE 27.3 ► Various Cell Lines with Transfected Transporters Used in Cell Layer Permeability Assays

Transporter	Transporter species	Parental cell line	Cell line developer[a]	Reference
PepT1	Human	MDCK	Rutgers/BMS	[9,24]
BCRP	Human	MDCKII	Pfizer	[25]
OAT-K1	Rat	LLC-PK1	Kyoto	[26,27]
MDR1	Human	MDCKII	NCI	[28]
MDR1	Human	LLC-PK_1	NCI, Takeda	[29]
MDR1	Monkey	LLC-PK_1	Takeda	[29]
MDR1	Canine	LLC-PK_1	Takeda	[29]
mdr1a	Mouse	LLC-PK_1	NCI, Takeda	[29]
mdr1b	Mouse	LLC-PK_1	NCI, Takeda	[29]
MDR1a	Rat	LLC-PK_1	Takeda	[29]
MDR1b	Rat	LLC-PK_1	Takeda	[29]
MRP1	Human	MDCKII	NCI	[30]
MRP2	Human	MDCKII	NCI	[30]

[a] Rutgers University (Rutgers), Bristol-Myers Squibb Co. (BMS), Netherlands Cancer Institute (NCI), Takeda Pharmaceutical Company and Kanazawa University, Kyoto University Hospital.

Professor Dr. Piet Borst from Netherlands Cancer Institute has kindly shared transfected cell lines with researchers (Table 27.4). These cell lines have been provided without charge to academic laboratories and for a licensing fee to companies. Contact information: Professor Dr. P. Borst, Division of Molecular Biology, Netherlands Cancer Institute, Telephone: +31-20-512 2087, Fax: +31-20-669 1383, e-mail: p.borst@nki.nl.

TABLE 27.4 ► Cell Lines from the Netherlands Cancer Institute

Transfected cell line	Inserted transporter gene
2008 Parental	
2008 MRP1	Human MRP1 cDNA
2008 MRP2	Human MRP2 cDNA
2008 MRP3	Human MRP3 cDNA
HEK 293	
HEK 293 MRP5	Human MRP5 cDNA
LLC Parental	
LLC MRP1	Human MRP1 cDNA
LLC MDR1	Human MDR1 cDNA
LLC MDR3	Human MDR3 cDNA
LLC Mdr1a	Mouse Mdr1a cDNA
LLC Mdr1b	Mouse Mdr1b cDNA
LLC Bcrp1	Mouse Bcrp1 cDNA
MDCKII Parental	
MDCKII MDR1	Human MDR1 cDNA
MDCKII BCRP	Human BCRP cDNA
MDCKII Bcrp1	Mouse Bcrp1 cDNA
MDCKII MRP1	Human MRP1 cDNA
MDCKII MRP2	Human MRP2 cDNA
MDCKII MRP3	Human MRP3 cDNA
MDCKII MRP5	Human MRP5 cDNA

Such cell lines allow the development of an assay that is predictive of the effect of a specific transporter on test compounds. An example is a specific Pgp efflux assay using MDR1–MDCKII (Madin Darby Canine Kidney cell line II transfected with human MDR1 gene, which codes for Pgp protein product). In such an assay, the efflux ratio often is higher than that in Caco-2 for Pgp efflux substrates. Pgp is a major concern of drug discovery because it reduces GI absorption, reduces blood–brain barrier (BBB) penetration, and is responsible for drug resistance in cancer cells. LLC, 2008, and HEK cells also have been transfected with transporter genes for specific transporter assays.

A typical MDR1–MDCKII transport assay with cell layer is performed as follows. Cells are seeded in a transwell plate (3×10^6 cells/cm^2) and cultured for 3 days. The transepithelial electrical resistance (TEER) values are checked (e.g., >200 ohm/cm^2) to ensure minimal paracellular permeation. A paracellular marker, such as atenolol, is included in the assay to check layer integrity. The growth medium is removed and replaced with 37°C reduced-serum medium (to minimize protein binding) containing the test compound (5–20 μM). The transwell plates are maintained at 37°C, with gentle shaking. Small volumes from the apical

and basolateral chambers are withdrawn at specific time points (e.g., 30, 60, 120 minutes) and quantitated using liquid chromatography/mass spectrometry (LC/MS) techniques.

27.2.2 Uptake Method for Transporters

The uptake assay is an alternative to the cell layer transport assay. Uptake assays measure the rate of test compound concentration increase inside cells that are living in standard solid-bottom culture plates, as opposed to the rate of test compound concentration increase in the basolateral chamber as a result of layer permeation. At an experiment time point, the medium is completely removed from a well, the cells are gently washed, and they are lysed using a detergent (e.g., TX-100, sodium dodecyl sulfate (SDS)) or organic solvent, combined with shaking or sonication. The concentration of test compound released into the lysate is measured, and the concentration in the cells is determined based on the total cell volume. The uptake experiment is a convenient format for higher-throughput assays because it does not require the complexity and high cost of the transwell system. The uptake experiment is a necessity when the cells do not form a confluent cell layer with tight junctions. Under these conditions, the test compound molecules in a transwell device would leak through by paracellular permeation. Uptake assays have been applied for Pgp efflux,[8] PepT1,[9] and BBB transporters.[10] A drawback of the uptake assay is that it does not guarantee the transcellular permeation of a test compound across the entire cell, as is necessary for absorption. Nonspecific binding to cell surface, rather than uptake, can complicate data interpretation.

Uptake assays have also proven beneficial for in vitro studies, in which the observed functional in vitro activity is correlated to the intracellular test compound concentration. This correlation is valuable to discovery project teams.

27.2.3 Oocyte Uptake Method for Transporters

BD Gentest has expressed several transporters in *Xenopus* oocytes (Table 27.2). The mRNA of a transporter is microinjected into each oocyte using a transcription vector.[11] Oocytes are useful because they are large, they are easy to handle, and they express in high abundance the transporters on the oocyte membrane. Oocytes are good for up to 1 week after injection. The assay is conducted by placing oocytes in suspension in the well of a plate and adding medium containing the test compound (Figure 27.2). Incubations occur for 30 to 120 minutes. At a specific time point, the oocytes are washed with ice-cold transport buffer and lysed with 10% SDS. The cell contents are assayed for the concentration of test compound, typically using LC/MS or scintillation counting techniques. Water-injected or uninjected

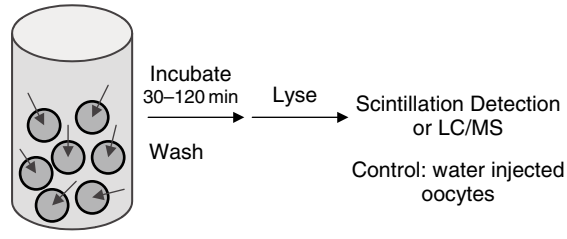

Figure 27.2 ► Schematic of the *Xenopus* oocyte transporter assay.

oocytes are used as controls. The extent and kinetics of uptake are calculated. Gentest Transportocytes are available in multiple species for OAT1, OAT2, OAT3, PEPT1, PEPT2, OATP1, OATP2, OATP4, OATP8, OATP1B3, OCT1, and NTCP. Oocyte transporter studies are not considered high throughput, but they could be beneficial for the study of selected compounds.

27.2.4 Inverted Vesicle Assay for Transporters

Transporter genes have been cloned into insect cells (*Spodoptera frugiperda*). From this expression system, vesicles are produced that contain the transporter in their membranes. With special treatment, the vesicle is inverted so that the normally extracellular face of the transporter is inside the vesicle. If the transporter is Pgp and the inverted vesicles are placed in a solution containing a Pgp efflux substrate, the compound will be taken up into the vesicle. At a specific time point, the vesicles are separated from the solution by filtration, washed, and lysed to release the compound. Quantitation uses scintillation counting or LC/MS. Solvo is a vendor of inverted vesicles (Table 27.2).

TABLE 27.5 ► Comparison of Three Assays Commonly Used In Drug Discovery for Pgp Effux[12]

	Pgp Assay		
Comparison	Cell Layer Efflux	ATPase	Calcein-AM
Activity Indicated for Test Compound	Pgp Efflux	ATPase Activation	Pgp Inhibition
Test Compound Diagnosis	Definitely a Pgp Efflux Substrate	May be a Substrate, Inhibitor or ATPase Activator	May be a Substrate or Inhibitor
Materials for Pgp Assay	MDR1-MDCKII Cell Line	MDR1-MDCKII Cell Line or Membrane Vesicles	MDR1 Membrane Vesicles from Sf9
Instrumentation Required	LC-MS-MS	UV Absorption Plate Reader	Fluorescence Plate Reader
Compounds Tested Per Week	20	200	200

27.2.5 ATPase Assay for ATP Binding Cassette Transporters

ATP binding cassette (ABC) transporters (e.g., Pgp) bind and hydrolyze ATP molecules as part of the transport process. ATP hydrolysis demonstrates that the test compound affects the ATPase activity of Pgp. The ATP hydrolysis reaction is shown in Figure 27.3. An ATPase assay measures ATP hydrolysis. The transporter material can be purchased from vendors in a convenient membrane-bound form that is produced from transduced insect cells. The inorganic phosphate released from ATP hydrolysis reacts with ammonium molybdate in the reaction solution to produce an intense color that is detected using an UV/Vis plate reader. Increasing color intensity is correlated with increasing ATPase activity. The

concentration at half-maximal ATPase activation is frequently used to estimate the test compound binding affinity to the ABC transporter. The assay is readily automated for high throughput analysis. One drawback is that the assay only demonstrates that the test compound affects the ATPase activity of Pgp, not that the compound actually is effluxed by Pgp. This has been demonstrated by Polli et al.[12] and is summarized in Table 27.5.

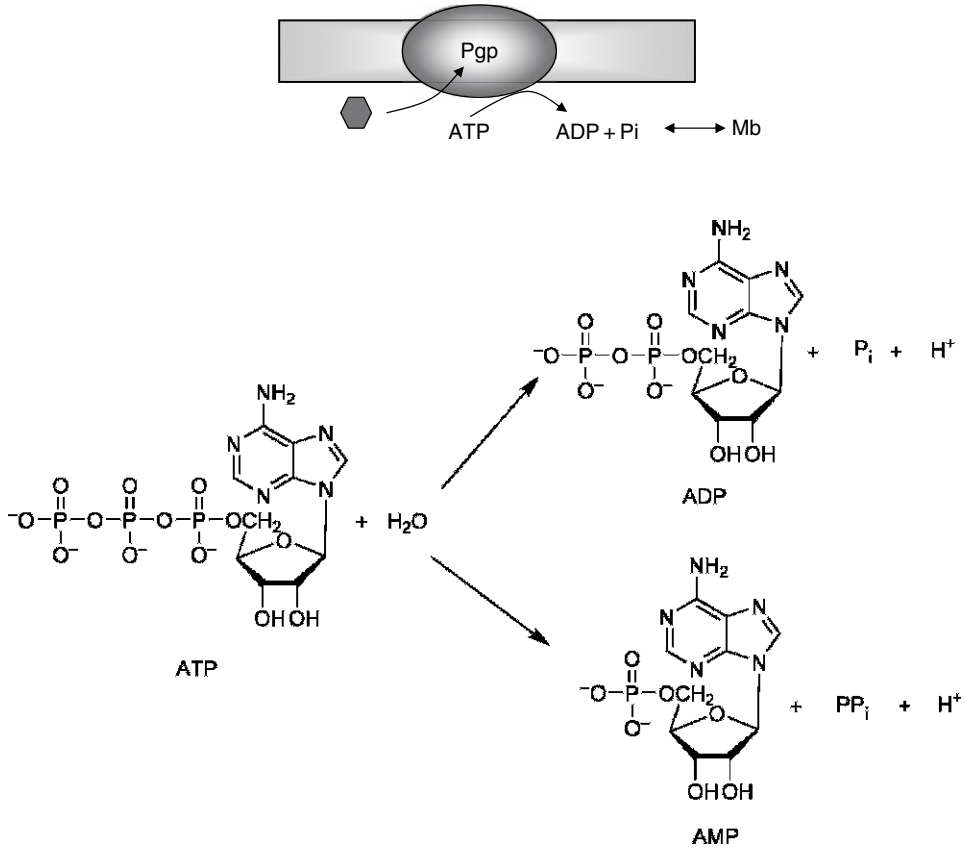

Figure 27.3 ► ATPase assay schematic. cDNA-expressed ABC transporter (e.g., Pgp) is provided on a membrane. Test compound, ATP, and ammonium molybdate are added. Binding a molecule of test compound to the ABC transporter results in ATP hydrolysis. The inorganic phosphate (Pi) reacts with molybdenum to produce a visible color.

27.2.6 Calcein AM Assay for Pgp Inhibitor

Calcein acetoxymethyl ester (calcein-AM; Figure 27.4) is a Pgp substrate, which limits its ability to enter the cells. Calcein-AM is rapidly hydrolyzed inside the cells to form calcein. Calcein is fluorescent and is readily detected using a fluorescence plate reader. If a coincubated test compound is a Pgp transport inhibitor, the test compound will inhibit Pgp efflux, and more calcein-AM will be able to enter the cells. This results in an increased intracellular concentration of calcein-AM, which subsequently is hydrolyzed to produce calcein and leads to increased fluorescence. Thus, a test compound that is a Pgp transport inhibitor causes a higher level of fluorescence than controls. A drawback of the method is that it determines inhibitors of Pgp transport, which may or may not be substrates of Pgp (see Table 27.5).

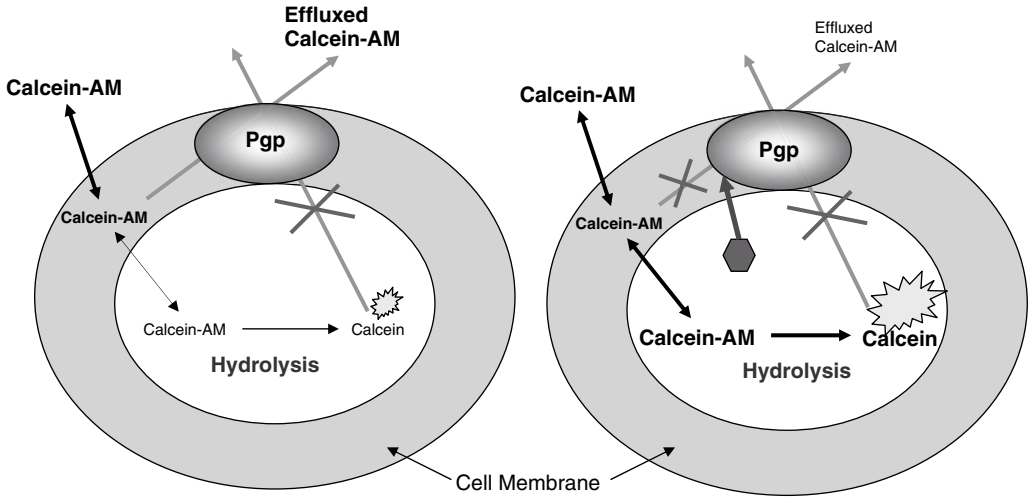

Figure 27.4 ► Calcein-AM fluorescence assay for Pgp efflux. Calcein-AM is a Pgp substrate and is effluxed from the cell (**left**), forming minimal fluorescent Calcein. In the presence of a Pgp inhibitor (hexagon), the efflux of calcein-AM is reduced, and more fluorescent calcein is produced (**right**).

27.3 In Vivo Methods for Transporters

It is valuable to follow up *in vitro* studies of key compounds with further study *in vivo*. Such studies confirm *in vitro* observations and provide greater confidence and understanding of the effect of the transporter in a dynamic living system. Two types of *in vivo* transporter experiments are commonly performed: genetic knockout and chemical knockout. Comparison of the compound performance in wild type animals to knockout animals provides strong evidence of the effect of a transporter and its extent.

27.3.1 Genetic Knockout Animal Experiments for Transporters

Mice strains have been developed in which the mdr1a, mdr1b, and mrp1 genes are knocked out, individually or in combinations.[13,14] These animals are commercially available. Examples of the use of this approach are as follows:

- For a neuroscience discovery project: Does the compound have a much higher BBB permeability in the knockout versus the wild-type?[15–18]
- For a compound that is poorly absorbed after oral dosing and has good solubility, passive diffusion permeability, and metabolic stability, does it have higher absorption in the knockout versus the wild-type?[19,20]

27.3.2 Chemical Knockout Experiments for Transporters

Coadministering or preadministering an inhibitor that is specific for the transporter can knock out the transporter. The inhibitor will specifically reduce the transporter activity. A difference in compound performance, pharmacokinetically or pharmacologically, with

inhibitor coadministration versus no inhibitor is evidence that the transporter is having an effect on the compound's in vivo ADME/Tox properties.[16,21,22]

A variation of this experiment is to saturate the transporters with the test compound. If increasing permeability or absorption is observed with increasing doses, an efflux transporter likely is being saturated.[23]

Chemical knockout experiments can be performed with the animal efficacy/pharmacology model. The results will be better correlated to observations in regular in vivo biology experiments.

Problems

(Answers can be found in Appendix I at the end of the book.)

1. Define efflux ratio. What does it indicate?
2. What other variations on the cell monolayer assay can be used to detect or confirm efflux?
3. When is an uptake method useful, compared to a transport assay using transwell devices?
4. What does the ATPase assay measure?
5. What does the calcein-AM assay measure?
6. What are in vivo Pgp assays useful for?
7. How do the genetic and chemical knockout experiments differ?

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Blood–Brain Barrier Methods

Overview

- *Several in silico predictions for blood–brain barrier (BBB) have appeared as a result of the importance of brain penetration for drug research on neurological disorders.*
- *Various in vitro BBB methods are used: PAMPA-BBB, Log P, ΔLog P, brain-plasma dialysis, IAM HPLC, surface activity, and cell layer permeability (MDCK, Caco-2, BMEC).*
- *Brain penetration is studied in vivo using in situ perfusion, brain uptake, brain to plasma ratio (B/P), CSF sampling, and microdialysis.*
- *It is necessary to differentiate BBB permeation, brain/plasma partitioning, and free drug.*

Brain penetration is a major barrier for some compound series that otherwise would have efficacy for diseases of the brain. As discussed in Chapter 10, brain penetration is limited by (1) blood–brain barrier (BBB) permeation and (2) brain distribution. Each of these is determined by multiple underlying mechanisms. Understanding the difference between BBB permeation and brain distribution can lead to more effective data interpretation and successful planning for structure modification to enhanced brain penetration.

Methods for BBB permeation include the following:

- In silico models
- In vivo brain perfusion or uptake
- Passive diffusion
- P-glycoprotein (Pgp) efflux
- Uptake transport
- Physicochemical properties that are components of passive diffusion

BBB permeation is the velocity of compound flux from blood into brain tissue through the BBB endothelium. This velocity is determined by passive diffusion, efflux transport, and uptake transport. In vivo methods (e.g., in situ perfusion, brain uptake) accurately determine total BBB permeability or the impact of a transporter on total permeability (e.g., Pgp knockout mice). However, they are expensive. In vitro methods are useful for screening larger numbers of compounds and obtaining data on specific underlying mechanisms. In vitro methods for passive diffusion (e.g., parallel artificial membrane permeability assay) [PAMPA]-BBB, MDCK) and underlying structural properties that affect it (e.g., polar surface area [PSA], molecular weight [MW], Log P, H-bonding) assist the optimization of passive BBB permeability. Pgp efflux potential can be assessed using in vitro Pgp methods

(e.g., MDR1-MDCKII, Caco-2). Screens expressing uptake transporters have been suggested as a means to support the enhancement of BBB permeation but have not been widely implemented.[1] Uptake transporter affinity usually is discovered by serendipity. Methods for uptake transporters are discussed in Chapter 27.

Methods for brain distribution include the following:

- In silico models
- In vivo B/P or Log BB
- Metabolic stability
- Plasma protein binding
- Equilibrium dialysis

In vivo pharmacokinetic (PK) methods for distribution of compound between plasma and brain (e.g., brain to plasma ratio [B/P]) are widely used. Chapter 10 discusses how the improvement of metabolic stability increases brain penetration. Methods for metabolic stability are discussed in Chapter 11.

Most companies performing neuroscience drug discovery research use a combination of methods to better determine the brain penetration characteristics of their leads. Combination of data from in vivo and in vitro methods can reveal and confirm predominant BBB mechanisms for a compound. General reviews of BBB methods are available.[2,3]

28.1 In Silico Methods for BBB

Considerable work has been invested in the development of in silico tools for predicting brain penetration.[4] As discussed in Chapter 10, brain penetration has many variables (e.g., Pgp efflux, passive diffusion, uptake, plasma protein binding, brain tissue binding, metabolic clearance), thus providing a complex challenge for in silico models.

28.1.1 Classification Models

Some in silico models seek simply to classify compounds as CNS+ (penetrates into the brain) or CNS- (does not appreciably penetrate into brain). Such methods have been reviewed.[5,6] At least five different computational classification methods have been used.[7–11] The classification of compounds in the training sets as CNS+ and CNS- is based on whether a compound showed in vivo CNS efficacy or had a B/P value above a certain level (e.g., B/P $\geq$ 1). Thus, the criteria are not quantitative. The average accuracy of the in silico models for validation set compounds for CNS+ classification was 90% and for CNS- classification was 74%. Such models are useful for an initial overview of structures, such as hits from high-throughput screening, compound library planning or assessment, and planning compound synthesis.

28.1.2 Quantitative Structure–Activity Relationship Methods

Quantitative structure–activity relationship (QSAR) models typically use brain distribution B/P or Log BB values for brain penetration with linear regression, partial least squares, and molecular descriptor mathematical methods.[11–17] The models developed using these

approaches are consistent with experimental evidence from in vivo measurements of the importance of structural physicochemical properties in determining brain penetration, including hydrogen bonding (or PSA), lipophilicity, molecular size/weight, and basicity/acidity. Results for six models have been summarized as having an average predictive performance of within 0.4 log unit (2.5-fold in B/P).[5,6]

Newer QSAR models[6] have used BBB permeability–surface area coefficient (PS, permeability · surface area) data.[12,13] These models also have major descriptors for lipophilicity and hydrogen bonding. One opportunity for models based on passive diffusion is to compare the predictions with measurements to obtain an initial indication if the compound is primarily permeable by passive diffusion (good agreement of measurement with model), highly affected by efflux transport (measurement is much lower than model), or affected by uptake transport (measurement is much higher than model).

Other in silico models for brain distribution use MolSurf[14] and free energy calculations.[15] A model for BBB permeation used VSMP systematic variable selection.[16] The physiologically based pharmacokinetic modeling approach was used to model BBB permeability as the time to reach brain equilibrium.[17]

28.1.3 Commercial Software

Commercial software for predicting BBB penetration is available. Examples are listed in Table 28.1. Software is useful for an initial prediction of brain penetration potential. When evaluating any software for purchase, it is important to test its predictions for compounds that have been studied in-house. This gives a realistic demonstration of the accuracy of the software model on compounds that are well known to the company. The training set for the software may differ greatly from the compound series being studied in a discovery project,

TABLE 28.1 ► Partial List of Commercial Software and Products for Predicting BBB Penetration

Software name	Company	Web site
Software		
ADMET Predictor	Simulations Plus	*www.simulationsplus.com*
Volsurf	Molecular Discovery	*www.moldiscovery.com*
Volsurf	Tripos	*www.tripos.com*
MedChem Explorer	Accelrys	*www.accelrys.com*
KnowItAll	Bio-Rad	*www.biorad.com*
ADME Boxes™ (Pgp)	Pharma Algorithms	*www.ap-algorithms.com*
Absolv	Pharma Algorithms	*www.ap-algorithms.com/absolv.htm*
QikProp	Schrodinger	*www.schrodinger.com*
CSBBB	ChemSilico	*www.chemsilico.com*
PreADME	Bioinformatics and Molecular Design Research Center	*www.bmdrc.org/02_R&D/04_chem.asp*
Admensa	Inpharmatica Ltd.	*www.inpharmatica.co.uk*
Products		
HTD 96	HTDialysis	*www.htdialysis.com/*
Dialyzer 96-well	Harvard Apparatus	*www.harvardapparatus.com*
Dialyzer Multi-Equil	Harvard Apparatus	*www.harvardapparatus.com*
IAM.PC.DD2	Regis Technologies	*www.registech.com*
Delta-8	Kibron	*www.kibron.com*
CT Bovial	Cellial Technologies	*www.cellial.com*

so prediction should be verified using in-house data for a few compounds in the series. It is advantageous if the software allows using a training set of in-house data to develop a custom "local" model or to modify the model.

28.2 In Vitro Methods for BBB

28.2.1 Physicochemical Methods for BBB

28.2.1.1 PAMPA-BBB Method [BBB Permeability]

This method predicts a classification of passive BBB permeability in high throughput. PAMPA-BBB follows the PAMPA format (see Section 26.2.3) but uses porcine brain lipid in dodecane as the artificial permeability membrane (Figure 28.1).[18] The performance of the method with 30 commercial drugs is shown in Figure 28.2. Compounds in the figure are CNS+ or CNS− if they were reported in the scientific literature as penetrating into the brain or not penetrating into the brain, respectively. The PAMPA-BBB method

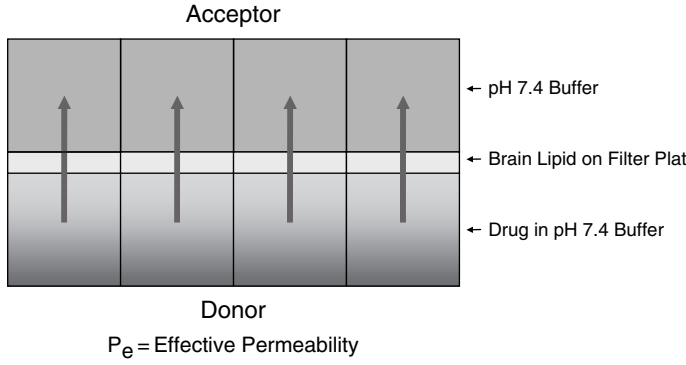

Figure 28.1 ► PAMPA-BBB assay diagram.

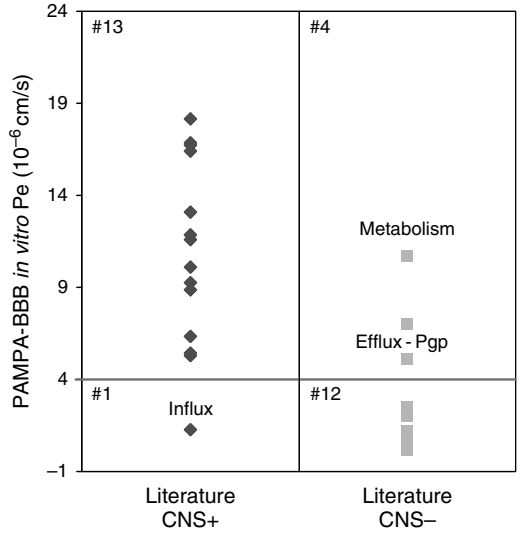

Figure 28.2 ► Application of PAMPA-BBB to 30 test compounds. (Reprinted with permission from [13].)

classified all of the CNS+ compounds correctly, except for one that in vivo penetrates into the brain by active uptake transport. It also classified all of the CNS− compounds correctly, except for two that in vivo are prevented from penetrating into the brain by efflux and one that in vivo is heavily metabolized and does not reach sufficient blood concentration to allow significant concentrations in the brain. PAMPA-BBB is a rapid, low-cost method for classifying compounds for predicted passive BBB permeability. Because PAMPA-BBB permeability indicates how fast a compound can permeate through the BBB, it does not necessary predict in vivo B/P because B/P relates to distribution properties of a compound in the static state rather than kinetic rate. PAMPA-BBB correlates well with in vivo perfusion data (Log PS or Log BB) and predicts well for acute in vivo clinical indications, which measures kinetic properties and not equilibrium static state properties of a given drug.

An example of the application of PAMPA-BBB is shown in Figure 28.3. In a discovery project, compounds from multiple compound series were evaluated in vitro for several features, including potency, structure diversity, BBB permeability, and metabolic stability. The data were used together in a holistic manner to prioritize compounds for in vivo dosing. In vivo results suggested where additional assays for other mechanisms should be performed to diagnose unexplained behavior. Finally, structures were modified to improve in vivo performance, as measured by efficacy and brain penetration. Several applications of the PAMPA-BBB assay have been reported in the literature.[19−21]

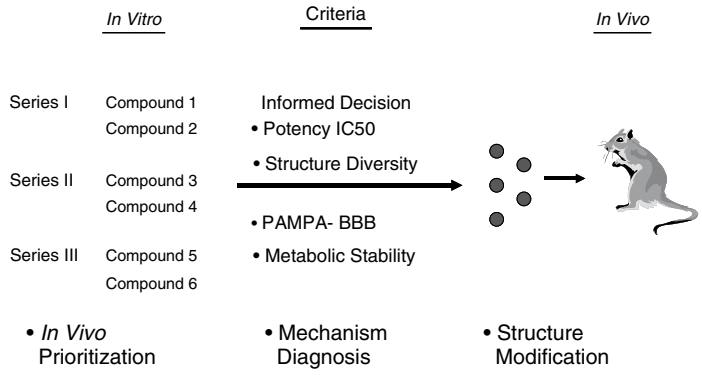

Figure 28.3 ► Applications of PAMPA-BBB assay.

28.2.1.2 Lipophilicity [BBB Permeability and Brain Distribution]

Lipophilicity affects both BBB permeation and brain distribution. Since the discovery of the BBB, it has been recognized that lipophilic molecules have greater access to the brain than hydrophilic molecules. The importance of lipophilicity, as measured by Log P or Log D, in predicting BBB permeability was discussed in Chapter 10. The optimal Log D for brain penetration is 1 to 3.[5,22−24] Lipophilicity affects BBB permeation by increasing lipid membrane permeability. It has been reported that in vivo brain permeability correlated to Log P and $MW^{-1/2}$.[25] Lipophilicity is also involved in partitioning between brain and blood, where it contributes to plasma protein binding and nonspecific binding to proteins and lipids in brain tissue. Lipophilicity could bring the compound into closer proximity with a brain target that is in a highly lipophilic environment. Log P and Log D can be calculated or measured, as discussed in Chapter 23. An example of the relationship of lipophilicity and BBB permeability is shown in Figure 28.4.[1]

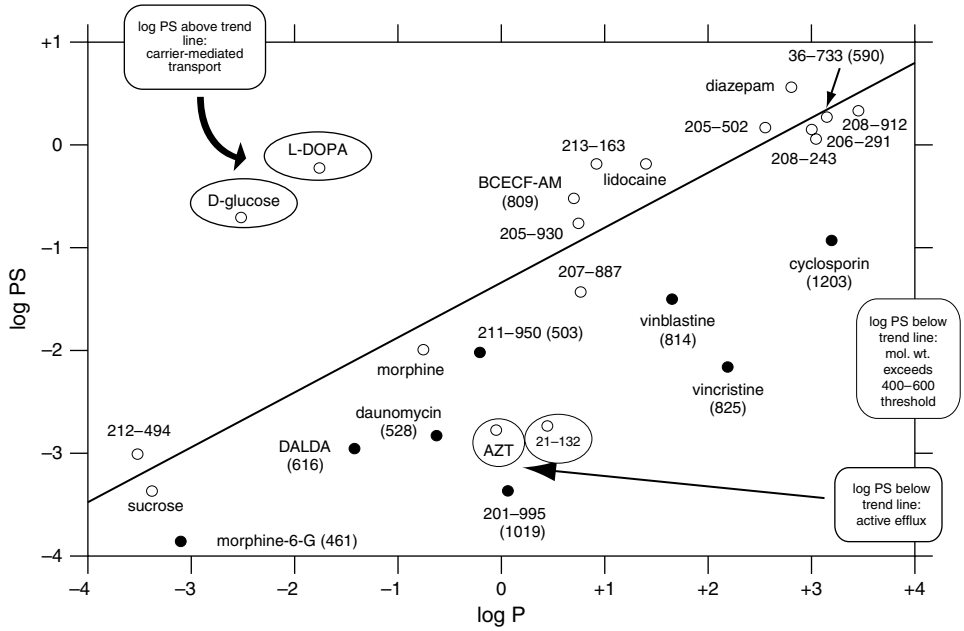

Figure 28.4 ► Lipophilicity (Log P) and the BBB permeability surface coefficient (Log PS) are correlated for compounds that permeate by passive diffusion. Compounds that are above the line tend to have enhanced uptake due to active transport. Compounds that are below the line tend to have efflux. (Reprinted with permission from [1].)

28.2.1.3 Equilibrium Dialysis [Brain Distribution]

This method predicts partitioning between plasma and brain homogenate by equilibrium dialysis.[26,27] Thus, it provides insights on brain distribution. Compound is dialyzed between plasma and buffer, then between brain homogenate and buffer. From this, the free drug in brain, free drug in plasma, and brain/plasma partitioning is calculated. By combining partitioning and Pgp efflux data, good correlations are provided to in vivo B/P. The assay is performed using 96-well format dialysis device from HTDialysis or Harvard Biosciences. Free drug in brain can be correlated to in vivo pharmacology.

28.2.1.4 ΔLog P [BBB Permeability]

ΔLog P is an assay for estimating the contribution of hydrogen bonds.[28] For this method, the Log P of a compound is measured by performing both octanol–aqueous buffer and cyclohexane–aqueous buffer partitioning. The aqueous buffer is at least 2 pH units away from the pK_a (i.e., below the pK_a of an acid and above the pK_a of a base). The basis of the method is that octanol forms hydrogen bonds with the test compound but cyclohexane does not; therefore, the ΔLog P value is mainly due to hydrogen bonding. The following calculation is used:

$$\Delta\text{LogP} = \text{Log P}_{\text{octanol}} - \text{Log P}_{\text{cyclohexane}}.$$

As ΔLog P increases, BBB permeability and brain distribution usually decrease. Optimal ΔLog P for brain uptake is < 2.[24,29] Young et al.[28] a reported that the brain to blood partition (Log BB) correlated to ΔLog P for 20 H_2 receptor histamine antagonists (see equation below):

$$\text{Log BB} = -0.485 \times \Delta\text{LogP} + 0.889.$$

28.2.1.5 Immobilized Artificial Membrane High-Performance Liquid Chromatography Column [BBB Permeability]

This method predicts permeability but not distribution. Immobilized artificial membrane (IAM) columns were described in Chapter 26. A lipid is bonded to the high-performance liquid chromatography (HPLC) stationary phase. The chromatographic retention time, using the IAM.PC.DD2 column, was shown to be a predictor of BBB permeability.[30,31] Estimation of B/P distribution values is not advised.

28.2.1.6 Surface Activity [BBB Permeability]

BBB permeability has been correlated to air/water interfacial tension partitioning coefficient K_{memb}.[32] This technique has been demonstrated using a high-throughput surface tension instrument from Kibron.

28.2.2 Cell-based In Vitro Methods [BBB Permeability]

Cell-based BBB assays have inherent appeal as in vitro models of BBB permeation because they are living and, presumably, closer to the BBB than physicochemical methods. However, they may not closely resemble the complex conditions at the BBB. They also consume considerably more resources. Most cell-based BBB assays use "transwell" procedures similar to Caco-2 (see Section 26.2.2). Compounds with high lipophilicity tend to depot in the cell monolayer and not move into the subsequent chamber (e.g., basolateral), so these should be tested in vivo. Cell-based permeability methods are not intended to correlate with in vivo B/P (distribution) values.

28.2.2.1 Microvessel Endothelial Cell Permeability [BBB Permeability]

The bovine microvessel endothelial cell (BMEC) method was developed as a model for BBB permeability. The microvessels of brain are isolated from fresh tissue and the endothelial cells are cultured.[33–35] When plated in trans-well culture plates they form monolayers with tight junctions. The correlation to the BBB is reasonable. A limitation of this assay is that the cells are used only as primary cultures. Fresh brain is obtained and the cells are immediately separated, cultured, and used soon in experiments. The expression level of transporters can vary from preparation to preparation. Porcine cultures also have been used.[29] An immortalized endothelial cell line has been produced.

Variations of this method are bovine brain endothelial cells (BBEC) and human primary brain endothelial cells (HPBEC).[2] Microvessels are isolated from brain and cultured. Endothelial cells grow out from the vessels and form microcolonies, which are harvested and plated. They have been cultured in proximity to rat astrocytes, which appear to release factors that enhance differentiation of BBB-like characteristics. The cultured endothelial cells are viable for a few passages.

Endothelial cell cultures have been used for detailed BBB studies but are not common for moderate- to high-throughput applications. It requires considerable resources to prepare endothelial cell cultures. The brain capillary endothelial monolayers in cell models typically are much leakier than BBB, with an electrical resistance more than 10-fold lower than BBB in vivo.[36] Furthermore, most BBB transporters or carriers are down-regulated, up to 100-fold in culture. For example, transport of L-dopa (a CNS drug for Parkinson's disease) was not detected in cell culture models because of the marked suppression of LAT1.[36]

Cellial Technologies supplies bovine brain capillary endothelial cell and glial cell kits, along with special culture media for cell layer BBB permeability studies (Table 28.1).

28.2.2.2 Caco-2 Method for BBB [BBB Permeability]

Caco-2 was developed as a permeation model for gastrointestinal absorption. Some companies have extended its use to screening BBB permeability. Caco-2 incorporates lipid bilayer membranes, Pgp efflux, and some other transporters. The limitations of using Caco-2 for BBB are that it does not form as tight junctions as brain endothelial cells, the lipid mixture of Caco-2 cells is not the same as BBB endothelial cells so it can exhibit different passive diffusion characteristics, and the profile of its transporters (which transporters and their expression levels) differs from those of the BBB.

A more limited, but powerful, application of Caco-2 and MDR1-MDCKII (below) is as a targeted Pgp efflux assay (see Chapter 27). Caco-2 expresses Pgp, and MDR1-MDCKII overexpresses Pgp. Therefore, they provide a means for predicting Pgp efflux, which has a crucial role in BBB permeability. The efflux ratio can be reliably used to evaluate the efflux potential of discovery compounds, diagnose BBB permeability limitations, and develop structure–Pgp efflux relationships to guide structural modification for the purpose of reducing Pgp efflux. Pgp efflux data also can be used in conjunction with other in vitro data to predict brain penetration. An example of this application is shown in Figure 28.5. PAMPA-BBB indicated that a compound should penetrate into the brain by passive diffusion (CNS+). However, an in vivo study showed poor brain penetration (CNS-). The compound was assayed for Pgp efflux using Caco-2. It was found to have an "efflux ratio" ($P_{B>A}/P_{A>B}$, see Section 27.2.1) of 7, which disappeared when cyclosporin A (CSA), a Pgp inhibitor, was coincubated with the compound in the Caco-2 assay media. Thus, the compound could enter the BBB by passive diffusion, but it was rapidly removed by Pgp efflux.

Pgp efflux data can be used in conjunction with equilibrium dialysis (see Section 28.1.3) to predict brain/plasma partitioning.[21,22]

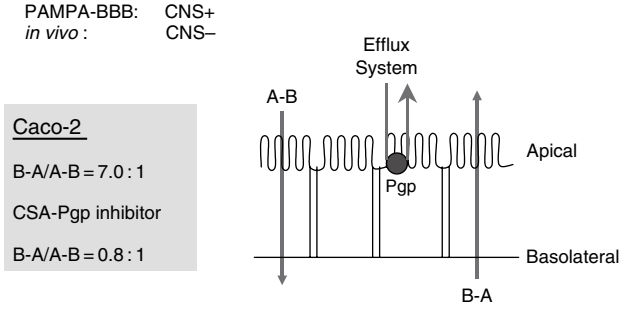

Figure 28.5 ► Diagnosis of BBB permeation mechanisms in a CNS project.

28.2.2.3 MDR1-MDCKII [BBB Permeability]

MDCK or MDR1-MDCKII cells are widely applied for BBB permeability prediction (but not brain distribution). MDCK cells have low transporter expression levels and have been used as a model of passive diffusion permeability.

MDR1-MDCKI and MDR1-MDCKII cell lines both have been transfected with human MDR1, the gene that codes for Pgp.[37] [This cell line is kindly provided by Professor Piet Borst, Netherlands Cancer Institute (NCI), Amsterdam, The Netherlands [see Section 27.2.1.2]. It is free to academic laboratories and can be licensed by companies for a fee. Another line of MDR1-MDCK cells is available from the National Institutes of Health, Bethesda, MD, USA.] Transfection with human MDR1 imparts overexpression of Pgp. A study of the (NCI) MDR1-MDCKII permeability of commercial drugs indicated that CNS drugs usually have a net apical to basolateral $P_{app} > 150$ nm/s.[37] Also, they have an efflux

ratio $P_{app,B>A}/P_{app,A>B} < 2.5$. Interestingly, a few CNS drugs had significant efflux ratios and still were active in the CNS, indicating that some Pgp-effluxed compounds still may sufficiently penetrate the brain if their $P_{app,A>B}$ is sufficiently high. MDCK cells form tighter junctions than Caco-2 cells.[38]

A productive use of the MDR1-MDCKII cell line is as a specific assay for Pgp efflux. It overexpresses Pgp; therefore, it is a more sensitive than Caco-2 for detecting Pgp efflux and reliably differentiating among series analogs to indicate structure–Pgp efflux relationships to guide chemists in structural modification.

28.2.2.4 TR-BBB and TM-BBB Cell Lines [BBB Permeability]

The TR-BBB and TM-BBB cell lines have been immortalized from microvessel endothelial cells by Terasaki.[39,40] These cell lines express membrane transporters with good fidelity to endothelial cells. They do not form tight junctions; therefore, they are used only for uptake studies (see Section 27.2.2), not transcellular permeation. The experiment is run using 24-well culture plates. The test compound is added to the cell medium over the cells and incubated. Following incubation, the cell medium is washed off, the cells are lysed, and the concentration of drug inside the cells is measured to determine how much compound permeated into the cell.

28.3 In Vivo Methods for BBB

28.3.1 B/P Ratio or Log BB [Brain Distribution]

The B/P experiment uses standard in vivo PK approaches and is widely implemented in companies. B/P provides brain distribution data (not BBB permeability) over several hours. In this experiment, multiple animals are dosed (time zero), and at designated time points three animals are sacrificed. A sample of blood is retained and the brain is removed. The compound concentrations in the plasma and brain homogenate are measured. The concentrations are plotted versus time (Figure 28.6). B/P is calculated as brain area under the curve (AUC)/plasma AUC. Log BB is the log of B/P. The figure shows a case where the compound has good brain penetration (CNS+), and $B/P = 1.23$. In the second case, the compound has poor brain penetration (CNS−), and B/P = 0.02. $B/P \geq 0.3$ are generally considered acceptable. Some lipophilic compounds produce $B/P \geq 10$. An advantage of this approach is that the B/P values are easy to use for ranking compounds in a discovery project. Another advantage is that this experiment provides other PK insights, such as C_{max} and the length of time that the compound concentration remains above a certain value that is active in vitro (e.g., EC50), to help understand the relationship of pharmacodynamics (PD) and PK.

B/P has limitations. First, the experiment requires considerable resources. The seven time point studies shown in Figure 28.6 require 21 animals per compound. Each animal is used for only one time point. The experiment requires significant compound material, animal scientist's time, bioanalytical scientist's time, and instrumentation. Second, B/P is a brain distribution value that is largely determined by nonspecific binding of drug molecules by brain tissue (i.e., partitioning between plasma and brain lipid) and much less by brain permeability.[36]

Third, the values from in vivo studies are total concentration. The experiment does not provide data for the free (unbound) drug concentration in the extracellular fluid (ECF) of the brain. Only free drug interacts with the receptor or enzyme to produce the pharmacological action.[36,41] (See Section 28.2.1.3 for a method for predicting free drug concentration in brain.)

It has been suggested that B/P measurements should be replaced by Log PS (Log BBB permeability-surface area coefficient) in drug discovery because BBB permeation is the greatest limitation to brain exposure.[36,41]

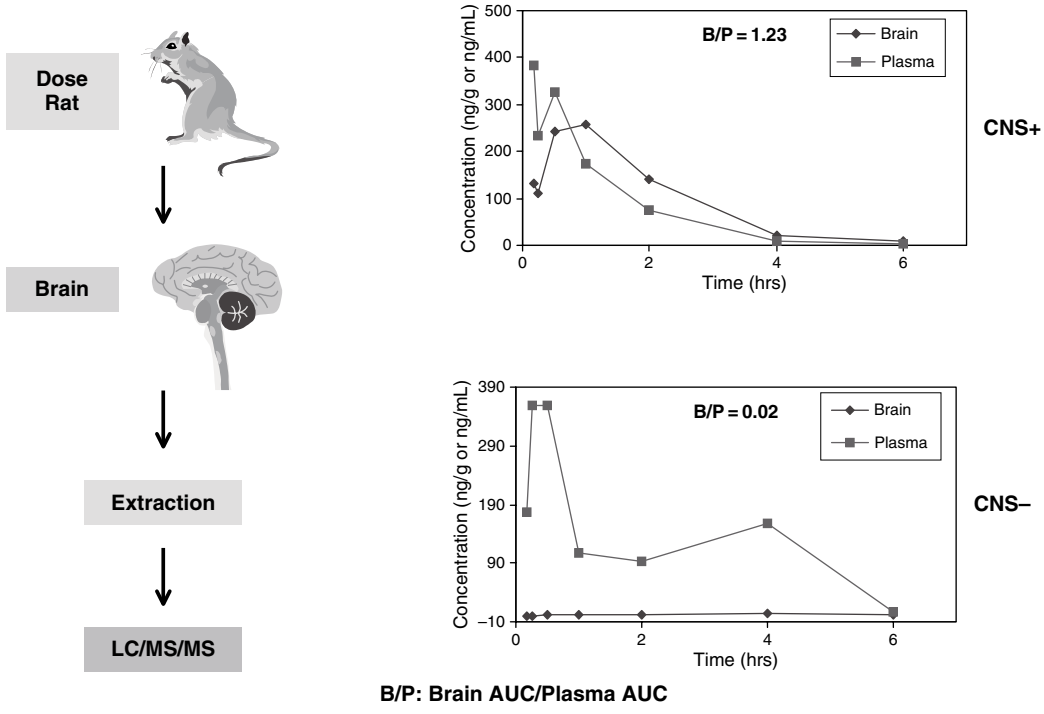

Figure 28.6 ► In vivo brain uptake experiment for B/P and Log BB determination.

Abbreviated in vivo brain penetration studies are often performed as part of an in vivo pharmacology study. During the study, a couple of dosed animals are sacrificed at one or two time point(s) to measure the concentration of the dosed compound in brain and plasma. From these samples, the total brain concentration and a single time point B/P value are obtained. The data allow a simple correlation of brain concentration (or B/P) data to pharmacological data. The information is valuable in early dosing studies. If pharmacological activity is observed, the study shows that a particular brain concentration is efficacious. If no activity is observed, the study confirms whether any compound reached the brain tissue or at what total brain concentration the compound is not efficacious. The limitation of such an experiment is that there can be differences in $t_{max,}$ $t_{½}$, and C_{max} between brain and blood, so single time points do not give a complete assessment of brain penetration or PK/PD relationships.

It is useful to perform an initial brain PK study prior to full pharmacology dosing experiments. This study is conducted with different dose levels or vehicles to determine what dose will achieve a desired brain concentration and duration for the subsequent pharmacology experiment.

28.3.2 Brain Uptake Index [BBB Permeability]

The brain uptake index method was one of the earliest techniques for studying in vivo BBB permeability. A quantity of radiolabeled drug is injected into the common carotid artery of an animal along with tritiated water. After 15 seconds, the experiment is stopped and the brain removed. The amount of compound in the brain is calculated relative to the amount of tritiated water (used as an internal standard). This method was used for many years but has been replaced by in situ perfusion, which allows greater control of the fluid composition for research purposes.

28.3.3 In Situ Perfusion [BBB Permeability, Log PS, μL/min/g]

The in situ perfusion method provides high-quality in vivo BBB permeability data.[42–49] The experimental setup is shown in Figure 28.7. A catheter is placed in the common carotid artery of an anesthetized animal, and the external carotid artery is ligated. The pterygopalatine artery remains open. The blood flow is stopped, and the syringe pump is switched in line. The perfusate, which contains dissolved drug, physiological electrolytes, oxygen, and nutrients, provides the fluid flow to the brain. The perfusate rapidly flows through the brain arteries and throughout the brain capillaries. The perfusate replaces the blood. During the perfusion, circulation to half of the brain is totally taken over. BBB integrity has been shown to remain high throughout the experiment. The perfusion is conducted for periods ranging from 5 seconds to several minutes, after which compound-free perfusate is used to purge the brain vessels. The brain is removed, and the compound concentration in the half of the brain that was perfused is measured. This experiment provides high-quality data for studying BBB permeation and specific mechanisms. The short time periods allow minimization of nonspecific binding. From this experiment, PS is determined.[50] Other methods for PS determination include carotid arterial injection and/or infusion methods, and quantitative intravenous injection methods.[12,36]

Coadministration of transporter inhibitor or application of the techniques to transgenic animals that lack a transporter allows study of the extent of transporter contribution to the penetration of a particular compound. For example, in situ perfusion was used with Pgp-deficient and wild-type mice to demonstrate that brain uptake of colchicine, a Pgp substrate, was reduced several-fold by Pgp.[43] The flexibility of selecting constituents of the perfusion fluid allows a wide range of studies of the BBB permeability effects of factors in the blood (e.g., proteins, electrolytes).

This method provides high-quality data, but it consumes significant resources for the surgery, experiment, and quantitation. It is useful for advanced BBB studies. When used by

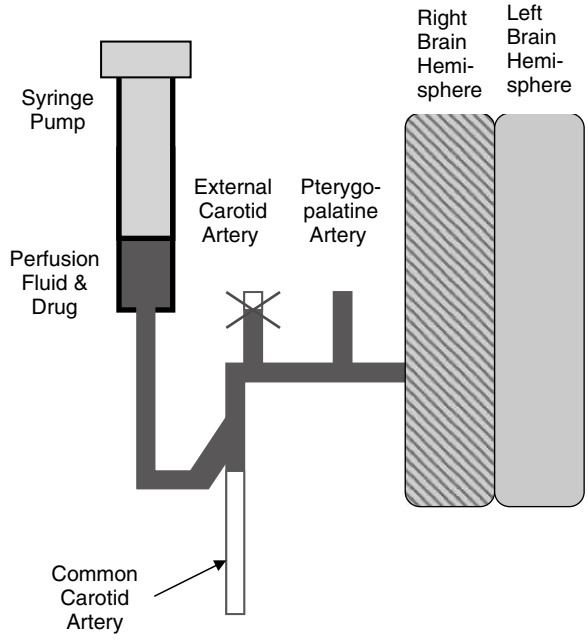

Figure 28.7 ► In situ brain perfusion experiment.

a pharmaceutical company, it typically is implemented for detailed studies that are critical to a discovery project.[51,52]

28.3.4 Mouse Brain Uptake Assay [BBB Permeability and Brain Distribution]

The in vivo mouse brain uptake assay (MBUA) is a short duration in vivo experiment.[2,53] The MBUA experiment is initiated with a 6.5 μmol/kg injection (50 μL) into the tail vein of a mouse. At 5 minutes, the animal is anesthetized, and a blood sample is collected and the brain removed. It is assumed that at 5 minutes there is minimal nonspecific brain tissue binding, back flow of test compound out of the brain, and brain metabolism. The concentration of compound in each sample is analyzed using LC/MS/MS. PS is calculated, and from this value BBB Papp is derived (based on a surface area of 240 cm^2 per gram brain in mouse). Running the same experiment for 60 minutes indicates whether the compound is cleared from the brain at the same rate as from the plasma, suggesting fast equilibration across the BBB, or more slowly from the brain, indicating that the compound accumulates in the brain. This method is rapid and uses a minimum of animals and laboratory resources. MBUA Papp data can be plotted versus calculated Log D at pH 7.4 (Clog D7.4). As shown in Figure 28.8., and Table 28.2, this provides insights on the predominant mechanism(s) of

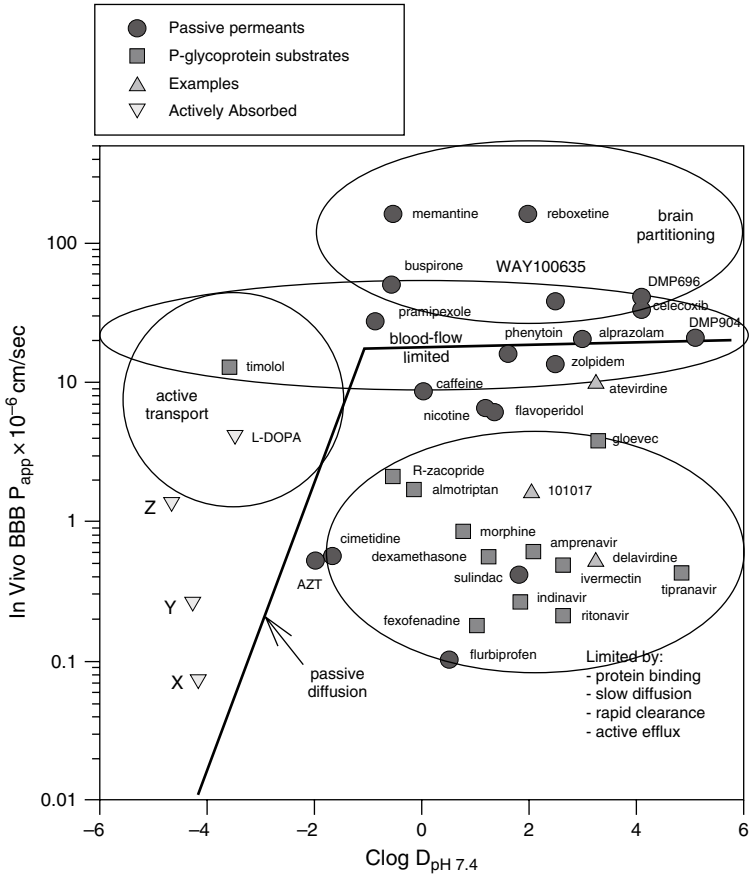

Figure 28.8 ► Mouse brain uptake BBB permeability data (Papp) can be compared to Clog $D_{pH\,7.4}$ to gain insights on potential mechanisms of BBB penetration. (Reprinted with permission from [49].)

TABLE 28.2 ► BBB Penetration Mechanisms Suggested by Plotting Location of Data in Figure 28.8

Predominant mechanism	Diagram location
Passive diffusion	Ascending diagonal line
High passive diffusion limited by blood flow	Horizontal line
High distribution (nonspecific binding) into brain	Above horizontal line
Hydrophobic compounds with high plasma protein binding, slow passive diffusion, rapid clearance, or active efflux	Below horizontal line
Hydrophilic compounds with active uptake	Plots to left of passive diffusion diagonal

a compound's BBB penetration. Raub et al.[53] have used this data, in combination with in vitro assays (i.e., plasma protein binding, MDR1-MDCKII) to discern and estimate multiple permeability mechanisms.

28.3.5 Microdialysis Method for BBB

The components in brain ECF can be sampled directly using microdialysis methodology.[54–57] This technique is common for measuring neurotransmitters and has been applied for measuring drug candidate concentrations in the ECF. A small probe having concentric tubes and tipped with a dialysis membrane is implanted into brain tissue (Figure 28.9) and allowed to equilibrate. Artificial ECF fluid is pumped through the probe at a low flow rate (e.g., 1 μL/min). Compounds below the MW cutoff freely diffuse across the membrane and are trapped in the flow of fluid out of the probe. The compound concentration in the probe ("recovery") is 5% to 20% of the concentration in the ECF, depending on the compound and flow rate. The probe is calibrated by placing it in solutions of known concentrations and measuring the dialysate.

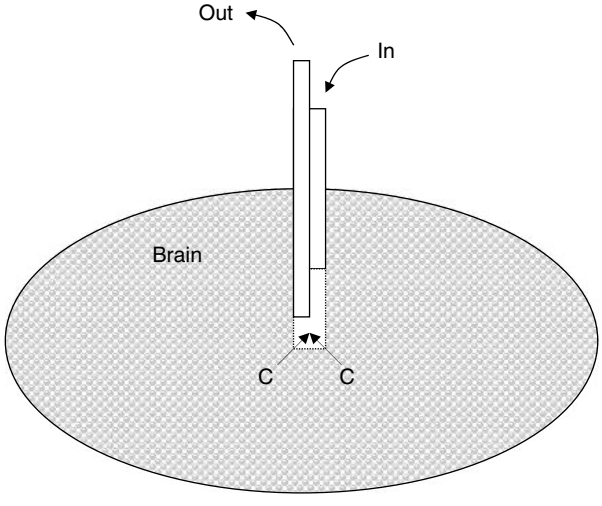

Figure 28.9 ► A microdialysis probe is implanted in brain. After equilibration, the ECF is sampled by perfusion with artificial ECF. Compound (C) and other components (protein is excluded) from the ECF are collected into the microdialysate and quantitated using LC/MS/MS.

Data from this method are useful and reliable because only free compound in the ECF is sampled. The limitations of the method are that it is relatively time-consuming to perform, it can disrupt the BBB by causing leakage from capillaries, and very lipophilic compounds can adsorb to the dialysis membrane, resulting in low recovery.

28.3.6 Cerebrospinal Fluid Method for BBB

Cerebrospinal fluid (CSF) is relatively easy to sample in vivo and thus is attractive as a means of assessing brain penetration. Use of CSF is controversial.[36] Compound gets into the CSF by crossing the blood–cerebrospinal fluid barrier (see Chapter 10), which has about 1/5000th the surface area of the BBB, or by being cleared from the ECF, which is slow. CSF is completely refreshed every 5 hours, so there is a rapid turnover. Doran et al.[58] sampled CSF for 31 commercial drugs as part of a major in vivo brain penetration study. Compound CSF concentrations have been predictive of pharmacological activity in some studies.[36,41]

28.4 Assessment Strategy for Brain Penetration

Several methods are available for assessing brain penetration. As the complexity of the data increases, the cost increases and the throughput decreases (Figure 28.10). Therefore, methods should be selected with care.

It is advisable to first assess discovery compounds for brain penetration using higher-throughput methods, such as in silico, physicochemical properties (especially PSA; see Section 10.3 for useful structural property rules), and PAMPA-BBB (Figure 28.11). These provide an initial prediction of passive BBB permeability, which is useful for early prediction of in vivo BBB permeability. This provides discovery scientists insights on whether their

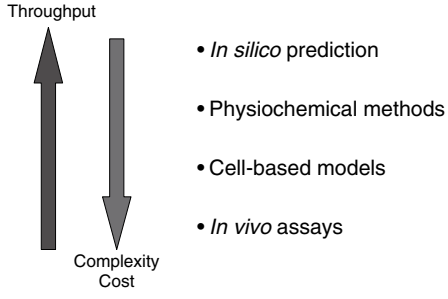

Figure 28.10 ► Several methods are available for BBB penetration.

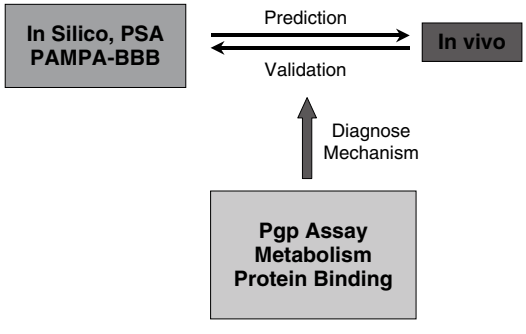

Figure 28.11 ► Screening strategy for BBB penetration.

project compounds are expected to have any problems with penetrating to the therapeutic target in the brain. Selected series examples then can be studied using in vivo studies. These results provide more in-depth assessment of penetration into the brain as well as feedback (validation) on whether the early assessment of passive brain penetration was correct. If the in vivo and in vitro brain penetration data differ significantly, the contributions of other mechanisms, such as Pgp efflux, hepatic clearance, plasma protein binding, and nonspecific brain tissue binding, can be assessed using other in vitro assays. The limiting mechanisms can be discerned, and structure modifications can be undertaken to improve the brain penetration properties of the compound series.

Problems

(Answers can be found in Appendix I at the end of the book.)

1. What is the difference between BBB permeation and brain distribution?
2. What structural properties affect brain penetration?
3. PAMPA-BBB provides what predictions of brain penetration?
4. In the equilibrium dialysis method, what are the fluids between which the compounds are dialyzed? Is this a BBB permeability or brain distribution method? What predictions are obtained from this experiment?
5. What does ΔLog P mainly indicate?
6. What does IAM predict?
7. Why is the microvessel endothelial cell method challenging to perform? What are the limitations of this method?
8. What BBB permeability insights can be gained using MDR1-MDCKII cells?
9. What is the most commonly used in vivo measurement for brain penetration? What are its limitations?
10. The brain uptake and in situ perfusion methods at short time points are good measurements of what?

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Chapter 29

Metabolic Stability Methods

Overview

- *It is difficult to predict metabolic rate using software, but predicting the labile sites is more successful.*
- *Various combinations of cell fractions, hepatocytes, and cofactors are used to study metabolism in vitro. Each studies a different profile of metabolic enzyme activities.*
- *Structure elucidation of major metabolites guides structure modification to increase stability.*

Metabolic stability is one of the most important properties in drug discovery. Stability often is a major liability for a lead series and needs improvement. Quantitative metabolic stability data are used to assess the extent of metabolic conversion, prioritize compounds for in vivo studies, and set priorities for discovery activities. Qualitative metabolite structure information indicates the sites of metabolic instability ("hot spots") that medicinal chemists use to plan structural modifications for improved stability. Reviews of metabolic stability[1–3] and hepatobiliary clearance[4] methods have been published.

Metabolic Stability Methods

Five types of methods are commonly used in discovery (Table 29.1):

- *Microsomal Stability Assay:* Uses liver microsomes and NADPH cofactor to assess phase I oxidations by CYP and flavin monooxygenases (FMO); applied to all discovery compounds as soon as they are synthesized.
- *Phase II Stability Assay:* Uses liver microsomes, S9, or hepatocytes with appropriate cofactors (e.g., uridine diphosphate glucuronic acid [UDPGA] to assess glucuronidation); applied to selected compounds with moieties susceptible to conjugation (e.g., phenols, aliphatic hydroxyls).
- *S9 or Hepatocyte Stability Assay:* Encompasses a broader range of metabolizing enzymes for assessment of microsomal and extramicrosomal (e.g., cytosolic, mitochondrial) metabolic reactions; applied to selected compounds, especially in late discovery.

- *Metabolite Structure Elucidation:* Uses spectroscopy to determine metabolite structures; applied to selected series examples during optimization or late discovery.
- *Metabolic Phenotyping:* Examines which enzymes or isozymes metabolize the compound; applied to selected compounds during optimization or late discovery.

TABLE 29.1 ► In Vitro Methods for Determination of Metabolic Stability

Method	Metabolic material	Cofactor	Throughput	Purpose
Microsomal	Microsomes[a]	NADPH	500 cpd/wk	Phase I stability ($t_{½}$)
Phase II	Microsomes, S9, or hepatocytes	UDPGA, PAPS	500 cpd/wk	Phase II conjugation stability ($t_{½}$)
S9	S9	NADPH, UDPGA, SAPS	500 cpd/wk	Phase I and II stability ($t_{½}$)
Hepatocyte	Fresh or cryopreserved hepatocytes	None	100 cpd/wk	Broader stability from phase I and II and permeability ($t_{½}$)
Metabolite structure elucidation (LC/MS or NMR)	Microsomes, S9, hepatocytes	NADPH, UDPGA, SAPS	5 cpd/wk	Sites of metabolic liability to modify structure; synthesize to test activity and safety
Metabolic phenotyping	rhCYP, individual enzymes	NADPH	100 cpd/wk	Identify isozymes(s) of metabolism to modify structure; plan clinical DDI studies

[a]Microsomes refer to liver microsomes.

A common strategy for using these assays is illustrated in Figure 29.1.

It is beneficial to measure the in vitro metabolic stability in species that are relevant for the project. Metabolic stability can be quite different in various species. It is efficient to initially obtain stability data in a single generic species (e.g., rat). This provides a common

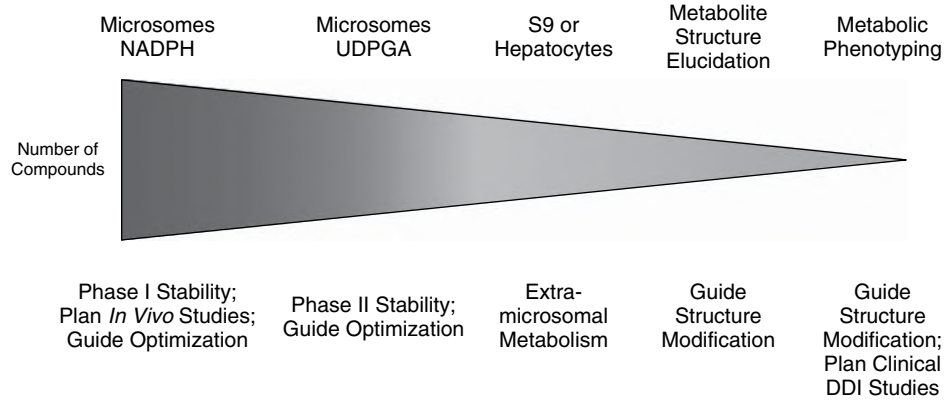

Figure 29.1 ► Metabolic assays and their corresponding applications in drug discovery.

point of comparison for all projects. Rat is also commonly used for toxicity screening, so data from this species are useful for planning and interpreting safety studies. In addition to rat, project teams evaluate metabolic stability in their efficacy species (e.g., transgenic mouse, cynomolgus monkey) in vitro to assist with achieving sufficient dosing levels for pharmacology proof of concept studies. In vitro metabolic stability in human is useful for projecting ahead to clinical studies. Metabolic stability data are useful for prospectively selecting compounds for in vivo dosing studies and for retrospectively diagnosing the causes of poor pharmacokinetic (PK) performance or lack of in vivo activity.

29.1 In Silico Metabolic Stability Methods

Three software companies offer tools for predicting metabolite structures and metabolic stability (Table 29.2). Metasite does a very good job at predicting sites of metabolic instability in compound structures.[5] Such tools can be used during structure design to determine if planned substructure modifications will be liable for metabolism or to suggest sites on the molecule to block (see Section 11.3) if the structures of major metabolites have not been spectroscopically elucidated.

TABLE 29.2 ► Commercial Software or Metabolic Stability

Name	Company	Purpose	Web site
Metasite	Molecular Discovery	Metabolite structures	*www.moldiscovery.com*
KnowItAll	Biorad	Metabolic stability	*www.biorad.com*
ADMENSA	Inpharmatica	Metabolic stability, metabolite structures	*www.inpharmatica.com*
Meteor	Lhasa	Metabolite structures	*www.lhasalimited.org*

29.2 In Vitro Metabolic Stability Methods

Metabolic stability assays incubate the test compound with a selected metabolic stability material. Detection methods are used to quantitate the remaining (unmetabolized) compound or to spectroscopically study the structures of metabolites.

29.2.1 General Aspects of Metabolic Stability Methods

29.2.1.1 Metabolic Stability Materials

Materials for in vitro metabolic stability methods are readily available because of extensive use of the assay. The activity levels of each batch of material should be prechecked using appropriate quality control compounds because activity can vary with isolation procedure, vendor, and liver source and among batches from the same laboratory.

Metabolic enzymes for metabolic assays are available from several sources. These materials are selected based on the data required by the discovery project team. It is important to know the differences between the materials for proper interpretation of the data. A partial list of vendors of materials is given in Table 29.3. Human-derived materials are generally preferred for in-depth studies in later discovery because they appear to more accurately predict human in vivo stability and are useful for projection to human clinical studies. In earlier stages of discovery, metabolic materials derived from animal species are used in running thousands of assays, for making generic species comparisons, and for planning and

TABLE 29.3 ► Partial List of Commercial Suppliers of Materials and Instruments for Metabolic Stability Studies

Company	Product	Web site
BD Gentest	Superzomes (rhCYP isozymes) Supermix (mix of rhCYP isozymes) Individual metabolic enzymes (other than CYP) Liver microsomes (various species and human) S9, cytosol NADPH regenerating system Fresh and cryopreserved hepatocytes	*www.gentest.com*
XenoTech	Bactosomes (rhCYP isozymes) Liver microsomes (various species and human) S9, cytosol, mitochondria Fresh and cryopreserved hepatocytes	*www.xenotech.com*
In Vitro Technologies	InVitroSomes (rhCYP isozymes) Liver microsomes (various species and human) Fresh and cryopreserved hepatocytes S9	*www.invitrotech.com*
ADMET Technologies	Human liver microsomes S9, cytosol Fresh and cryopreserved hepatocytes	*www.admettechnologies.com*
Invitrogen	Baculosomes (rhCYP isozymes)	*www.invitrogen.com*
Sigma-Aldrich	CYP isozymes Individual metabolic enzymes (other than CYP)	*www.sigmaaldrich.com*
Qualyst	B-Clear sandwich hepatocyte culture	*www.qualyst.com*
Instruments		
Perkin Elmer	Packard Multiprobe Robot	*www.perkinelmer.com*
Tecan	EVO Platform	*www.tecan.com*
Beckman Coulter	Biomek Laboratory Automation Workstation	*www.beckman.com*

interpreting experiments with efficacy species. Liver microsomes from rats are one third the cost of liver microsomes from humans.

- *rhCYP isozymes:* Individual recombinant human CYP (rhCYP) isozymes are available in bulk. They are produced from cDNA cloning of human CYP genes and transfection using baculovirus into insect cells in culture or cloning into bacteria. The isozymes are used individually or in mixtures.
- *Liver Microsomes:* These are the most frequently used metabolizing material in drug discovery. They contain the metabolizing enzymes that are bound to the endoplasmic reticulum (ER) membrane in the cells. Liver microsomes contain CYP and other metabolizing enzymes that have a major role in drug clearance. They are a convenient reagent for in vitro metabolic studies. They represent a major portion of the

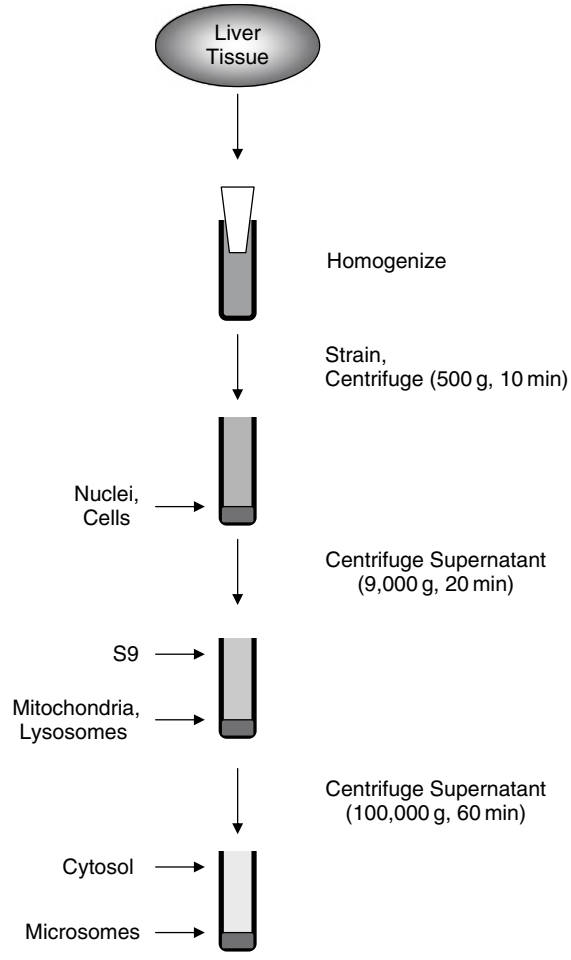

Figure 29.2 ► General scheme for preparation of subcellular fractions for metabolic stability studies.

metabolizing enzymes in living systems. Structural optimization guided by liver microsome metabolism has proven to be a very successful strategy in drug discovery. Liver microsomes are prepared by differential centrifugation of liver tissue homogenate (Figure 29.2). The S9 (supernatant) is centrifuged at 100,000*g* to obtain microsomes (pellet) and cytosol. Many of the important metabolizing enzyme classes in liver microsomes are listed in Table 29.4. Cytosol and mitochondria fractions can be separately purchased from vendors.

► *S9:* This material contains a broader set of metabolizing enzymes than liver microsomes (Table 29.4). S9 is the supernatant obtained from differential centrifugation at 9,000*g* of strained liver homogenate. This fraction contains both the cellular cytosol and the ER membranes with their respective metabolic enzymes. S9 has about 20% to 25% of the CYP activity of liver microsomes because the liver microsomes make up about one fifth of the S9.

► *Hepatocytes:* These are liver cells and contain an ensemble of all the metabolizing enzymes. They are prepared from fresh livers by treatment with calcium-free chelating agent, followed by collagenase to dissociate the cells from the liver matrix. Hepatocytes

TABLE 29.4 ► Major Metabolizing Enzyme Activity Classes and Subcellular Liver Fractions in Which Class Members are Commonly Found

Metabolizing activity[a]	Liver microsomes	Cytosol	S9	Mitochondria
Cytochrome P450	X		X	
Alcohol dehydrogenase		X	X	
Aldehyde dehydrogenase		X		X
Monoamine oxidase				X
Diamine oxidase		X		X
Flavin-monooxygenases	X		X	
Reductases	X	X	X	
Esterases	X	X	X	
UDP-Glucuronosyltransferases	X		X	
Sulfotransferase		X	X	
N-Acetyltransferase		X	X	X
Amino acid conjugations	X		X	X
Glutathione-*S*-transferases	X	X	X	
Methyltransferases	X	X	X	

[a]These activities represent classes of enzymes. The specific enzyme that metabolizes a particular compound may or may not be present in this fraction. The degree of an enzyme's expression varies with fraction, tissue, species, and individual.

can be used freshly prepared (primary culture) or frozen in liquid nitrogen ("cryopreserved").

► *Liver slices:* "Precision-cut" liver slices are sections of whole liver tissue. They represent all of the natural liver metabolizing system, including transporters, enzymes, and cofactors.[6] They are used for in-depth studies of selected compounds.

29.2.1.2 Detection Methods for Metabolic Stability

Detection of test compounds after incubation usually is performed using liquid chromatography/mass spectrometry (LC/MS) techniques following the incubation and sample preparation.[7–11] The sensitivity, selectivity, and cost of LC/MS are necessary because the assays are commonly performed at a low compound concentration (e.g., 1 μM). The measurement of 1% of test compound remaining after incubation requires a lower limit of quantitation of 10 ng/mL. The selectivity of high-performance liquid chromatography (HPLC) separation and MS or MS/MS analysis is required because the sample matrix is quite complex and can interfere with detection of the test compound. The matrix contains microsomal components, incubation cofactors, and compound metabolites that must be resolved to obtain a reliable signal for the trace levels of test compound. Recent LC/MS methods for use in metabolic stability assays include trap and inject (no HPLC),[7,8] fast chromatography (1–2 minutes per injection) using fast ("ballistic") gradients,[9,10] or "ultraperformance liquid chromatography" (UPLC). Multichannel HPLC with a switching ion source (MUX) has been used,[11] but it can be challenging because each channel (usually four) analyzes a different compound and must use unique MS/MS conditions, so the constant switching creates a lot of overhead time and requires additional instrument maintenance. MS/MS analysis, using a unique precursor–product ion pair, provides high levels of selectivity and sensitivity. All MS/MS instrument vendors now offer automated procedures for the unattended selection of optimum MS/MS conditions (i.e., precursor and product ions, ion source voltages, collision energy), which

saves considerable time for the laboratory scientist and works well. Electrospray ionization (ESI) is used for most compounds. Atmospheric pressure chemical ionization (APCI) is useful for compounds that do not ionize well using ESI.

29.2.2 In Vitro Microsomal Assay for Metabolic Stability

An overview of the metabolic stability assay incubation is shown in Figure 29.3. The test compound is dissolved in dimethylsulfoxide (DMSO) and a small volume is diluted into the solution containing liver microsomes. This solution is added to buffer (5 mM EDTA, 100 mM potassium phosphate buffer, pH 7.4) containing NADPH regenerating system (glucose-6-phosphate, $NADP^+$, and glucose-6-phosphate dehydrogenase). The final microsomal protein concentration typically is 0.5 mg/mL. The mixture is incubated at 37°C, and aliquots are removed at specific time points and quenched. For a single time point assay, the entire incubation solution is quenched. Quenching is accomplished by adding at least one volume of cold acetonitrile, which inactivates the enzymes and precipitates the microsomal material (e.g., protein, lipid). The incubate is centrifuged, and the precipitate pellets to the bottom. The supernatant is injected into an LC/MS/MS system to quantitate the test compound remaining after incubation. Specific LC/MS/MS conditions are used for each test compound. The results are reported as percent remaining and $t_{½}$ (half-life). Half-life is calculated from first-order kinetics. A fully automated assay method has been described.[12,13]

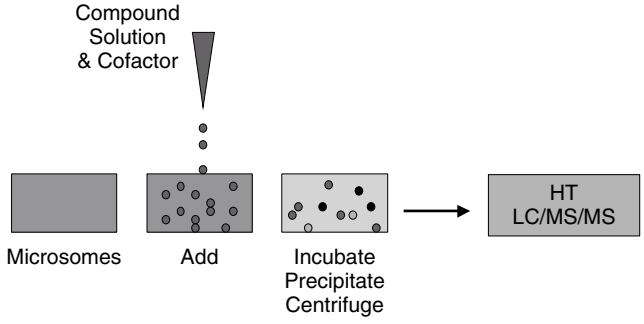

Figure 29.3 ► Microsomal stability assay overview. (Reprinted with permission from [28].)

The concentration of the test compound used in the assay differs among laboratories from 0.5 to 15 μM[10–13]; however, the concentration has a dramatic effect on the results.[12] For example, propranolol (Figure 29.4) in rat liver microsomes had 76% remaining after 5 minutes at 10 μM compared to 1% remaining at 1 μM. Apparently, the enzymes are saturated at higher concentrations. The effect is compound dependent. Thus, when comparing (rank ordering) compounds for metabolic stability, it is important that all the data derive from the same assay conditions. The test compound concentration should be low and physiologically relevant. A good concentration for the assay is 1 μM or lower.

Figure 29.4 also illustrates that fact that metabolic rates differ among species. For propranolol, the metabolic rate followed the order: rat > mouse > human. Therefore, it is important to select a species that is relevant to the discovery project and to rank order compounds using data from the same species.

Several microsomal stability assays have been reported.[9–12] With time, improvements to the quality and throughput of the method have been achieved. A 96-well plate assay using the high-throughput aspects outlined has been described.[12] High throughput can be reliably achieved using a "single time point" approach,[13] as opposed to the multiple time

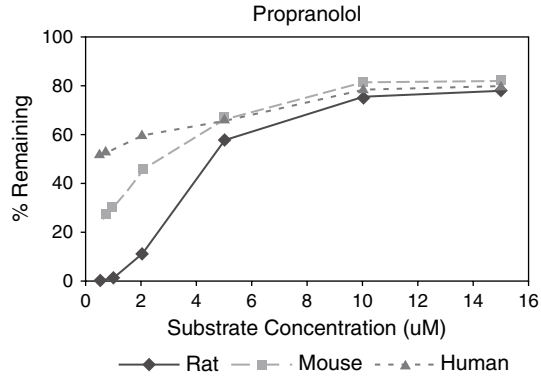

Figure 29.4 ► Species and substrate concentration dependence of percent propranolol remaining after 5 minutes of incubation with liver microsomes. (Reprinted with permission from [12].)

points of many methods. Microsomal stability reactions follow first-order kinetics, in which there is a linear relationship between a plot of log % remaining and linear time. Thus, $t_{½}$ can be determined using a linear fit of two points: the first is log (100% remaining) at t_0 and the second is log (% remaining) at the time the reaction is quenched (e.g., 15 minutes). Two points define the line. It is common practice in many laboratories to measure percent remaining at multiple time points. This is not necessary for determination of $t_{½}$. Multiple time points only add data points for increased precision and extend the maximum predictive half-life. If the method is reliable, duplicate measurements at t_0 and t_{15min} provide sufficient precision for discovery stability assessment. An example of the agreement of multiple time point data with single time point data (15 minutes) is shown in Figure 29.5 and Table 29.5. A single-point assay of 15 minutes allows highly unstable compounds with $t_{½}$ values in the range from 1 to 5 minutes to be measured and provides an upper limit of 30 min $t_{½}$. This is a good working range for discovery, which is primarily concerned with being alerted to and stabilizing the highly unstable compounds. Concerns decrease at $t_{½}$ greater than 30 minutes.

It is important to know the limitations of the assay in reporting $t_{½}$. It has been shown that the logarithmic relationship of $t_{½}$ to percent remaining and the error of the method set limits on the maximum and minimum limits of reliable predictive half-life values.[13] For an assay having a single time point at 15 minutes, it is reliable to report $t_{½}$ values from 0 to

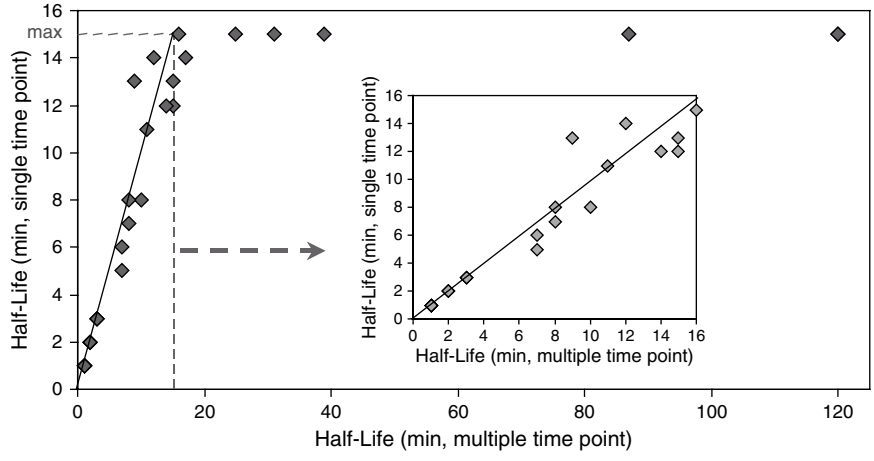

Figure 29.5 ► Correlation of single time point and multiple time point data for drug discovery compounds.

TABLE 29.5 ► Agreement of Single Time Point HT Microsomal Assay Results with Multiple Time Point Literature Results[3]

	$T_{½}$ (min)	$t_{½}$ (min)
Compound	Wyeth	Literature
Midazolam	3	4
Verapamil	6	10
Diltiazem	15	21
Zolpidem	<30	44
Tenoxicam	>30	38

30 minutes. Above 30 minutes, the logarithmic amplification of the error makes differences in the values insignificant. If an assay runs for 30 minutes, the maximum predictive half-life should be no more than 60 minutes.

It is important to optimize microsomal stability assays in order to produce reliable data for low-solubility compounds. Many drug discovery programs have a lead series that is highly lipophilic and, therefore, has low solubility. These types of compounds are not properly assayed by many in vitro assays. Many scientists use well-behaved drug compounds during method development and validation. These do not have the property limitations of many drug discovery compounds (e.g., low solubility, high lipophilicity). Low-solubility compounds precipitate or adhere to the walls of the well. Precipitation eliminates interaction of the test compound with the enzymes, so the compound is not metabolized. When the quenching organic solvent (e.g., acetonitrile) is added to the reaction, the precipitated compound is redissolved and is quantitated as a high percentage of original compound material remaining. Therefore, the compound falsely appears to be stable. It has been shown that metabolic stability methods can be improved for low-solubility compounds by the following critical method conditions and steps[14]:

1. Diluting test compound DMSO stock solutions into organic solvent, not aqueous buffer, to reduce precipitation at higher concentrations (10–20 μM)
2. Adding the diluted test compound into diluted liver microsomes, not into aqueous buffer, to utilize the microsomal lipid and protein for solubilization and to bring the compound into close proximity to the membrane-bound metabolic enzymes
3. Maintaining the final incubation solution at the highest organic solvent and type of solvent that can be tolerated by the assay without affecting the results (e.g., 0.8% acetonitrile/0.2% DMSO rather than 0.2% DMSO alone)

These precautions in method conditions and protocol will keep insoluble test compounds in solution and provide a more accurate measurement of metabolic stability.

Higher throughput in metabolic stability assays is achieved using automated instruments. Robotics using a multichannel robot allows unattended liquid handling with reproducible pipette volumes, timing, and protocol. A diagram of the automation scheme is shown in Figure 29.6. The robot takes a 96-well plate containing stock solutions of each test compound in DMSO (10 mg/mL) and dilutes it to an "optimization plate," which is used for automated MS/MS method optimization, as well as an "incubation plate," which is used for the stability assay. The robot configuration for the microsomal stability assay is shown in Figure 29.7. The assay produces a "sample plate," which is taken to the LC/MS/MS for quantitation. The MS/MS method, developed using the optimization plate and the automated method optimization procedure, is used to quantitate the test compound percent remaining after

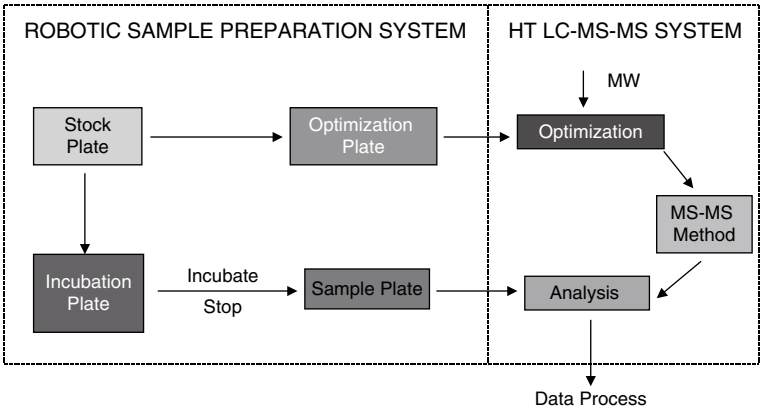

Figure 29.6 ► Automation scheme for microsomal stability. (Reprinted with permission from [12].)

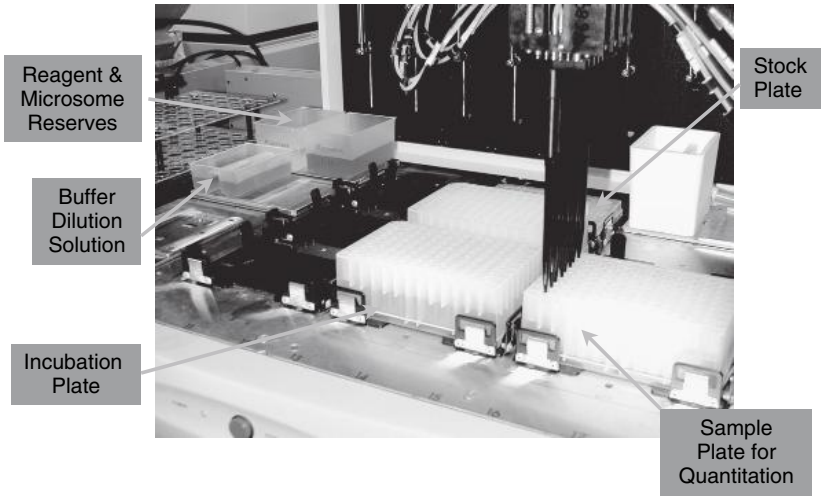

Figure 29.7 ► Microsomal stability assay setup using a multichannel robot.

incubation. Figure 29.8 shows a high throughput LC/MS/MS quantitation system that uses an HPLC with a short analysis time, an autosampler that can manage up to twelve 96-well plates, and an MS/MS instrument for quantitation.

It is important to qualify each batch of liver microsomes used in the assay. For example, results from liver microsomes of three vendors are shown in Figure 29.9. The enzyme activity in each vendor's material differed greatly. Results can vary among batches from the same vendor or laboratory (Figure 29.10). It is good practice to measure the activity of each new batch using consistent quality control standards and to adjust the method in order to obtain consistent data.

29.2.3 In Vitro S9 Assay for Metabolic Stability

The S9 stability assay conditions are nearly identical to the microsomal assay (e.g., NADPH, buffer, EDTA, Mg).[15,16] S9 contains all of the metabolizing enzymes in liver microsomes plus some additional enzymes found in the cytosol (Table 29.4). However, in S9 the microsomal enzymes are present at 20% to 25% of their concentrations in liver microsomes, so there

Figure 29.8 ► High-throughput LC/MS/MS system for quantitation.

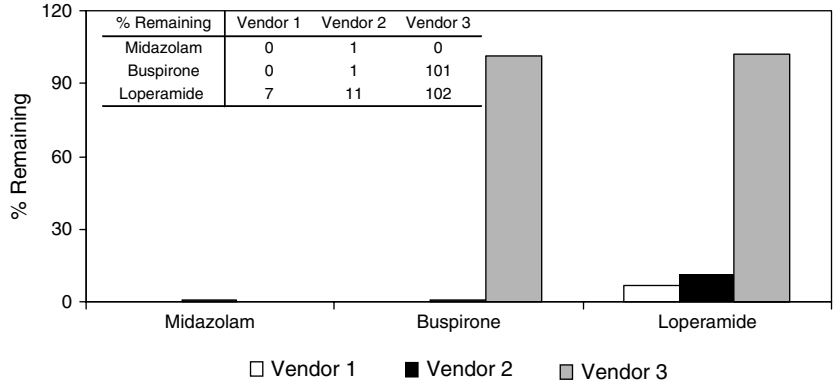

Figure 29.9 ► Vendor-to-vendor variation in findings from rat liver microsomes. (Reprinted with permission from [12].)

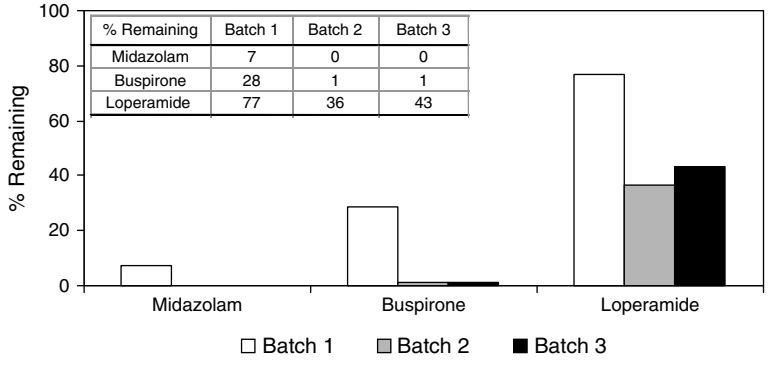

Figure 29.10 ► Batch-to-batch variation in findings from rat liver microsomes from the same vendor. (Reprinted with permission from [12].)

is a lower rate of metabolism for ER-bound enzymes. One application of S9 used 2.5 mg/mL protein compared to the normal 0.5 mg/mL protein for microsomal stability assays.[15]

An advantage of S9 is that it accesses extramicrosomal enzymes, such as sulfotransferase, alcohol dehydrogenase, and *N*-acetyltransferase. It is especially useful for compounds having moieties that are prone to metabolic reactions catalyzed by extramicrosomal enzymes. (An alternative is to mix microsomes and cytosol.) As shown in Figure 29.1, S9 typically is used for more detailed study of a selected set of compounds. S9 also is often used for producing metabolites for structure elucidation to ensure a broad coverage of possible metabolic reactions.

29.2.4 In Vitro Hepatocytes Assay for Metabolic Stability

Metabolic stability studies can be conducted using living hepatocytes.[16–19] Isolated hepatocytes closely parallel the conditions of in vivo liver cells,[19] having a complete ensemble of metabolizing enzymes of all isoforms, cofactors, cellular components, and membrane permeation mechanisms (e.g., passive, transporter). Hepatocytes are used immediately as primary cell cultures, are directly prepared from livers, and can be cryopreserved for later use.[17] They also can be used as "sandwich cultured hepatocytes" for uptake and biliary excretion studies.[4] Hepatocytes are available from several suppliers (Table 29.3). They are considerably more expensive than microsomes. Cryopreserved hepatocytes are convenient because they do not depend on the immediate availability of fresh livers. They can be stored for later use, thawed just before the assay time, and used in suspension. It is prudent to check hepatocytes for activity levels of key metabolizing enzymes because expression of metabolizing enzyme can vary as a result of isolation and with the characteristics of the donor.[19] Pooled hepatocytes from several donors are available. Hepatocytes should be used within a few hours before activity diminishes.

Hepatocytes contain all of the enzyme cofactors and physiological concentrations, so they do not require supplementation with NADPH and other cofactors. Some discovery scientists believe that hepatocytes are better correlated to expected in vivo metabolism than other metabolic stability materials. A much lower hepatocyte $t_{½}$ than microsome $t_{½}$ may indicate significant extramicrosomal or phase II metabolism for the compound.

Fresh cells are more apt to adhere to the cell culture surface. Otherwise, the cells are used in suspension. As with S9 and phase II assays, hepatocytes are used for selected compounds for more detailed studies, although high-throughput approaches using 96-well plates are being used.[18] A commercial product (Qualyst, Table 29.3) is a sandwich-cultured hepatocyte system that predicts in vivo hepatic uptake, metabolism, and biliary clearance in one assay.[4]

29.2.5 In Vitro Phase II Assay for Metabolic Stability

Phase II metabolic stability assays are performed using liver microsomes, hepatocytes, or S9.[20,21] Use of this assay is suggested when a compound has a moiety that is susceptible to phase II conjugation (e.g., phenol, aliphatic hydroxyls) to evaluate the stability of a compound against glucuronidation. Some drugs are primarily cleared by glucuronidation.[19,21] Sulfation usually is studied using S9 because the sulfotransferase enzyme is located in the cytosol. Sulfation is rapid compared to glucuronidation but has low capacity and is saturable.

Phase II metabolic reactions in microsomes, cytosol, and S9 require cofactors. UDPGA is required for glucuronidation, and 3′-phosphoadenosine-5′-phosphosulfate (PAPS) is necessary for sulfation.

The UDP-glucuronosyltransferases (UGTs) have many isozymes. UGTs are found on the inside of the ER membranes and remain inside the liver microsomes prepared from ER membranes. The membrane may reduce access of some compounds to the UGTs. Use of the pore-forming peptide alamethicin at 50 μg/mL of protein in the assay medium is thought by some researchers to increase the rate of in vitro glucuronidation without affecting the enzyme activity. Other researchers find little difference use of Mg^{2+} at 1 mM also increases the rate of glucuronidation.[20]

Glucuronidation is a rapid reaction relative to CYP oxidation. Therefore, if a compound must be hydroxylated by CYP prior to glucuronidation, the CYP reaction is the rate-limiting step and glucuronidation follows immediately. A glucuronidation assay with liver microsomes uses the same conditions and detection as a microsomal assay, except UDPGA is added to the medium at 1.9 mg/mL. Another method uses [^{14}C]UDP-glucuronic acid in the incubation, separates the metabolite(s) from the unreacted UDPGA by solid-phase extraction, and measures the ^{14}C-labeled metabolites using a scintillation detector.[22]

29.2.6 Metabolic Phenotyping

This assay examines which isozymes metabolize the compound (i.e., differentiates among CYP isozymes).[23] This experiment is performed by incubating the test compound with individual isozymes under the same conditions as the microsomal stability assay. The percent remaining of test compound after incubation is quantitated using LC/MS techniques. The relative $t_{½}$ for each isozyme (Figure 29.11) indicates whether the compound is a substrate and the rate of the reaction. Metabolic phenotyping has also been performed with UGTs.[22]

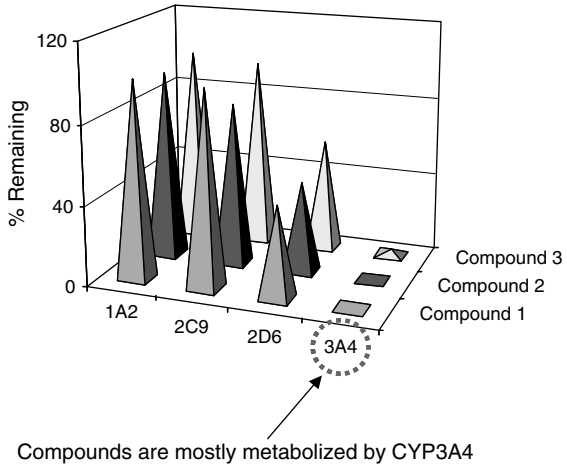

Figure 29.11 ► Metabolic phenotyping. Incubation of three compounds with individual rhCYP isozymes indicates which isozyme(s) metabolizes the compound and to what extent.

Metabolic phenotyping has been performed using the microsomal assay and adding an inhibitor that is specific for an isozyme. An increase in $t_{½}$ in the presence of the inhibitor is an indication that the isozyme is important in the metabolism of the compound. However, once this isozyme has been inhibited, it is possible for one or more other isozymes to metabolize an increased portion of the compound (colloquially termed *metabolic switching*) and result in a similar $t_{½}$.

Metabolic phenotyping has been used together with structural knowledge of the isozyme's active site or substrate specificity (see Chapter 11) to guide structure modifications intended

to make the molecule less prone to reaction at the particular isozymes. Metabolic phenotyping data also are necessary for early human clinical studies because they indicate which CYP inhibitors may interact with the new drug (see Chapter 15). Possible drug–drug interactions must be studied using co-dosing experiments during clinical development. It is advantageous if a new clinical candidate is metabolized by three isozymes or more so that if one metabolic route is blocked by a coadministered inhibitor drug, then clearance can occur by "metabolic switching" to another uninhibited isozymes.

29.2.7 In Vitro Metabolite Structure Identification

During the active chemical synthetic phase of discovery optimization, it is useful to determine the structures of the major metabolites of a lead so that they can be synthesized. This can enable testing for activity and toxicity. Metabolite structures also can enable synthetic modifications to block metabolism and increase stability. A selected series lead is incubated with liver microsomes or another metabolic material, and the structures of the metabolites are elucidated using spectroscopy.

A rapid profile of the major metabolites can be obtained using microsomal incubation, followed by LC/MS/MS analysis (Figure 29.12).[24–27] First, the parent compound is analyzed to obtain the HPLC retention time, molecular ion (e.g., MH^+), and MS/MS product ion spectrum (Figures 29.12 and 29.13). Modern instruments allow these data to be obtained from a single HPLC injection using automated "data-dependent" analysis. The MS/MS product ion spectrum is interpreted using the scientist's experience and software (e.g., Mass Frontier from Thermo Fisher) to assign specific product ions to specific substructures of the molecule. A microsomal extract control containing the compound and incubation components but quenched at time zero is analyzed. The actual microsomal incubation extract is injected, and components that appear in the incubated sample and not in the microsomal extract control likely are metabolites (Figure 29.14). Their molecular weights determine the mass difference from the parent compound. This can be interpreted in terms of common metabolic reactions (e.g., +16 for hydroxylation or *N*-oxidation, +32 for dihydroxylation, –14 for demethylation). Interpretation of the MS/MS product spectra of each metabolite (Figure 29.15) demonstrates the mass shifts in specific substructures and indicates the substructure that was metabolized. In some cases, the metabolite is unambiguously elucidated (e.g., dealkylation).

In other cases, such as that shown in Figure 29.15, the specific position of hydroxylation is not determined and NMR is required.[24–27] In these cases, the microsomal incubation

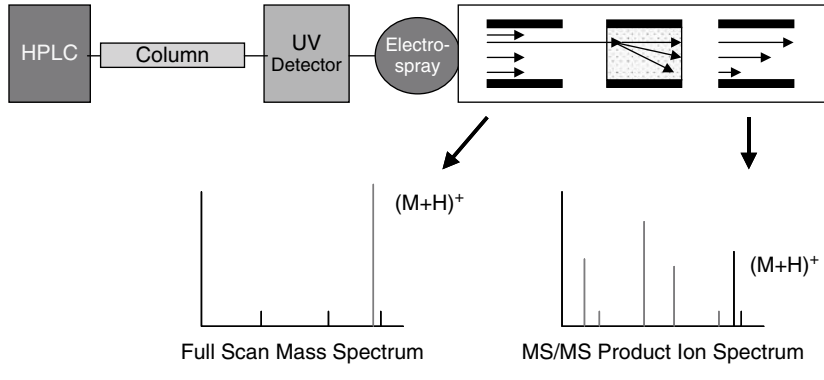

Figure 29.12 ► Diagram of LC/MS/MS system and its use in determining the molecular weight of metabolites and MS/MS product ion spectra.

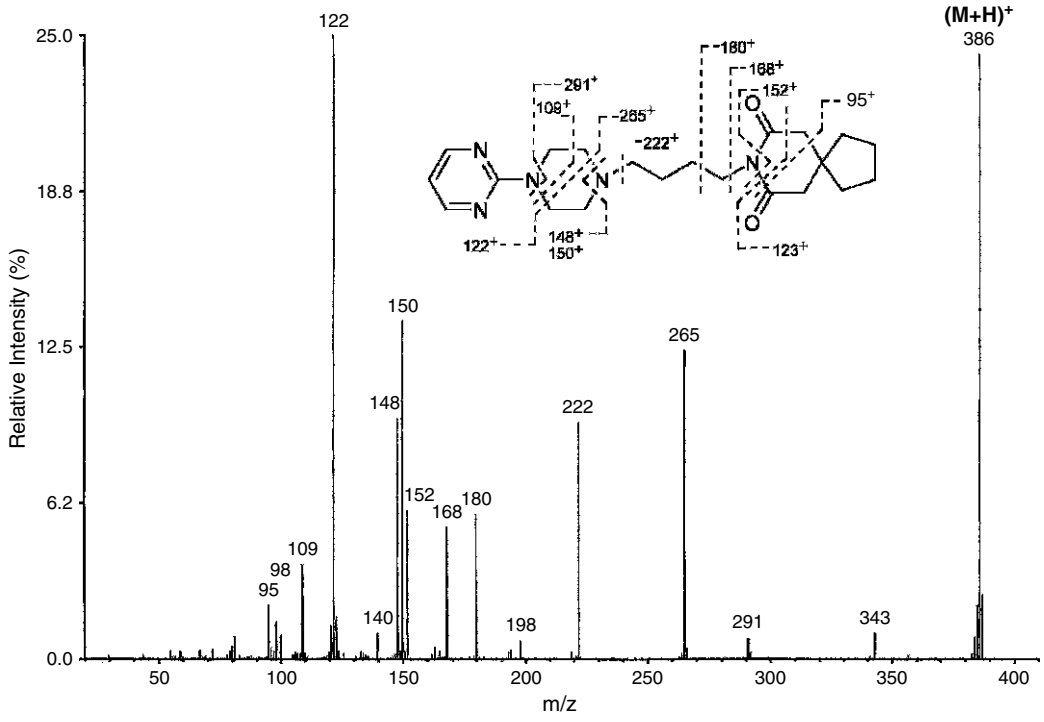

Figure 29.13 ► MS/MS product ion spectrum of buspirone and assignment of substructures to MS/MS product ions. (Reprinted with permission from [24].)

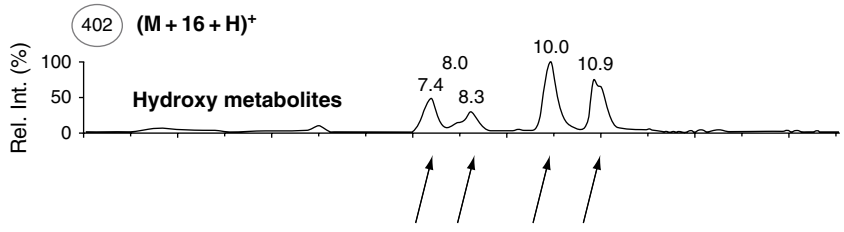

Figure 29.14 ► Selected ion chromatogram of m/z 402 of microsomal incubation of buspirone. Four (M+16) hydroxyl buspirone metabolites are indicated. (Reprinted with permission from [24].)

is repeated at a larger scale. The specific metabolite is isolated using HPLC. The HPLC peak can be monitored using UV detection or MS detection of the specific metabolite molecule ion. Fractions from multiple injections are collected in order to obtain 10 to 50 μg of the metabolite. The mobile phase is removed using low heat and vacuum or inert gas impingement.

Often ^{1}H-NMR is sufficient for determining the site of metabolism. The spectrum of the compound is first obtained and the resonances assigned. Then the spectrum of the metabolite is obtained and changes in the resonances examined. Figure 29.16 shows an example where the hydroxylation was determined to be at C_{17} based on the lack of the C_{17} proton in the ^{1}H-NMR spectrum of the metabolite.

An iterative strategy for optimizing the metabolic stability of a lead series is shown in Figure 29.17. Metabolic assays provide a quantitative stability assessment of the compound. If its stability is low, the specificity for the metabolizing isozyme(s) can be determined

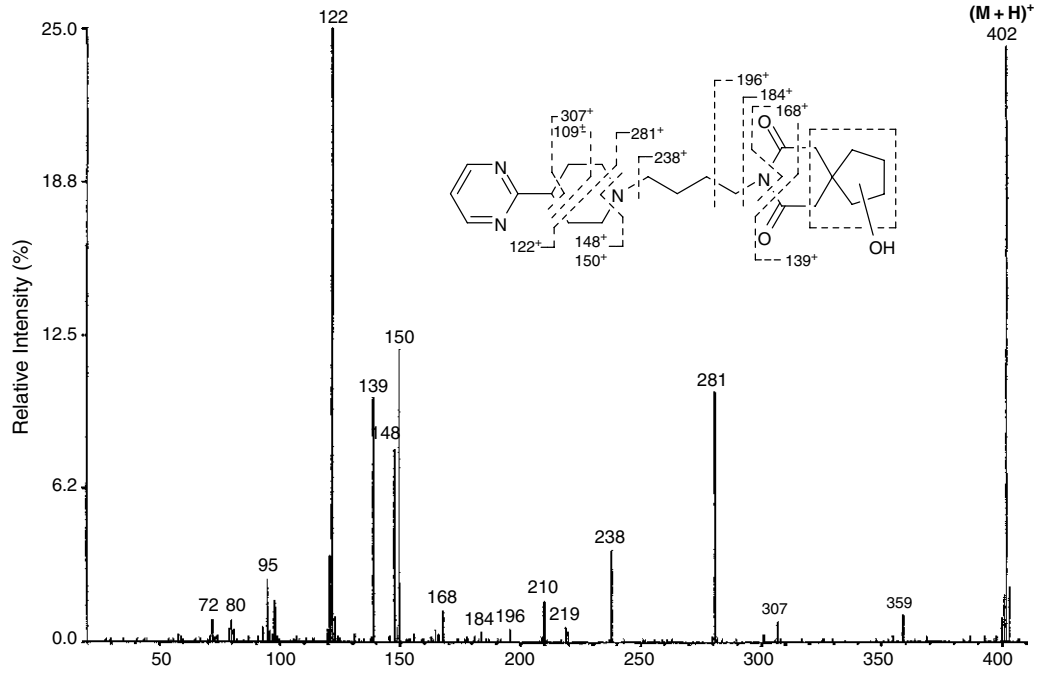

Figure 29.15 ► MS/MS product ion spectrum of a (M+16) buspirone metabolite. (Reprinted with permission from [24].)

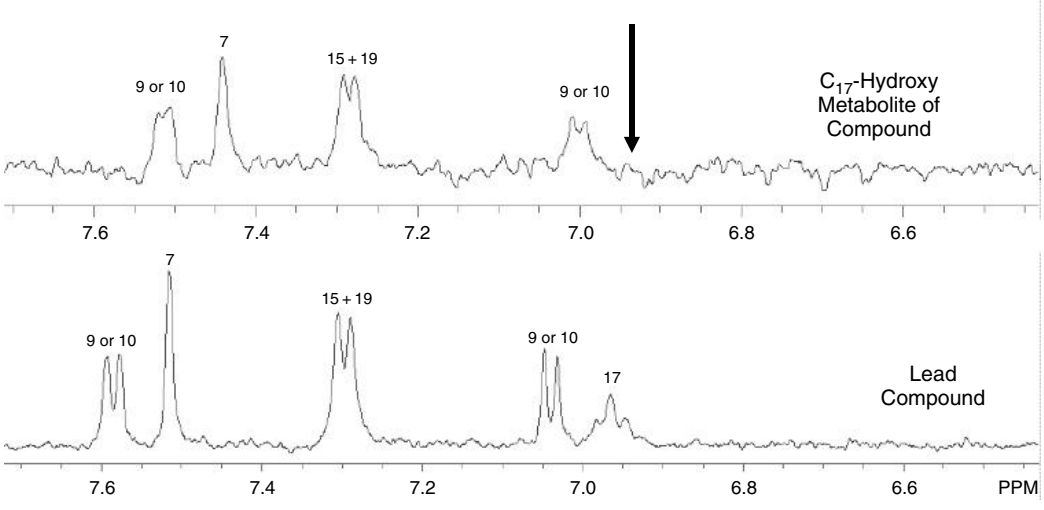

Figure 29.16 ► H-NMR spectra of the aromatic region for a discovery lead and 10 µg of isolated hydroxyl metabolite. The proton signal at C_{17} in the spectrum for the compound was not present in the spectrum for the metabolite, indicating the hydroxylation had occurred at C_{17}.

and the metabolite structures elucidated. The structure can be modified to block metabolic hotspots, and the new compound can be assayed for its stability. The metabolites themselves also can be synthesized to test them for pharmacological activity, for activity at other targets where side effects may be produced, and for safety testing.

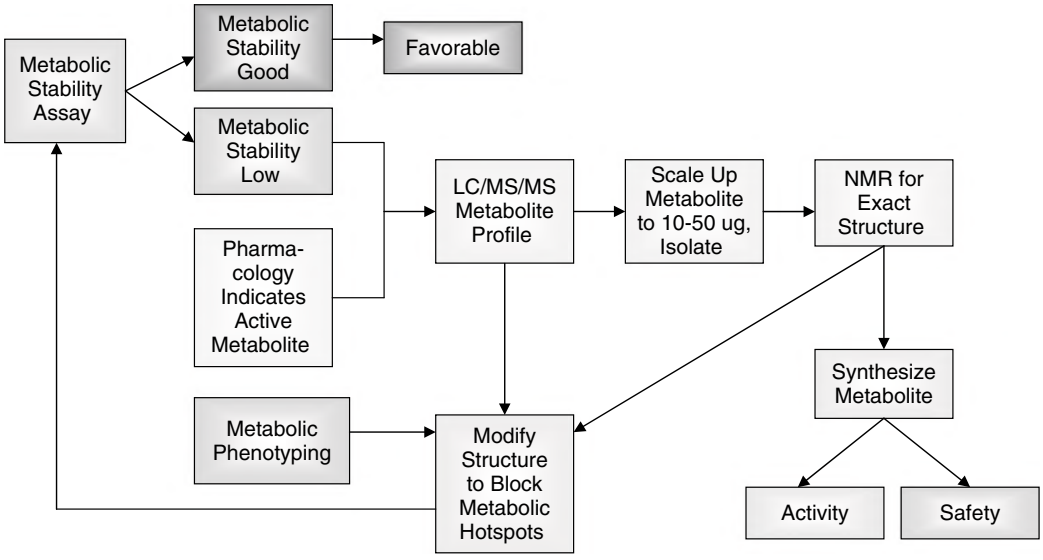

Figure 29.17 ► Strategy for metabolic stability assessment and optimization. Metabolites also can be synthesized to test their activity and safety.

Problems

(Answers can be found in Appendix I at the end of the book.)

1. What does S9 or hepatocyte incubation provide compared to microsome incubation?
2. When is it useful to screen for phase II metabolic stability?
3. Which metabolic stability materials include CYP enzymes? Which contain sulfotransferase enzymes?
4. What cofactor provides the energy for the CYP reaction?
5. Why is it best to screen metabolic stability at low concentration ($\sim$1 μM)?
6. Why is it useful to measure metabolic stability in multiple species?
7. Why is it possible to screen metabolic stability using only time zero and one additional concentration time point?
8. Why is it important to check the activity of each batch of metabolic stability material (e.g., microsomes)?
9. What are the cofactors for glucuronidation and sulfation?
10. What are the cofactors for the hepatocyte metabolic stability assay?
11. What is metabolic phenotyping useful for?
12. Why is structure elucidation of major metabolites useful?
13. When are LC/MS/MS and NMR used for structural studies of metabolites?

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Chapter 30

Plasma Stability Methods

Overview

- *Compounds are incubated with plasma in vitro to predict in vivo stability.*
- *Identification of decomposition products indicates the hydrolytically labile sites.*

Hydrolysis in plasma can be a major route of clearance for compounds having certain hydrolytically labile functional groups. Incubation with plasma in vitro can quickly determine if a compound is susceptible to plasma degradation and to what extent. It also supports structure modification studies to reduce plasma degradation. Identification of the plasma decomposition products indicates which group in the molecule is being attacked. It also is important for pharmacokinetic (PK) studies to determine if a compound is stable in plasma after the sample is taken and stored.

Plasma is a fraction of blood that is prepared as follows. Blood is collected into a vessel containing an anticoagulant (e.g., heparin) and mixed immediately. The liquid is centrifuged to remove cells, and the clear fluid is the plasma. It can be used immediately or stored frozen at –80°C for later use. It can be transported frozen on dry ice.

Several in vitro methods for plasma stability have been published. The emphasis of these articles has often been on high-throughput instrumentation using direct plasma injection liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) with restricted access high-performance liquid chromatography (HPLC) columns,[1,2] automated HPLC column switching,[3] and robotic sample preparation.[4] Conditions for plasma stability assays are quite diverse with regard to compound concentration (3 μM to 6 mM), percent organic solvent (0%–5%), and dilution of plasma with buffer before use (0- to 4-fold).[5–11]

Di et al.[12] investigated the effects of method conditions. They found no significant difference in percent remaining for most compounds over the concentration range from 1 to 20 μM, indicating that plasma stability is not sensitive to drug concentration up to 20 μM. Saturation of plasma enzymes has been reported at 100 μg/mL.[9] A test compound concentration of 1 μM is suggested because it reflects an average plasma concentration after dosing. Percent dimethylsulfoxide (DMSO) up to 2.5% had little effect on the assay. At higher percentages of DMSO, the solvent may denature the enzyme or interfere with protein binding, causing a decrease in activity. Dilution of plasma with buffer does not reduce the hydrolytic rate until it is diluted to 20% to 40% plasma in buffer. Plasma appears to have high catalytic capacity. Plasma pH increases to pH 8 to 9 during long-term storage have been reported; therefore, it is advisable to dilute plasma with pH 7.4 buffer so that stability results are not complicated by decomposition due to a basic pH. Plasma enzyme activity does not appear to decrease for at least 22 hours at 37°C. A 3-hour incubation time is suggested as consistent with in vivo dosing experiments that often are conducted out to 6 hours in discovery. Plasma should be centrifuged (e.g., 3,000 rpm, 10 minutes, 10°C) to remove particulates prior to the assay. Overall, plasma stability assays are relatively tolerant

of varying experimental conditions compared to microsomal stability assays[12]; however, consistent use of optimized conditions is prudent.

Plasma for in vitro stability studies is readily available from many suppliers (Table 30.1). Activity can vary greatly with vendor and batch (Figure 30.1) and is compound dependent.[12] A 2- to 20-fold difference can occur. This could alter the compound ranking in a discovery project if assays are performed with different plasma batches. Careful quality control (QC) and adjustment of incubation time to produce the same extent of degradation are suggested for each new plasma batch that enters the laboratory. Alternatively, some compounds can be rerun to "bridge" datasets from different plasma batches. The best solution is to purchase a large batch of plasma to use for an extended time.

TABLE 30.1 ► Partial List of Commercial Suppliers of Plasma for Stability Studies

Company	Product	Web site
Bioreclamation	Animal plasma	*www.bioreclamation.com*
Innovative Research	Animal and human plasma	*www.innov-research.com*
Taconic	Animal plasma	*www.taconic.com*

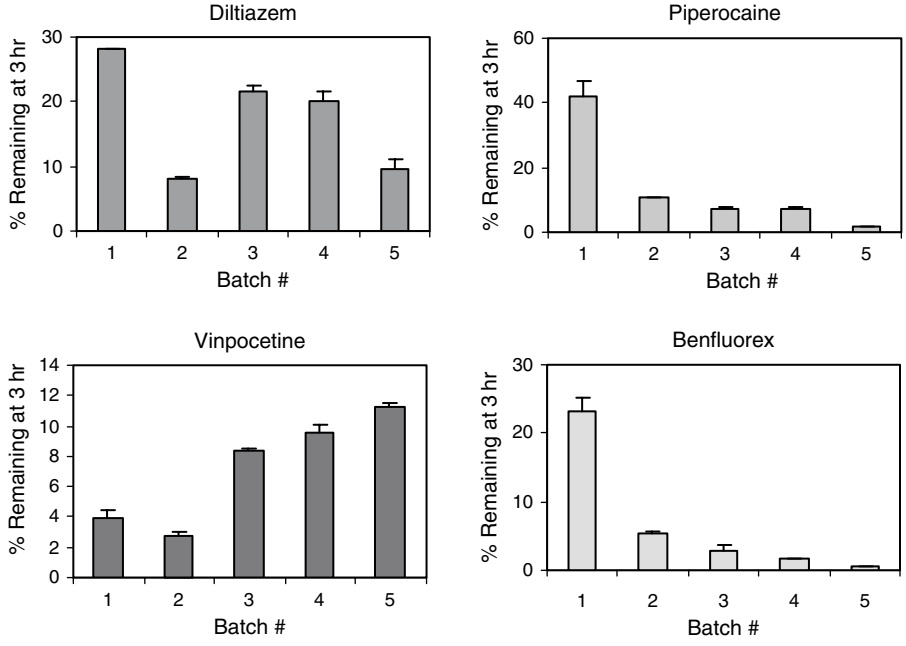

Figure 30.1 ► Batch-to-batch variation in plasma from the same vendor.

Detection and quantitation of test compounds after plasma stability incubation are performed using LC/MS techniques (see Chapter 29). Plasma contains many components that may interfere with quantitation; thus, a detection instrument with good selectivity is important.

Few companies assay plasma stability for all new compounds because it is only a problem for compounds having functional groups that are susceptible to hydrolysis. The most common plasma for use in a generic assay is Sprague-Dawley male rat plasma (heparinized and filtered). Because plasma stability varies with species, many discovery project teams

may prefer to use a relevant animal species for their particular project. This often is the efficacy species used for the project's pharmacology experiments. Human plasma stability is important to obtain when planning for human clinical studies. Proper safety precautions should be followed when using human plasma to avoid disease.

A diagram of the plasma stability assay is shown in Figure 30.2.[12] To each well is added 195 μL of plasma that has been diluted 1:1 with phosphate buffer (pH 7.4). Five microliters of DMSO stock compound solution 40 μM is added to obtain a final test compound concentration of 1 μM in the plasma and final DMSO content of 2.5%. The plate is sealed with a plate mat, vortexed, and placed on a 37°C shaker (gentle) for 3 hours. The reaction is quenched with 600 μL of cold acetonitrile, followed immediately by mixing and centrifugation. The time zero samples are quenched immediately after the sample is added to plasma. The supernatant (400 μL) is transferred to a 96-well plate for LC/MS analysis. As discussed for metabolic stability assays, it is sufficient to use a single time point concentration plus the initial (time zero) concentration for the calculation of $t_{½}$. No problems are anticipated for low-solubility compounds in this assay because of the high protein and DMSO concentrations, which help solubilize compounds. Use of a laboratory robot for the assay is efficient if sufficient samples are to be assayed.

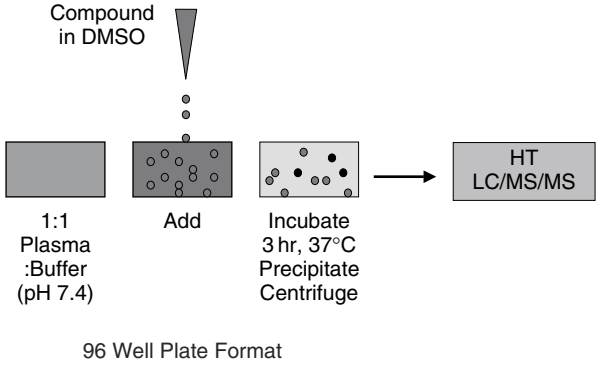

Figure 30.2 ► Plasma stability assay overview.

It is important to co-assay QC controls with each set of test compounds. These ensure that the assay is operating properly and that the activity of the plasma is consistent with established criteria. Four suggested QC compounds are shown in Figure 30.3, and others have been discussed.[12] The assay described here produces consistent QC results over a range of experimental dates.

As with metabolites, it is useful to obtain structural information about plasma degradation products. This provides useful guidance during the active chemical synthetic phase of discovery optimization. Structural modifications can be made to reduce or eliminate plasma hydrolysis reactions. When more than one site in the molecule might be hydrolyzed, these data indicate which site(s) is labile. Often, LC/MS (single-stage) analysis with molecular weight determination of the degradants is sufficient because plasma hydrolysis reactions occur at predictable sites in the molecule, and the molecular weights of the putative products are easily calculated. Because hydrolysis can produce acidic, basic, or neutral products, it is useful to operate the mass spectrometer in alternating positive and negative ion polarities to ensure detection of all of the degradation products. For more detailed structural studies, the LC/MS/MS and NMR techniques, as described for metabolite structure elucidation (see Section 29.2.6), can be used.

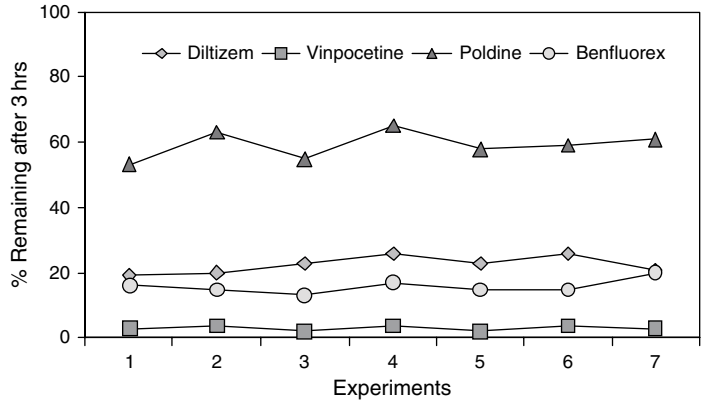

Figure 30.3 ► QC standards and results over multiple experiments are reproducible. (Reprinted with permission from [12].)

Problems

(Answers can be found in Appendix I at the end of the book.)

1. What is in plasma that makes compounds unstable?
2. Before using plasma from a new preparation or vendor batch, it is good to do what?
3. Are the following statements true or false?: (a) plasma stability assays are highly sensitive to % DMSO, buffer dilution, and substrate concentration, (b) plasma stability varies with species, (c) degradation in plasma will increase clearance in vivo, (d) stability in plasma is important for accurate PK study results, (e) QC controls are not important for plasma stability assays because the results are so consistent.

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Solution Stability Methods

Overview

- *Decomposition in various in vitro and in vivo solutions can confuse research if it is not recognized.*
- *Reactions can occur in bioassay matrix, dosing solution, or gastrointestinal fluids.*
- *Compounds can be incubated in relevant solutions in well plates and analyzed by liquid chromatography/mass spectrometry.*

Solution stability encompasses a broad range of drug barriers. Chapter 13 discussed the most frequently encountered solution stability issues: biological assay buffers, pH, and in vivo gastrointestinal fluids. (Plasma stability was discussed in Chapters 12 and 30.) Because of the diversity of solution decomposition situations and conditions, the testing of all new company compounds using generic high-throughput solution stability assays is not common. Instead, assays often are performed to prospectively check compounds that contain groups that might be unstable or to retrospectively diagnose unexpectedly poor performance in an in vitro biological assay, pharmacokinetics, or in vivo pharmacology. Solution stability assays are customized to the specific physicochemical conditions (e.g., pH, temperature, time, ionic strength, light), solution components (e.g., enzymes, buffer, modifiers, excipients, oxygen), and protocol that are relevant to the team's solution stability questions. In-depth methods are performed on late discovery compounds that are being considered for clinical development, in order to meet established criteria for advancement. In vitro methods for chemical stability have been reviewed.[1,2]

31.1 General Method for Solution Stability Assays

Solution stability assays have a unique problem with quenching the reaction. If the solution is injected into a high-performance liquid chromatograph (HPLC) column immediately at a particular incubation time point, the concentration of the test compound is accurately measured. However, if a period of time passes before the solution is injected, then the compound continues to be exposed to the solution conditions and can continue to react. In the past, stability studies were performed individually for a few compounds, and they could be immediately manually injected. However, with larger numbers of discovery compounds and more frequent study of solution stability, assays must be automated and performed in 96-well plates. Injection of the solution in the last well in the plate can occur 7 hours after injection of the solution in the first well (with a 5-minute analysis cycle). Thus, the incubation time incrementally lengthens for later samples in the plate, and different wells have different incubation times. Short-duration stability studies that simulate intestinal transit time or in vitro assays are particularly affected. For a pH stability study, one might assume that the reaction can be quenched by titration to pH 7.4, but compounds still may be unstable at neutral

pH, and they continue to be exposed to solution components (e.g., enzymes, excipients). For most solutions (e.g., bioassay buffer), there is no way to effectively quench the reaction.

One answer for this problem is to use a multichannel HPLCs that simultaneously analyze many samples.[3] Nanostream (*www.nanostream.com*) and Eksigent (*www.eksigent.com*) offer instruments that can simultaneously analyze 8 or 24 samples by HPLC with UV detection. This allows studies of 24 solution conditions or compounds at the same time. These systems are not directly coupled to a mass spectrometer (MS) for online liquid chromatography (LC)/MS analysis.

Another approach is to start the incubation of each sample at a different time preceding the time zero injection. The samples are spaced according to their HPLC run time so that they will have the same incubation time at the time they are injected. This process is complicated and time consuming for 96-well plates.

Others groups have frozen samples at –80°C to stop the reaction. Individual samples are thawed just prior to HPLC injection. In this way, the samples have the proper incubation time.[4] This process requires manual manipulation and is time consuming.

An efficient and widely applicable method that solves the quenching problem is use of a conventional HPLC instrument that has an autosampler (e.g., Agilent 1100) capable of programmed liquid handling.[5] Into the autosampler are placed two 96-well plates, one containing dimethylsulfoxide (DMSO) stock solutions of the test compounds and the other containing wells filled (e.g., 180 µL) with the stability solution (Figure 31.1). The autosampler is programmed using the system software (e.g., Chemstation) to perform the following steps:

1. Transfer test compound (e.g., 20 µL) from the DMSO stock to the stability solution
2. Thoroughly mix the combined solution
3. Incubate the samples at a programmed temperature (e.g., 37°C)
4. Remove solution at programmed time points
5. Inject solution into the HPLC
6. Manage the other samples in the same manner by interlacing them

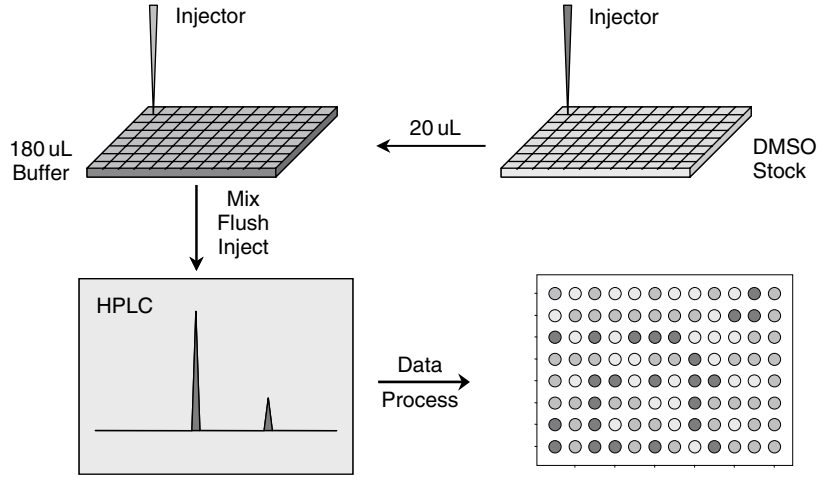

Figure 31.1 ► Application of a programmable HPLC autosampler to perform the liquid handling, incubation, and injection steps of the solution stability assay and obtain reliable time point data. (Reprinted with permission from [15].)

Programmed injections provide the exact time point for each data point, which is useful for accurate kinetic analysis and avoids the need to quench the reaction. This system is readily interfaced to a MS, which is valuable for high sensitivity and spectroscopic structural analysis. Structural data for the reaction products facilitate rapid identification of the labile sites in the structure and complement the kinetic stability results. Figure 31.2 shows a typical instrument used for this assay.

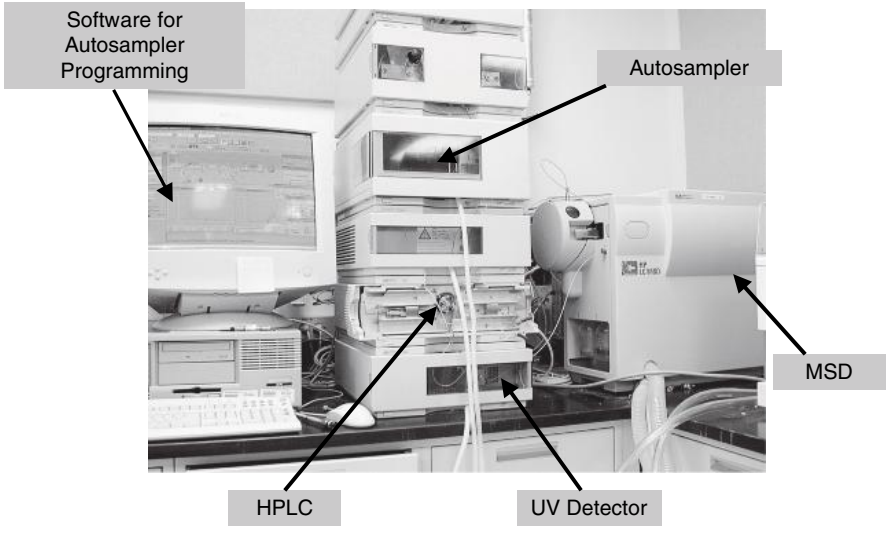

Figure 31.2 ► Conventional LC/UV/MS instrument (Agilent 1100) with a programmable autosampler that can be used for solution stability assays as shown in Figure 31.1.

This method has been demonstrated to be robust for stability studies in biological assay buffers, simulated gastric fluids, dosing vehicle, and pH stability studies.[5] Minimal analyst time and intervention are required. The assay can run unattended overnight, on weekends, or on holidays. It can be flexibly applied for custom assay design, such as simultaneous testing of 1 to 96 compounds, one to multiple time points, and different solution conditions on the same plate. Examples of applications of this method include single time point pH solution stability screening (Figure 13.7), high-throughput single time point study of the stability of 96 compounds in bioassay buffer (Figure 13.8), and kinetic analysis of one compound at multiple pHs (Figure 13.6). The last application is an example of the use of high-throughput methods to provide enhanced detail for one compound, as opposed to the more conventional use of high-throughput methods to provide single time point data for a large number of compounds. Kinetic analysis of the data can be performed using Scientist software (Micromath Research, St. Louis, MO, USA).

31.2 Method for Solution Stability in Biological Assay Media

If erratic results are obtained from bioassays, the cause may be low compound solubility or instability in the bioassay buffer. A set of project compounds can be rapidly screened, using the method discussed in Section 31.1, to check compound stability. The assay should mimic as closely as possible the conditions and protocol of the biological assay to ensure meaningful results. The assay conditions should ensure compounds are completely soluble in stability assays, either using co-solvent or by lowering the screening concentration. If compounds

precipitate during the assay, the area counts for the test compound will decrease and lead to inconclusive results. Alternatively, percent purity can be used to monitor formation of degradation products, which is less sensitive to solubility and precipitation. An example is shown in Figure 13.8.

31.3 Methods for pH Solution Stability

Compounds in drug discovery encounter a wide range of pHs. Many assay buffers and physiological fluids are at pH 7.4. Oral dosing exposes compounds to pH 1 to 2 in the stomach, pH 4.5 at the beginning of the small intestine, pH 6.6 as an average pH for the small intestine, and pH 5 to 9 in the colon. These are useful pHs for profiling discovery compounds.

An integrated generic method profiles solution stability at multiple pHs (e.g., 2, 7, 12) and oxidation with 3% hydrogen peroxide in a 96-well plate using a Gilson 215 robot.[6] Compounds were incubated at 100 μM, and low-solubility compounds were kept in solution using 1:1 acetonitrile to buffer. (The reaction rate at this high organic content may be low compared to 100% aqueous because of a low dielectric constant. An alternative is to reduce the test compound concentration, allowing a lower organic content.) Samples were immediately injected into the HPLC at their time points.

The decomposition of compounds under acidic conditions in the stomach have been screened in solution at pH 2 for 75 minutes to simulate the time spent in the stomach.[7] These data were combined with solubility data for a "liberation ranking" as part of prospectively predicting oral bioavailability classification prior to in vivo dosing.

pH stability studies have been used for in vitro evaluation of prodrugs.[8] Solution stability studies used buffers at 0.02 M concentration with acetate (pH 5.0), phosphate (pH 3.0, 6.9, 7.4), borate (pH 8.5 and 9.75), and hydrochloric acid (pH 1). Constant ionic strength was maintained because of the effect of ionic strength on reaction rate. The experiment was run at 100 μM in an HPLC vial, with samples withdrawn at selected time points and immediately injected into the HPLC column.

The programmable autosampler technique described in Section 31.1 is very useful for automated custom pH solution stability studies.[5] Buffers of pHs that are relevant to the pH stability issues of the particular project team are placed into wells of the 96-well plate. The autosampler adds test compound solution to the wells, mixes, incubates at 37° C, and injects into the LC/MS system at programmed time points. The data from such an experiment are shown for a β-lactam compound in Figure 13.6. Such an assay provides reliable kinetics that can be used during discovery and development.

31.4 Methods for Solution Stability in Simulated Gastrointestinal Fluids

Important insights can be obtained from incubating discovery compounds with simulated gastrointestinal fluids. These include simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). These materials are specified in the United States Pharmacopeia (USP).[9]

The components of SGF are as follows:

- Pepsin (an acidic protease)
- pH 1.2 adjusted using HCl
- NaCl

SGF simulates stomach fluid and incorporates acidic and enzymatic hydrolysis conditions. The components of SIF are as follows:

- Pancreatin (mixture of amylase, lipase, and protease from hog pancreas)
- pH 6.8 using monobasic phosphate buffer adjusted with NaOH

SIF mimics the pH and hydrolytic enzymes in the intestine.

The main purpose of these assays is the prediction or diagnosis of stability after oral dosing. The data guide medicinal chemists in structural modifications to improve GI stability for optimizing bioavailability and prioritization of compounds for in vivo studies.

The stability of compounds with these materials is readily determined using the solution stability assay system described in Section 31.1. Incubation in these fluids is a rapid way to determine if a compound will be stable under conditions found in the GI. The compounds shown in Figure 13.9 have a range of stabilities in SIF and SGF, with compounds 1 and 3 having favorable profiles.

31.5 Identification of Degradation Products from Solution Stability Assays

The structure elucidation of decomposition products from solution stability experiments follows the same methodology as discussed for plasma stability (see Chapter 30) and metabolic stability (see Section 29.2.6). Molecular weight data from a single-stage MS attached to the HPLC provides valuable information for the reaction products. The hydrolytically labile sites in a molecule usually are obvious; thus, the molecular weight of putative decomposition products can be rapidly calculated and compared to the results. Such information is superior to quantitative data alone because it can guide medicinal chemists in making structure modifications to improve stability.

31.6 In-Depth Solution Stability Methods for Late Stages of Drug Discovery

In late stages of drug discovery, in-depth assessment usually is undertaken of the solution stability of just a couple of potential development candidates from a project. Standard assays are used for the scrutiny of all compounds so that the data are comparable across all projects and are applicable to established advancement requirements. The assays often are similar to those suggested by the Food and Drug Administration for regulatory filings in new drug applications[10,11] but may not be as rigorous. The following are examples of stability experiments that often are performed in aqueous solutions to model drug barriers:

- pH: Aqueous buffers (37°C, pH 1–12)
- Oxidation: 3% hydrogen peroxide in pH 7.4 buffer for 10 minutes
- GI: Simulated intestinal fluid (USP, 37°C, 1–24 hours)
- GI: Simulated gastric fluid (USP, 37°C, 1–24 hours)
- GI: Simulated bile/lecithin mixture (USP, 37°C, 1–24 hours)
- Plasma: Human plasma (37°C, 1–24 hours)

- Light: High-intensity cool white fluorescent light (200 watt h/m^2, 1.2 million lux hour, room temperature, 1–7 days)
- Temperature: Heat (30–75°C, 1–7 days)

For studies over a long time, vials can be prepared for each condition and placed into an HPLC autosampler. Samples of each reaction condition are injected at time points. This is convenient because of the low number of compounds being evaluated. Kinetics can be calculated and used to predict long-term stability. Co-solvent can be added to help dissolve insoluble compounds.

Problems

(Answers can be found in Appendix I at the end of the book.)

1. List some of the solution conditions under which compounds may be unstable or which may accelerate decomposition.
2. What is a difficulty of solution stability assays?
3. What approach can be used to effectively and efficiently run solution stability assays?
4. What conditions should be used for solution stability studies?
5. In addition to percent remaining at a certain time point, what additional data can be obtained from solution stability studies and how can they be used?
6. For what purposes can solution stability data be used?

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CYP Inhibition Methods

Overview

- *CYP inhibition is assessed by coincubating the compound, substrate, and isozyme.*
- *Reduction in substrate metabolic rate indicates inhibition by the compound.*
- *Substrate concentration should be at the K_m for the isozyme. Less than 10% of substrate and compound should be metabolized for accurate measurement.*
- *Double cocktail method uses a mixture of substrates and of recombinant CYP isozymes with liquid chromatography/mass spectrometry.*

Cytochrome P450 (CYP) inhibition is a major cause of drug–drug interactions (DDI). Because DDI can lead to toxicity, considerable attention has been given to methods for measuring CYP inhibition. Most pharmaceutical companies have implemented CYP inhibition testing during the discovery process. A working group of experts from Pharmaceutical Research and Manufacturers of America (PhRMA) companies has published a consensus paper on DDI testing.[1] Measurement of CYP inhibition during discovery provides early warning of potential safety issues. It is used with other data to select leads and helps develop structure–CYP inhibition relationships that are used by medicinal chemists to overcome CYP inhibition by structural modification.[2–9]

In vitro CYP inhibition data are important in late discovery for planning human clinical studies.[10–12] In vitro CYP inhibition data indicating minimal inhibition are allowed by the Food and Drug Administration (FDA) for concluding that the clinical candidate lacks CYP inhibition potential and does not require clinical DDI studies. Approaches have been developed to predict in vivo DDI using in vitro data,[13] but other researchers have shown this to be a difficult task.[14]

Leaders in the field continue to work on optimum standardized methods for CYP inhibition testing.[15] For efficient discovery research and for planning of clinical DDI studies, high-quality in vitro data are crucial.

32.1 In Silico CYP Inhibition Methods

Two software companies offer tools for predicting CYP inhibition (Table 32.1).

Several groups have studied the in silico prediction of CYP inhibition.[16–24] The greatest current use of in silico CYP inhibition methods appears to be screening compound libraries for compounds with potential problems and guidance of medicinal chemists during the structure optimization stage on modifications that may yield reduced CYP inhibition.[25] Computational docking to individual CYP isozymes structures using information from contemporary reports[26] should enhance the in silico predictions of the future.

TABLE 32.1 ► Commercial Software for CYP Inhibition

Name	Company	Web site
KnowItAll	Biorad	*www.biorad.com*
ADMENSA	Inpharmatica	*www.inpharmatica.com*

32.2 In Vitro CYP Inhibition Methods

CYP inhibition methodology is an active area of research, and several methods have been reported.[2] Assays can be distinguished by four method elements: (1) CYP material, (2) substrate compounds, (3) detection method, and (4) strategy of analysis.

Commonly used *CYP materials* are recombinant human CYP isozymes (rhCYP) and human liver microsomes (HLM). rhCYPs come from individually cloned human CYP isozyme genes that are transfected using baculovirus into insect cells and produced in bulk. They can be purchased as individual CYP isozyme reagents from suppliers (Table 32.2) and in kits with substrates, buffers, and NADPH. HLM also are commercially available. They are prepared from human livers that are pooled from many individuals, so they represent a broad human population. The livers are tested for human disease to ensure safety. HLM contain all of the natural human CYP isozymes at the natural concentrations.

TABLE 32.2 ► Some Commercial Suppliers of Materials and Equipment for CYP Inhibition Studies

Company	Product	Web site	Detection
BD Gentest	P450 inhibition kits Superzomes Human liver microsomes	*www.gentest.com*	Fluorescence
Invitrogen	VIVID Screening Kits Baculosomes	*www.invitrogen.com*	Fluorescence
Promega	P450-Glo Screening Systems	*www.promega.com*	Luminescence
In Vitro Technologies	Human liver microsomes	*www.invitrotech.com*	
XenoTech	Human liver microsomes	*www.xenotech.com*	
BMG Labtech	Fluorescence plate readers	*www.bmglabtech.com*	Fluorescence

Differences in IC_{50} values have been reported in the literature between data generated using rhCYP with fluorescent probes (lower IC_{50}) and drug probes with HLM. This difference can be attributed to the effects of HLM, including protein binding,[27] substrate turnover,[28] and test compound metabolism[28] (see Section 32.2.3).

The *substrate compounds* for CYP inhibition assays typically consist of two types: fluorogenic substrates and drug substrates. *Luminogenic* and radioactive substrates also have been used. Fluorogenic substrates have very low fluorescence, but their metabolites are fluorescent. Examples of fluorogenic substrates are listed in Table 32.3 and shown in Figure 32.1. When the fluorogenic substrate is metabolized by the CYP isozyme, fluorescence intensity increases and is quantitated using a fluorescence plate reader. If the test compound reduces the fluorescence signal compared to control, it is an inhibitor. Some fluorescent substrates can be metabolized by more than one CYP isozyme; therefore, they must be used with only one isozyme at a time. Drug substrates used for CYP inhibition assays are selected because

TABLE 32.3 ► Examples of Fluorogenic Substrates

Isozyme	Abbreviation	Substrate/metabolite
3A4	BFC	7-benzyloxy-4-trifluoromethylcoumarin 7-hydroxy-4-trifluoromethylcoumarin
2D6	AMMC	3-[2-(*N*,*N*-diethyl-*N*-methylamino) ethyl]-7-methoxy-4-methylcoumarin (3-[2-(*N*,*N*-diethyl-*N*-methylammonium) ethyl]-7-hydroxy-4-methylcoumarin)
2C9	MFC	7-methoxy-4-trifluoromethylcoumarin 7-hydroxy-4-trifluoromethylcoumarin
1A2	CEC	7-ethoxy-3-cyanocoumarin 7-hydroxy-3-cyanocoumarin

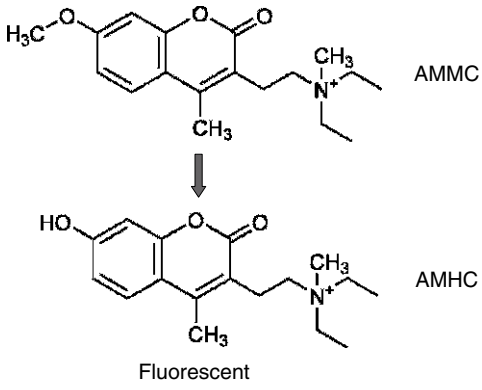

Figure 32.1 ► Structures of fluorogenic substrate AMMC and its metabolite AMHC used for the fluorescent inhibition assay for CYP2D6. (Reprinted with permission from [45].)

they are specifically metabolized by one CYP isozyme. As the drug substrate is metabolized by the CYP isozyme, the concentration of its metabolite increases and is quantitated using liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS). If the test compound reduces the concentration of drug substrate metabolite compared to control, it is an inhibitor. The luminogenic substrates (Figure 32.2) are derivatives of luciferin. The metabolites react with a proprietary luciferin detection reagent and produce light. Reduction in the light produced when test compound is coincubated compared to control is an indication that the test compound is an inhibitor.[29,30] Luminogenic substrates can have interference from test compounds that affect luciferase enzymatic activity. Radioactive substrates are more expensive to purchase and produce radioactive waste.

Detection methods include fluorescence, LC/MS/MS, radioactivity, and luminescence.[31] Fluorescence and LC/MS/MS are the two most commonly used methods. Fluorescence detection can be performed using a plate reader in 96- or 384-well formats and therefore is much higher throughput than LC/MS/MS. Fluorescence-based methods are commonly used during the lead selection and optimization stages, when thousands of compounds require testing. LC/MS/MS detection is medium throughput and typically is used during late discovery and development to provide the most definitive data. An advantage of LC/MS/MS is that a cocktail of substrates can be used in a single well for measurement of inhibition at multiple CYP isozymes. The selectivity of LC/MS/MS distinguishes between the metabolites of each substrate. This parallel processing increases the throughput by the number of separate substrates analyzed. The luminescence method is reported to have high sensitivity, no

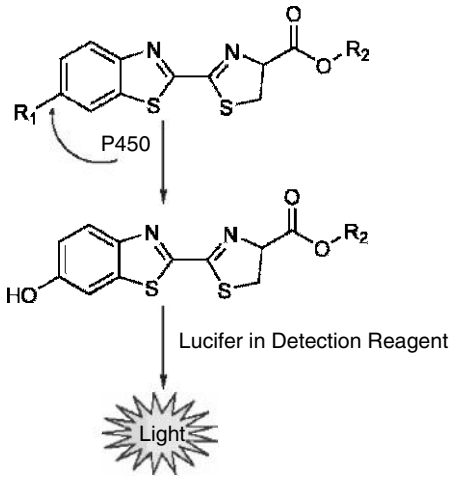

Figure 32.2 ► Structures of general luminescent substrate and its metabolite that are used for the luminescent inhibition CYP inhibition assay. Different R_1 and R_2 groups are used for different CYP isozymes.

fluorescence interference, and low false-positive rate. With radioactive assays, the radioactive metabolite must be separated from the radioactive substrate using high-performance liquid chromatography (HPLC) and quantitated with a radiometric detector, or a scintillation proximity assay is used.[32]

The CYP materials, substrate materials, and detection methods have been combined using four major assay strategies:

1. *Fluorescent Assay:* Individual rhCYP isozymes, individual fluorescent substrates, fluorescent detection
2. *Single Substrate HLM Assay:* HLM, individual specific drug substrates, LC/MS/MS detection
3. *Cocktail Substrate HLM Assay:* HLM, cocktail of specific drug substrates, LC/MS/MS detection
4. *Double Cocktail Assay:* Cocktail of rhCYP isozymes, cocktail of drug substrates, LC/MS/MS detection

Each of these methods is summarized in Table 32.4 and discussed in the following. Many companies use different assays at different stages of discovery and development. Therefore, it is important that medicinal chemists verify that data from the different methods are consistent.[31,33–37] Some studies show good correlation of methods and some show weak correlation, depending on the assay conditions and materials used. Differences in data between methods are primarily affected by differences in the concentration of CYP material, which affects substrate turnover and test compound metabolism[28] (see Section 32.2.3).

A general diagram of CYP inhibition methods is shown in Figure 32.3. The CYP material is added to buffer, NADPH cofactor, and substrate in the well of a 96- or 384-well plate. A small volume of test compound dissolved in dimethylsulfoxide (DMSO) is added, and the solution is incubated at 37°C for a specific time (e.g., 20 minutes). The reaction is quenched by the addition of acetonitrile, and the substrate metabolite concentration is measured. A reduction in metabolite production compared to control indicates inhibition by the test compound. If a single concentration of test compound is used (e.g., 3 μM), then a percent inhibition value is produced.[38] Testing at multiple concentrations of test compound

TABLE 32.4 ► Methods for Determination of CYP Inhibition

Method	CYP material	Substrate material	Detection method	Throughput for four isozymes
Fluorescent	Individual rhCYP isozyme	Individual fluorescent substrate	Fluorescence	500 cpd/wk
Single substrate HLM	HLM	Isozyme-specific drug	LC/MS/MS	50 cpd/wk
Cocktail substrate HLM	HLM	Cocktail of isozyme-specific drugs	LC/MS/MS	200 cpd/wk
Double cocktail	Cocktail of rhCYP isozymes	Cocktail of isozyme-specific drugs	LC/MS/MS	200 cpd/wk

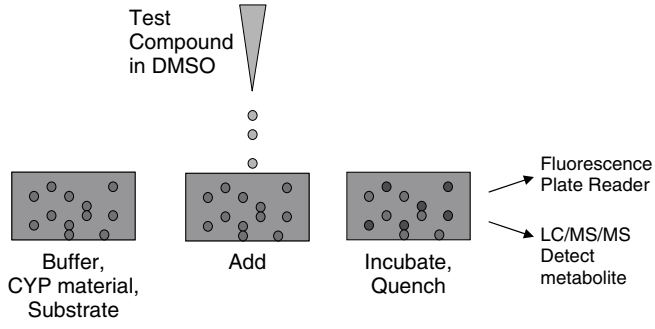

Figure 32.3 ► General diagram of an in vitro CYP inhibition assay.

provides an IC_{50} value. K_i values are produced from assays at multiple concentrations of test compounds and substrates.

32.2.1 Fluorescent Assay for CYP Inhibition

The fluorescent assay uses individual rhCYP isozymes, individual fluorescent substrates, and fluorescent detection. It is one of the most common techniques for higher-throughput CYP inhibition assessment.[33,35,36,39–46] The method scheme is shown in Figure 32.4. The fluorescent "probe" substrate forms its fluorescent metabolite at a predictable rate. When an inhibitory test compound is coincubated, it reduces the rate of production of the fluorescent metabolite. The CYP material for this assay is individual rhCYP isozymes. Following the incubation, the reaction is quenched, and the plate is placed in a fluorescence plate reader for quantitation.

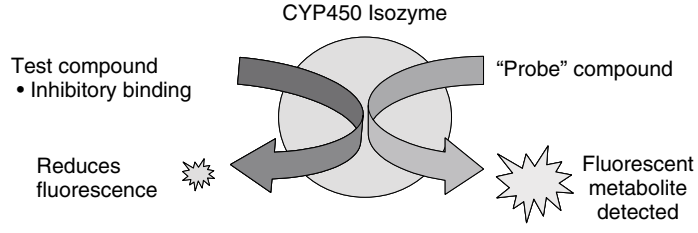

Figure 32.4 ► Scheme of the in vitro fluorescent CYP inhibition method using individual rhCYP isozymes, individual fluorescent substrates, and fluorescent detection.

Cloning and expression of individual human CYP isozymes has provided access to commercial supplies of each rhCYP isozyme, which can be prepared at a specific concentration with cofactors, substrate, and inhibitor for the generation of reliable enzymatic data under initial rate conditions. Furthermore, the design and development of substrates that produce fluorescent metabolites have enabled rapid plate reader quantitation of the reaction for high throughput.

A shortcoming of the fluorescent method is revealed if the test compound is fluorescent. The data for such compounds indicate negative inhibition values. It is advisable to assay these test compounds using a nonfluorescent detection method, such as LC/MS/MS.

32.2.2 Single Substrate HLM Assay for CYP Inhibition

The single substrate HLM assay uses HLM, individual specific drug substrates, and LC/MS/MS detection.[47] The scheme of the assay is shown in Figure 32.5. A reduction of substrate metabolite (e.g., hydroxyl midazolam) signal, relative to control, indicates inhibition (Figure 32.5). This method does not have the problem of fluorescent interference (see Section 32.2.1). The concentrations of substrate and enzyme can be set up so that this method does not have the problems of substrate depletion and test compound observed in the cocktail substrate HLM assay (see Section 32.2.3).

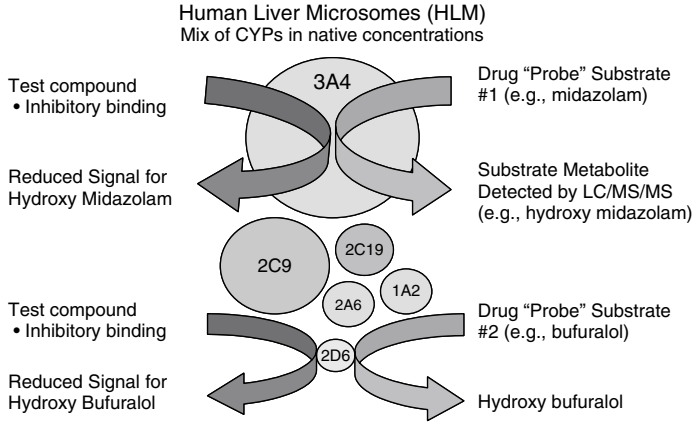

Figure 32.5 ► Scheme of the in vitro CYP inhibition methods using HLM, drug substrates, and LC/MS/MS detection. For the single substrate HLM method, only one drug substrate is used. For the cocktail substrate HLM method, a mixture of drug substrates is used.

The specificity of LC/MS/MS quantitation provides confidence of accuracy and reduction in interference. However, LC/MS/MS is more expensive and slower than a plate reader. The determination of definitive K_i values, late in discovery, often is performed using this method by varying both inhibitor and substrate concentrations. Higher throughput can be achieved by pooling the supernatants from incubations of single substrates and then analyzing the single pool in one LC/MS/MS analysis.[48]

32.2.3 Cocktail Substrate HLM Assay for CYP Inhibition

The cocktail substrate HLM assay uses HLM, a cocktail of specific drug substrates, and LC/MS/MS detection. The assay has higher throughput than the single substrate HLM assay. Each substrate in the cocktail is specifically metabolized by a single CYP isozyme and detected with specific LC/MS/MS conditions (i.e., retention time, precursor ion, product

ion). Therefore, the inhibition at each isozyme is independently assessed. For these reasons, the assay design is efficient and specific.[49,50]

There are three concerns with this method. First, the native abundance of each of the CYP isozymes varies widely in HLM (Table 15.2). For example, CYP3A4 makes up 28% of the total CYP material, whereas CYP2D6 is only 2%. Because this assay typically is run with similar initial drug substrate concentrations, the substrates for the high abundance CYP3A4 and CYP2C9 isozymes are depleted to a much greater extent (35% and 40% depletion, respectively) than those for low abundance CYP2D6 (1%–4% depletion). This is shown in Table 32.5.[51] The high substrate depletion for CYP3A4 and CYP 2C9 exceed ideal initial rate conditions for enzyme kinetics.

TABLE 32.5 ► Examples of High Substrate Depletion and Inhibitor Metabolism by the Cocktail Substrate HLM Assay at CYP3A4

Assay	Cocktail substrate HLM assay	Cocktail substrate HLM assay	Fluorescent assay	Double cocktail assay	Human liver microsome stability
Total protein concentration	0.5 mg/mL	0.1 mg/mL	NA	NA	0.5 mg/mL
3A4 concentration	42 pmol/mL	8.4 pmol/mL	5.0 pmol/mL	0.78 pmol/mL	42 pmol/mL
Substrate	Midazolam	Midazolam	BFC	Midazolam	NA
Test compound	IC_{50} (μM)	IC_{50} (μM)	IC_{50} (μM)	IC_{50} (μM)	Percent remaining of test compound at 20 min
Clotrimazole	0.46	0.041	<0.01	0.005	25
Ethynylestradiol	>90	>100	1.2	7.6	0
Miconazole	5.4	0.49	0.21	0.23	43
Nicardipine	>20	2.1	0.24	0.39	3
Fluconazole	>100	19	24	13	97
Terfenadine	>20	18	1.2	1.9	58
Verapamil	>40	26	3.9	5.9	21
Erythromycin	>50	28	15	23	65
Nifedipine	>100	24	11	12	10
Clomipramine	>100	28	6.9	14	87
Ketoconazole	0.60	0.14	0.05	0.05	89
Percent conversion of substrate	35%	16%	NA	1%	NA

Reprinted with permission from [28].
NA, Not applicable.

The second concern with this method is that many test compounds are highly metabolized by the HLM. This is shown in Table 32.5, with percent remaining of some test compounds after the 20-minute incubation in the range from 0% to 50%. Thus, the actual concentration of inhibitor is changing and unknown during the incubation.

A third concern with this method is nonspecific protein binding to the HLM material, which sequesters test compound away from the enzymes.

The three aspects of concern regarding this method (high substrate depletion, high inhibitor metabolism, nonspecific protein binding) can produce falsely high IC_{50} values for some discovery test compounds by underestimating their CYP inhibition potentials.[51] Table 32.5 shows that IC_{50} values from the cocktail substrate HLM assay approach those of

the fluorescent method if the total HLM protein concentration is reduced. However, operating the method at lower HLM concentration can reduce the substrate metabolite below the detection limit of LC/MS/MS.

It has been suggested that HLM is a better model of living systems. However, CYP inhibition is often used in discovery like a selectivity filter, and the concerns discussed can confuse interpretation of the data. No other selectivity experiment (e.g., nearest target neighbors) includes the decomposition of compound during the assay or the high nonspecific binding of test compound that restricts it from interacting with the enzyme.

32.2.4 Double Cocktail Assay for CYP Inhibition

The double cocktail assay uses a cocktail of rhCYP isozymes, cocktail of drug substrates and LC/MS/MS detection.[37,51] The method has high throughput, high specificity, and high sensitivity for CYP inhibition, and it overcomes problems of the other methods. The "double cocktail" name comes from the cocktail of rhCYP isozymes (first cocktail) and the cocktail of specific drug substrates (second cocktail).[51] This method is very cost effective because it uses small amounts of rhCYP isozymes. Substrate metabolites are quantitated using LC/MS/MS (Table 32.6).

TABLE 32.6 ► Commonly Used Drug Substrates for CYP Inhibition Assays and LC/MS/MS Conditions for Quantitation of the Substrate Metabolite[49]

Drug substrate	Drug metabolite	CYP isozyme	Precursor ion (m/z)	Product ion (m/z)
Midazolam	1′-Hydroxymidazolam	3A4	342	203
Bufuralol	1′-Hydroxybufuralol	2D6	278	186
Diclofenac	4′-Hydroxydiclofenac	2C9	312	231
Ethoxyresorufin	Resorufin	1A2	214	214
S-Mephenytoin	4′-Hydroxymephenytoin	2C19	235	150
Coumarin	7′-Hydroxycoumarin	2A6	163	107
Paclitaxel	6α-Hydroxypaclitaxel	2C8	870	286

The assay has the advantages of parallel incubation and parallel LC/MS/MS analysis of multiple substrate metabolites. Moreover, the rhCYP isozymes can be mixed at low concentrations that are optimized for enzyme kinetics. Therefore, the reaction conditions each can be controlled to keep the assay under initial rate conditions, with low substrate depletion, low test compound metabolism, and low protein binding. High specificity is provided by the LC/MS/MS analysis.

32.3 CYP Inhibition Assessment Strategy

An efficient strategy for CYP inhibition assessment in drug discovery is shown in Figure 32.6:

- The fluorescent assay at a single test compound concentration (3 μM) is used during "hit-to-lead" and optimization phases. Thousands of compounds are tested initially using the high-throughput characteristics of the fluorescent assay.
- Inhibition >50% triggers a fluorescent assay IC_{50} study for more definitive data on lead compounds of interest.

- Negative inhibition, caused by test compound fluorescence, triggers retesting using the double cocktail method.
- Compounds considered for development are tested for IC_{50} using the double cocktail method. A K_i value should be produced and compared to C_{max} (see Chapter 15).

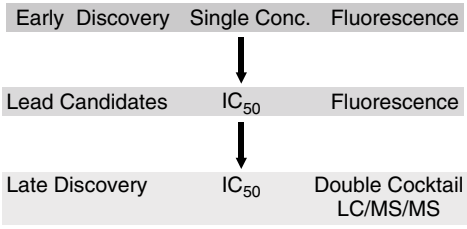

Figure 32.6 ► Strategy for CYP inhibition at various discovery stages.

Problems

(Answers can be found in Appendix I at the end of the book.)

1. What two types of materials are commonly used for CYP inhibition assays?
2. What are some negative effects of using microsomes in CYP inhibition assays that can affect the data?
3. What four types of substrates (probes) have been used for CYP inhibition assays? Which are most common?
4. CYP inhibition assays measure which of the following?: (a) increased production of substrate metabolite, (b) decreased production of substrate metabolite, (c) increased production of inhibitor metabolite.
5. What is a drawback of the fluorescent assay?
6. What is an advantage of the cocktail substrate HLM assay?
7. What is a drawback of the cocktail substrate HLM assay?
8. How does the double cocktail assay improve on the drawbacks of the cocktail substrate HLM assay?

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Plasma Protein Binding Methods

Overview

- *Literature and commercial in silico predictors of plasma protein binding are available.*
- *Equilibrium dialysis is the gold standard method. Ultrafiltration, ultracentrifugation, microdialysis, and plasmon resonance methods also are used.*
- *Red blood cell binding is important to measure for some compounds.*

The importance of free (unbound) drug for distribution into disease tissues and clearance organs has prompted many drug companies to implement plasma protein binding (PPB) measurement methods. Several of these methods have been developed in higher-throughput 96-well format.

33.1 In Silico PPB Methods

33.1.1 Literature In Silico PPB Methods

In silico models for PPB have been reported.[1–4] PPB was found to increase with increasing lipophilicity, increasing acidity, and increasing number of acid moieties.[1] PPB decreases with increasing basicity and number of basic substructures. One group used the chemical structure to calculate the ionization state and lipophilicity (Log P) for predicting PPB.[2]

33.1.2 Commercial In Silico PPB Methods

Several commercial software products for predicting PPB are available. Many of these products are listed in Table 33.1.

TABLE 33.1 ► Partial List of Commercial Software for Plasma Protein Binding

Name	Company	Web site
QikProp	Schrodinger	*www.schrodinger.com*
ADMET Predictor	Simulations Plus	*www.simulations-plus.com*
ADME Boxes Distribution	Pharma Algorithms	*www.ap-algorithms.com*
KnowItAll	Biorad	*www.biorad.com*
CSPB	ChemiSilico	*www.chemsilico.com*
DSMedChem Explorer	Accelrys	*www.accelrys.com*
Wombat database	Sunset Molecular Discovery	*www.sunsetmolecular.com*

33.2 In Vitro PPB Methods

In vitro PPB methods have been reviewed.[4,5] Several methods have been developed and described. Concomitant studies of PPB using more than one method have indicated the comparability of results from several of these methods,[6–8] with equilibrium dialysis generally considered the leading method and reference for other methods.

33.2.1 Equilibrium Dialysis Method

Equilibrium dialysis is considered the "gold standard" method for PPB.[4,9,10] It has been implemented in many formats, from individual chambers to 96-well plates. In this method, two chambers are separated by a dialysis membrane (Figure 33.1). The membrane has a certain molecular weight (MW) cutoff (e.g., 30 kDa). In one chamber is placed plasma with added test compound; in the other chamber is placed the same volume of blank buffer. The plasma protein molecules and bound compounds cannot pass through the membrane, but the free drug molecules can pass through. The chamber is incubated for 24 hours at a constant temperature. The concentration of free drug reaches equilibrium on each side of the membrane. The fraction unbound in plasma ($f_{u,plasma}$) is determined by measuring the concentration of drug in the buffer chamber and dividing by the total drug concentration in the plasma chamber.

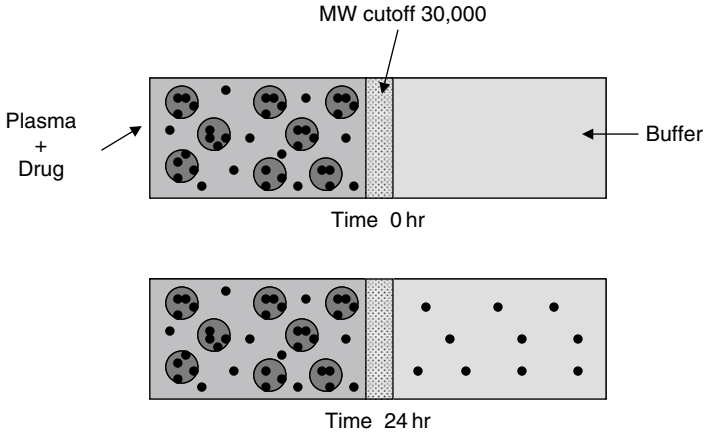

Figure 33.1 ► Equilibrium dialysis method for plasma protein binding.

A 24-well apparatus for measuring PPB by equilibrium dialysis has been described.[4] Throughput can be enhanced using a mixture of up to five compounds per dialysis cell (each at 10-μM concentration) in a cassette-type experiment. At this concentration, the albumin is at only a fraction of saturation. Two different 96-well apparatuses that work well have been described.[9,10] Several devices, from single-chamber to 96-well format, are available from commercial vendors (Table 33.2). An example of application of the method was measurement of the PPB of docetaxel.[11]

TABLE 33.2 ► Commercial Instruments and Supplies for Plasma Protein Binding

Method	Product name	Company	Web site
Equilibrium dialysis	HTD 96	HTDialysis	*www.htdialysis.com/*
Equilibrium dialysis	Dialyzer 96 well	Harvard Apparatus	*www.harvardapparatus.com*
Equilibrium dialysis	Dialyzer Multi-Equilib.	Harvard Apparatus	*www.harvardapparatus.com*
Equilibrium dialysis	Rapid Equilib. Dialysis (RED)	Pierce	*www.piercenet.com*
Equilibrium dialysis	Serum Binding System	BD Gentest	*www.bdgentest.com*
Ultrafiltration	Centrifree (single sample)	Millipore	*www.millipore.com*
Ultrafiltration	MultiScreen Ultracel-PPB	Millipore	*www.millipore.com*
Immobilized HPLC	Chiral-AGP, -HSA	Chrom Tech	*www.chromtech.com*
Microdialysis	Microdialysis probe	CMA/Microdialysis	*www.microdialysis.com*
Microdialysis	Microdialysis probe	BASi	*www.bioanalytical.com*

33.2.2 Ultrafiltration Method

In the ultrafiltration method, the test compound is added to plasma in a container and mixed. An aliquot of this solution is loaded into the upper chamber of an ultrafiltration apparatus (Figure 33.2) that has a membrane with a certain MW cutoff (e.g., 30 kDa). Individual sample vials and a 96-well ultrafiltration device are available (Table 33.2). The device is centrifuged (e.g., 2,000*g*, 45 minutes). The solution is ultrafiltered through the membrane by the force of the centrifugation. Unbound test compound moves with the liquid through the membrane into the receiver chamber, whereas compound bound to plasma protein remains in the loading chamber. Typically less than one fifth of the filtrate is collected. The concentration of test compound in the receiver is quantitated, and the fraction unbound (f_u) is calculated as this ultrafiltrate concentration divided by the total initial concentration. It is useful to perform mass balance studies to calculate the recovery to ensure lack of significant nonspecific binding to the apparatus.

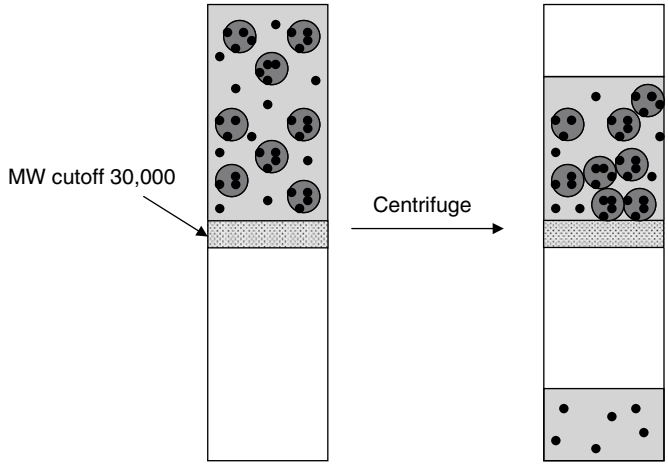

Figure 33.2 ► Ultrafiltration method for plasma protein binding.

33.2.3 Ultracentrifugation Method

In the Ultracentrifugation method test compound is added to plasma and incubated as above. A sample is transferred to an ultracentrifuge tube and centrifuged at high sedimentation rate (e.g., 6 hours at 160,000*g*, 15 hours at 100,000*g*). The concentration of test compound is measured in the original plasma and in the second layer of the supernatant.[8,12] Some researchers are concerned that the long period and conditions of the sedimentation may upset the equilibrium.

33.2.4 Immobilized Protein High-Performance Liquid Chromatography Column Method

High-performance liquid chromatography columns that have human serum albumin or α_1-acid glycoprotein bonded to the silica stationary support particles are available for purchase.[13,14] The test compound is injected onto the column and is moved along the column by the mobile phase. The test compound binds to the immobilized protein molecules to a degree that depends on its binding affinity. Binding slows the compound's progress through the column; thus, the retention time correlates to the affinity of the compound for the protein. Some researchers are concerned that the method does not provide good resolution for binding greater than 95%. Binding kinetics (association and dissociation rate constants) also can be determined by measuring the peak width and position.[15] A comparison of the most commonly used plasma protein binding methods is provided in Table 33.3.

TABLE 33.3 ► Comparison of Plasma Protein Binding Methods

Method	Advantage	Disadvantage
Equilibrium dialysis	Temperature controlled Widely used	Time to reach equilibrium Stability of protein and drug
Ultrafiltration	Technically simple, rapid, and inexpensive Widely used	Uncontrolled temperature Absorption of drugs to filter Dissociation of the bound drug
Ultracentrifugation	No absorption to membrane	Time: long (12–15 hr) Sedimentation, back diffusion Expensive equipment
Immobilized protein columns	Technically simple and inexpensive	Binding limited to albumin Limited physiological relevance
Circular dichroism	Technically rapid	Limited to specific albumin binding Limited physiological relevance

33.2.5 Microdialysis Method

Microdialysis is discussed in Chapter 28. Briefly, an isotonic solution bathes the inner surface of a dialysis membrane, which is placed into a fluid or tissue. The membrane is incorporated in a small probe and, after flowing past the membrane (e.g., 1 μL/min), the fluid is collected and analyzed. By placing this probe into plasma containing the test compound, the free drug is trapped in the dialysis fluid while the plasma proteins and drug binding to plasma protein stays in the sample.[16,17] Good success has been reported in

determining percent binding compared to other methods, but the method is considered time consuming.

33.2.6 Other PPB Methods

Many other methods for analysis of drug–protein complexes have been reported.[5] Some methods measure specific properties of the drug–protein complex:

- Fluorescence spectroscopy
- Circular dichroism/optical rotatory dispersion (CD/ORD)
- NMR
- Electron spin resonance (ESR)
- Microcalorimetry
- Surface plasmon resonance (Biacore)[18]

Surface plasmon resonance can be used to measure the rate of association (k_a) and dissociation (k_a), in addition to the percent binding.

Other methods are chromatography based:

- Size exclusion chromatography
- Frontal analysis chromatography
- Capillary electrophoresis

33.3 Red Blood Cell Binding

Some compounds bind to red blood cells (RBCs). It is important to account for this binding because it can be a significant percentage of the compound in blood. RBC-bound drug is not measured in pharmacokinetics (PK) experiments because the RBCs are removed prior to storage of the sample for later analysis. A study of red cell binding can be performed retrospectively if there is difficulty in accounting for the mass balance of a compound in a PK study. It also can be performed prospectively as a screening method if the lead series is known to have an RBC binding issue.

The test compound is added to whole blood and incubated (e.g., 2 hours at 37°C). The blood samples are transferred to measurement of sedimentation (MESED™) devices, and the RBCs are separated from plasma. Each fraction then is analyzed using liquid chromatography/mass spectrometry techniques.[19]

Problems

(Answers can be found in Appendix I at the end of the book.)

1. Which PPB method is generally considered to be most reliable?
2. How is the unbound fraction determined using the equilibrium dialysis method?
3. If a compound is highly bound to red blood cells, what will be the PK consequences?
4. Why is a membrane filter with a high MW cutoff necessary in some PPB methods?

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Chapter 34

hERG Methods

Overview

- *hERG blocking in silico models are being developed within companies and are increasingly commercially available.*
- *Patch clamp methods are most successful for in vitro predictions of hERG blocking*
- *In vitro assays should be followed up with in vivo ECG studies of long QT.*

Regulatory agencies insist on having experimental hERG data for all compounds moving into clinical development because of the risk of death from arrhythmia in a small portion of the population. Pharmaceutical companies need to develop a strategy for hERG block assessment during drug discovery to avoid investment in a chemical series that has significant risk.

For this reason, several methods have been developed to profile potential hERG channel blocking by discovery compounds. As with other properties, there is an emerging trend to begin screening for potential hERG blocking by applying in silico tools to hits from high-throughput screening. This initiates awareness of potential issues for the project team. However, because in silico predictions currently have low correlation to in vivo electrophysiology, they should not be used to eliminate potentially promising pharmacophores. The hERG screening strategy continues throughout discovery using in vitro methods, as part of the optimization process, which allows high throughput and fast turnaround of data.[1] Manual patch clamp is the most definitive in vitro assay. It is used for detailed study of the effects of drugs on the K^+ ion channel. Patch clamp often is referred to as the "gold standard" method. The method has been accelerated to higher throughput by instrument companies and is the most definitive of the high-throughput in vitro assays. Ultimately, in vivo electrophysiology methods using electrocardiography (ECG) studies must be performed for selected compounds moving toward development. Whereas in vitro studies indicate the likelihood of hERG block, the in vivo ECG will indicate the most important element, the production of long QT, and initiation of torsades de pointes arrhythmia. In vivo hERG methods are the most expensive and time consuming, but they produce the most definitive data. The Food and Drug Administration (FDA) requires that human ECG studies be conducted in the clinic for many drug candidates. As a general strategy, it is useful for drug research companies to have tools for in silico, in vitro, and in vivo hERG testing to meet the needs at each stage of discovery. Various methods for hERG are listed in Table 34.1.

TABLE 34.1 ► Methods of Screening for hERG Blocking

Method	Type assay	Comments
Membrane potential dye	HT in vitro	HT, LCP
Binding	HT in vitro	
Rubidium flux	HT in vitro	
HT patch clamp	HT in vitro	
LT patch clamp	In-depth in vitro	LT, expensive
Purkinje Fibers	In-depth in vitro	LT, expensive
Electrocardiogram	In-depth in vivo	LT, expensive

HT, High throughput; LCP, low correlation to patch clamp; LT, low throughput.

34.1 In Silico hERG Methods

Diverse compounds block the hERG K^+ channel. Evidence is emerging about the binding site(s) of drugs in the hERG channel and should eventually enable inexpensive and useful predictions.

Structural features associated with hERG blocking are discussed in Chapter 16. Various organizations have developed proprietary in silico methods for early prediction of hERG blocking potential. There is currently no commercial software for hERG blocking prediction based on compound structure. However, commercial software is likely in the near future as databases grow.

34.2 In Vitro hERG Methods

In vitro hERG methods can be classified into two types. Indirect methods (membrane potential-sensitive dye, ligand binding, rubidium efflux) measure effects associated with K^+ ion channel activity. These methods are favorable for high-throughput analysis but have low correlations to patch clamp and in vivo electrophysiology. The other type of method, high-throughput patch clamp, directly measures ion channel activity and has emerged as the in vitro method of choice. All in vitro hERG methods are cell based and use cell lines, such as CHO and HEK293, which have been transfected with the hERG gene and express K^+ channels on their membranes. The implementation of hERG screening in drug discovery has been reviewed.[1]

34.2.1 Membrane Potential–Sensitive Dye Method for hERG

Membrane potential–sensitive dyes are used in many ion channel experiments and have been adopted for hERG screening.[2] Cells transfected with the hERG gene have a more negative membrane potential than do wild-type cells as a result of the potassium channel activity, which lowers the internal K^+ ion concentration. If a compound blocks the hERG potassium channel, the membrane potential of the transfected cells increases, which can be monitored using a membrane potential–dye.[3]

The structure of one dye, $DiBAC_4(3)$, is shown in Figure 34.1. This dye interacts with cytoplasmic components to produce fluorescence, but no fluorescence is produced in the extracelluar solution. The cells are preincubated with dye in the extracellular solution, then the test compound is added and the fluorescence is monitored using a fluorometric plate reader (e.g., FLIPR [Molecular Devices Corp.] or POLARstar [BMG Labtech]) for 3 to 15 minutes.

The dye permeates in and out of the cell in response to the membrane potential. $DiBAC_4(3)$ is negatively charged, so there is a higher concentration in the cell when there are more K^+ ions in the cell. If the test compound blocks the K^+ channel, the fluorescence increases. The method is diagrammed in Figure 34.2, *A*.

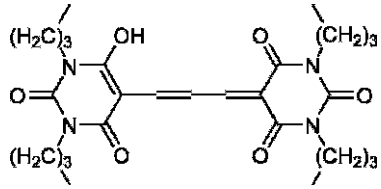

Figure 34.1 ► $DiBAC_4(3)$ membrane potential–sensitive dye for FLIPR assay.

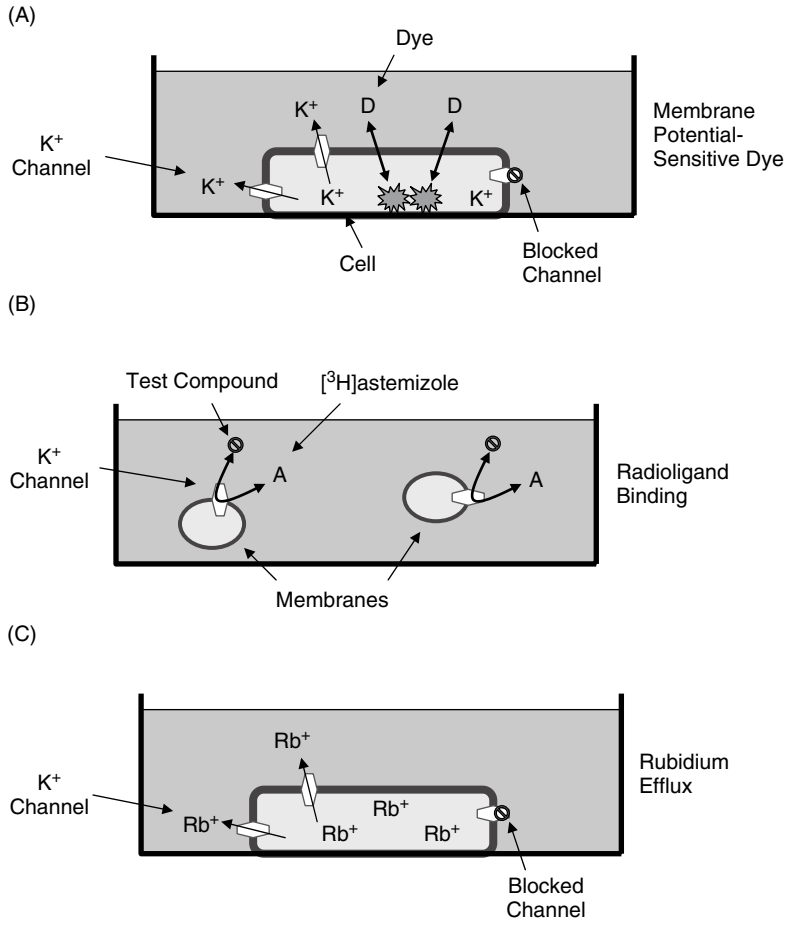

Figure 34.2 ► Diagrams of high-throughput in vitro methods for hERG blocking.

The drawback of $DiBAC_4(3)$ is slow response time.[3,4] A new dye, FLIPR membrane potential dye (FMP, Molecular Devices Corp.), responds 14-fold faster than $DiBAC_4(3)$, is less sensitive to temperature, and does not require a wash step; thus, it is more useful for high-throughput automation and screening. Dye methods are susceptible to fluorescence quenching and test compound interference, which can be compensated for by running the

assay also on wild-type cells. The IC_{50} values average fivefold higher than by the patch-clamp method and do not have a high correlation.[3] It is not a functional assay, so other ion channels and cellular factors can compromise the results. The method can detect potent inhibitors and does not produce false–positive results, but it does produce false–negative results for less potent inhibitors.[5] An advantage of the method is high throughput using conventional high-throughput fluorescence plate readers, speed, and low cost.

34.2.2 Ligand Binding Method for hERG

Binding of test compounds to hERG channel protein can be screened using a radioligand binding assay.[6,7] In this assay, membranes are prepared from HEK293 cells transfected with the hERG gene (Figure 34.2, *B*). The membranes are incubated with a radiolabled compound that is known to bind to the hERG channel (e.g., [^{3}H]dofetilide, [^{3}H]astemizole) and the test compound. The radioligand and test compound compete for binding to the hERG channel. After incubation at 37°C for 30 to 60 minutes, the membranes are filtered and washed multiple times with cold buffer. Bound radioligand is detected by scintillation counting, and the binding compared to control is plotted against test compound concentration. The stronger the affinity of the test compound for the hERG channel, the lower will be the radioligand response in the filtered membranes.

There is good correlation between receptor binding K_i values and patch-clamp IC_{50} values, with correlation $r^2 = 0.91$. In some laboratories, binding results are many-fold less than obtained by the patch-clamp method.[6,7] In other laboratories, the results are consistent and robust and the method is considered the best indirect in vitro hERG blocking method in side-by-side tests.[5] It is not a functional assay.

34.2.3 Rubidium Efflux Method for hERG

The rubidium (Rb) efflux method (Figure 34.2, *C*) is a functional assay based on hERG channel opening. Rb is found at only trace levels in cells and media, is the same size and charge as K^+, and is permeable through the K^+ channels. CHO cells transfected with hERG gene are preincubated with Rb-containing media to establish an equilibrium of Rb between, inside, and outside the cells. Then the media is removed and the cells are washed multiple times with buffer to remove Rb from the extracellular solution. Buffer containing K^+ (to manipulate the membrane potential and open the channels) and test compound is added. Following incubation, the media is removed. The cells are washed and lysed. The media and lysate are separately measured for Rb concentration using either scintillation counting of ^{86} Rb[8] or atomic absorption spectroscopy of Rb.[9] If the K^+ channel is blocked by the test compound, the Rb^+ stays in the cells; if the K^+ channel is not blocked, the Rb^+ permeates through the channel into the medium. From the measured values, the percentage of Rb permeation is calculated.

As with the other high-throughput methods, IC_{50} values often are higher in the Rb efflux method than in the patch-clamp method. The underprediction by the Rb efflux method is greater for more potent inhibitors.[9] This appears to result from the presence of Rb, which reduces the affinity of the compound for the hERG channel and reduces activation of the channel. The method appears to be useful for screening for stronger hERG inhibitors in early discovery.[5]

34.2.4 Patch-Clamp Method for hERG

The "gold standard" of in vitro assays for hERG blocking is the patch-clamp technique. This method is widely used for the study of all ion channels. A diagram of the patch-clamp

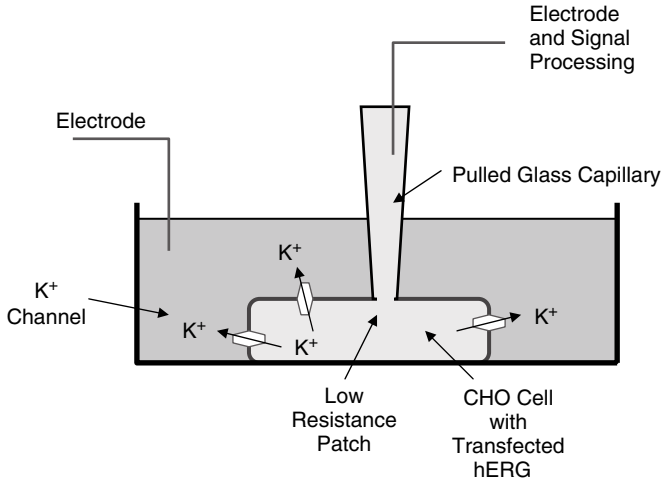

Figure 34.3 ► Diagram of patch-clamp method for hERG blocking.

method is shown in Figure 34.3. A glass capillary is melted, pulled to about 1-μm diameter, and cut off. The glass capillary is filled with buffer that is compatible with the cell cytoplasm, and an electrode is inserted. The capillary is attached to a micromanipulator and, while observing with a microscope, the tip is placed in contact with the membrane of a CHO or HEK293 cell that expresses the hERG K^+ ion channel. A negative pressure (vacuum) is applied, and the cell membrane is "patched" or held tightly to the capillary and forms a high resistance seal. Further application of vacuum causes the small portion of membrane at the tip of the capillary to be pulled into the capillary tip and ruptured, forming a "whole-cell patch." Most of the cell remains outside the capillary. The cell cytoplasm and the capillary fluid mix, establishing an electrical connection. The electrodes are used to set ("clamp") the membrane potential at a specified voltage. In response to a positive voltage applied by the investigator, the K^+ ion channel proteins undergo conformational change and open. Ions move out of the cell through the ion channels, creating a current. The membrane potential is held at a constant level by the electronics, and the current needed to maintain this voltage is recorded. This current is directly related to the total ion current of all the K^+ ion channels in the cell.

If a test compound blocks the hERG K^+ ion channel, then the current is reduced relative to control. An example of a patch-clamp profile for a hERG blocking experiment is shown in Figure 34.4. For the control, changing the membrane potential from –80 to +40 mV opens the channels and K^+ ion current flows. When the membrane potential then is reduced to –50 mV, ion current has a momentary spike due to the mechanics of the channel and then reduces to the unopen state. When hERG blocker thioridazine is added to the assay medium, the channel is blocked and the ion current is greatly reduced compared to control.

The detail provided by such data allows in-depth study of the function of the ion channel under hERG block. Patch clamp provides complete control of the crucial experimental conditions, such as membrane potential, which allows full ion channel function. Other in vitro methods cannot control this, except crudely with the K^+ ion concentration. Patch clamp also is able to monitor ion channel events on the millisecond level, whereas other techniques monitor events that are indirectly related to the ion channel. The method is more sensitive to hERG blocking than are other methods and consistently provides reliable data, even for compounds of higher IC_{50}.

The major problem with the patch-clamp method for drug discovery is the considerable time and skill needed for conducting each experiment. Therefore, the method is not

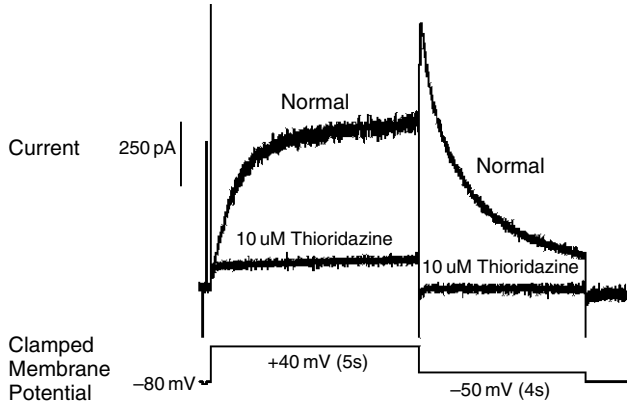

Figure 34.4 ► Examples of ion current profiles from patch clamp for K^+ ion channels.

amenable to high throughput in this configuration. This experiment is normally performed for detailed study of a few selected compounds.

34.2.5 High-Throughput Patch-Clamp Method for hERG

High-throughput patch clamp is achieved by modifying the patch-clamp experiment (Figure 34.5). Instead of pressing a glass capillary against the membrane, cells are seeded into a well that contains one or more small pores on the bottom. A vacuum is applied to draw a cell to the pore and hold it there. The electrical connection is established by further applying vacuum to burst the membrane. Alternatively, a modifier (e.g., amphotericin B, nystatin) is added to the fluid below the pore, which produces small openings in the cell membrane ("perforated patch"), allowing good electrical conductance. Membrane potential is controlled across the cell membrane. Several experiments can be performed in parallel using multiple wells for greatly enhanced throughput. This technique has become the method of choice for hERG block screening. Several commercial instruments now are available (Table 34.2). These instruments achieve both high throughput and highly informative data. High-throughput patch-clamp instruments have been reviewed.[10]

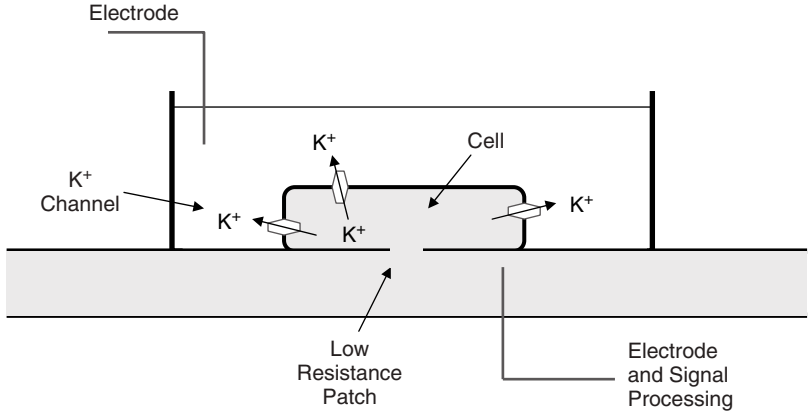

Figure 34.5 ► Diagram of planar patch-clamp method used for high-throughput hERG block screening.

TABLE 34.2 ► Commercial Instruments for Measurement of hERG Blocking

Method	Product name	Company	Web site
Fluorescent dye	FLIPR	Molecular Devices Corp.	*www.moleculardevices.com*
Rb efflux assay	ICR 12000	Aurora Biomed	*www.aurorabiomed.com*
Patch clamp	PatchXpress 700A	Molecular Devices	*www.moleculardevices.com*
Patch clamp	Flyscreen 8500	Flyion	*www.flyion.com*
MT patch clamp	Qpatch HT	Sophion Bioscience	*www.sophion.com*
MT patch clamp	CytoPatch	Cytocentrics	*www.cytocentrics.com*
MT patch clamp	NPC-16 Patchliner	Nanion	*www.nanion.de*
HT patch clamp	IonWorks Quatro	Molecular Devices	*www.moleculardevices.com*
HT patch clamp	PatchXpress 7000A	Molecular Devices	*www.moleculardevices.com*

Note: Medium throughput (MT)

34.3 In Vivo hERG Methods

The ultimate method for assessing and studying hERG block by discovery compounds is ECG using animal models (e.g., dog, monkey). The method produces data like the ECG traces shown in Figure 16.3. Electrodes are placed on the surface of the heart. The effects of the test compound of lengthening the QT interval and inducing torsades de pointes are directly observed. The method is definitive, but it is labor intensive, slow, low throughput, and expensive. Other in vivo methods involve Purkinje fiber action potential, isolated perfused heart ECG, perfused ventricular wedge, and repolarization reserve. Electrophysiologists conduct these studies.

Problems

(Answers can be found in Appendix I at the end of the book.)

1. In silico hERG blocking tools are best used for what?
2. Which in vitro hERG blocking method is generally considered to be most predictive?
3. Which hERG blocking method does the FDA require in humans, if there is a potential problem?
4. Dye methods for hERG rely on which of the following?: (a) membrane potential, (b) K^+ transporters, (c) interaction with cytoplasmic components to produce fluorescence, (d) interaction with extracellular components to produce fluorescence.
5. What is a drawback of the hERG membrane potential dye and Rb methods?
6. Which are true about the ligand binding hERG method?: (a) radiolabeled hERG protein is used, (b) the radiolabeled ligand is first incubated with the hERG protein and then test compound is added, (c) higher detected radioactivity correlates to lower test compound hERG binding, (d) radiolabeled hERG is used.
7. In the "efflux method," Rb is a surrogate for what? Why is Rb used?
8. In the patch-clamp method, rupturing the cell membrane provides which of the following?: (a) a delivery route for introducing the test compound into the cell, (b) a way to introduce the hERG gene, (c) an electrical connection between the inside and outside of the cell, (d) a means of controlling the transmembrane potential, (e) a way to introduce hERG inhibitor into the cell.

9. What is measured in patch clamp?
10. What is a drawback of the standard patch-clamp method for use in drug discovery?
11. In high-throughput patch-clamp methods, which of the following is true?: (a) the throughput is much higher than manual patch clamp, (b) a known competitive hERG blocker standard is added to the media, (c) the patch is created with a pore on the bottom of the well and negative pressure is applied, (d) the automated commercial instruments require less operator time and skill.

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Toxicity Methods

Overview

- *In silico tools predict potentially toxic substructures and metabolites.*
- *In vitro assays test many toxic mechanisms, including metabolite reactivity, induction, mutagenicity, and cytotoxicity. These findings alert teams to potential problems.*
- *In vivo acute and chronic dosing studies, in combination with study of physiological function and histology, are necessary for advanced candidates. The complexity of studies increases as candidates progress through development.*
- *Toxicometabonomics, toxicoproteomics, and toxicogenomics provide higher-throughput approaches that can recognize in vivo toxic responses soon after dosing and indicate underlying toxic mechanisms.*

As with other absorption, distribution, metabolism, excretion, and toxicity (ADME/Tox) properties, toxicity uses a combination of in vitro and in vivo assays to obtain data for key variables. At the in vitro level, a large number of compounds are examined for key indicators of toxicity. At the in vivo level, detailed studies are performed for medically defined end points that are clear indicators of health. Toxicity is so critical to the success of developing a new drug that tremendous attention and priority are accorded to it. The predictability of each method is thoroughly examined. New in vitro methods should produce a minimum of false-negative results so that unexpected toxicities do not appear at a later stage. False-positive results also are problematic because they place a stigma on the compound series. Indications of toxicity are a significant deterrent to further work on a compound or lead series but might be overcome during discovery if the mechanism can be determined. This chapter introduces discovery scientists to some of the terms and methods used in discovery and preclinical toxicity studies. For greater depth of understanding, detailed reviews, books, and experts should be consulted.

Toxicity studies during discovery focus on key mechanisms of toxicity. As in other areas, there is a hierarchy of assays and resources applied to compounds:

- Earlier studies use higher-throughput in silico and in vitro methods for indications of toxicity.
- Toxicity indications are followed up with sophisticated diagnostic testing of selected compounds.
- Lead or preclinical compounds are subjected to standard advanced toxicity procedures:
  - Off-target selectivity screening
  - Animal dosing experiments

Some organizations have found that shifting toxicology resources into discovery allows the early identification of toxicity issues.[1] These groups suggest the following advantages of early toxicity testing:

- More informed decisions during discovery for deprioritization of toxic leads or modification of leads to eliminate toxicity.
- Any issues are more robustly understood going into the investigational new drug stage.
- Saving resources on development of candidates that would fail because of toxicity.
- Dosing studies in phase I development can be better planned by having these data.

35.1 In Silico Toxicity Methods

The expense of in vivo toxicity testing and the large number of compounds to be assessed in drug discovery have encouraged the development of in silico and in vitro techniques. The ability of certain chemical moieties and structural templates to predictably induce certain types of toxic responses has led to quantitative structure–activity relationship (QSAR) studies for the purpose of predicting toxic responses using in silico algorithms.[2,3] Several limitations to the development of in silico models include the quality and quantity of the toxicological data, the intricacy of each type of toxic mechanism, and the multiplicity of toxic mechanisms. A listing of commonly used in silico tools is provided in Table 35.1.

TABLE 35.1 ► Commercial Products for Toxicity Methods

Product	Type	Company	Web site
DEREK	in silico	LHASA Ltd.	*www.lhasalimited.org*
OncoLogic	in silico	LogiChem Inc.	*woo.yintak@epa.gov*
Hazard Expert	in silico	CompuDrug	*www.compudrug.com*
MCASE	in silico	MultiCASE Inc.	*www.multicase.com*
TOPKAT	in silico	Accelrys	*www.accelrys.com*
LDH kit	in vitro	Roche Applied Science	*www.rocheappliedscience.com*
GreenScreen GC	in vitro	Gentronix	*www.gentronix.co.uk*

35.1.1 Knowledge-Based Expert System In Silico Toxicity Methods

General classifications and expert opinions have been combined into knowledge-based methods. These provide rules for the evaluation of new chemical entities based on structural features as well as their associated toxicity probabilities. Thousands of compounds have been studied, and thousands of rules have been developed.

DEREK is an expert system. Rules are agreed upon by a committee and incorporated into the model. Predictions, rule(s), and literature references are shown on the screen, so the user can follow up. DEREK can be linked to METEOR for inclusion of metabolite toxicity predictions. The DEREK interface reportedly is easy to use, and the system can be operated in batch mode. DEREK predicts many types of toxicity.

OncoLogic, which was developed by the U.S. Environmental Protection Agency, uses a large array of rules. The user interacts with the software to optimize the assessment. Carcinogenicity predictions and mechanisms are provided.

HazardExpert evaluates compounds by toxicity associated with structural fragments. The software makes bioavailability and bioaccumulation estimations for inclusion in the toxicity predictions. It links to MetabolExpert to make predictions for compound metabolites.

35.1.2 Statistically Based In Silico Toxicity Methods

In these programs, parameters are calculated for structures and substructure connectivity. Computational models are derived using statistical methods.

TOPKAT uses a QSAR-based model. Structures are evaluated using electro-topological structural descriptors and fit with statistical linear regression and linear free energy relationships to produce the models.

MCASE divides the structure into active and inactive moieties for pattern recognition. It provides a text output of predictions for each moiety. It interacts with META to make predictions for compound metabolites.

In a comparison study, four in silico packages were used by the National Toxicity Program to predict the rodent carcinogenicity of 44 compounds. The overall accuracies were as DEREK: 59%; TOPKAT: 57%; COMPACT: 53%; MCASE: 49%.[3,4] In a subsequent carcinogenicity study of 30 compounds, the overall accuracies were OncoLogic: 65%; COMPACT: 43%; DEREK: 43% and MCASE: 25%.[2,3] By comparison, the in vitro Ames Assay is 85% accurate for carcinogenicity. In general, the best predictions are provided by expert systems. Significant uncertainty still exists for in silico predictions.

35.2 In Vitro Toxicity Assays

As in other areas of drug discovery, in vitro assays are being increasingly applied for toxicity screening.[5] This strategy allows study of a greater number of discovery compounds, specific toxicity mechanisms,[6] and low compound expenditure, and reduces animal usage. In vitro methods also are applied at later stages to investigate specific toxicity questions. The following sections discuss many of the in vitro toxicity methods encountered by discovery scientists. Toxicity studies during earlier phases of discovery have been termed *predictive toxicology* or *exploratory toxicology*. The International Committee on Harmonisation (ICH) has provided extensive guidance for many of the in vitro toxicity assays.[7] Ultimately, all in vitro assays are judged by their predictivity of in vivo toxicity results.

35.2.1 Drug–Drug Interaction

Coadministered drugs can interact in many ways and result in toxic effects. The usual outcome of this interaction is a change in the pharmacokinetics of the effected drug. The effected drug increases in the bloodstream to a toxic concentration.

35.2.1.1 CYP and Other Metabolizing Enzyme Inhibition

A drug may inhibit the metabolism of a second coadministered drug. Much of the focus in this area has been on drug–drug interactions at cytochrome P450 (CYP) isozymes. Methods for CYP inhibition are discussed in Chapter 32. Other metabolizing enzymes (e.g., monoamine oxidase (MAO)) can be similarly inhibited.

35.2.1.2 CYP Induction (Pregnane X Receptor and Isozyme Expression)

Some drugs are CYP isozyme inducers. This triggers an increase in the expression of a CYP isozyme. If a second drug is primarily metabolized by this isozyme, its metabolism will occur at a higher rate than normal, resulting in an unexpectedly low concentration of the effected drug in the bloodstream.

A higher-throughput method for detecting compounds that are CYP 3A4 isozyme inducers is the pregnane X receptor (PXR) assay. The inducer drug activates PXR, which binds to PXR response element (PXRE) in the 3A4 gene. A PXRE-luciferase reporter gene as been engineered in a special cell line to assay this mechanism.[8] When the inducer binds to PXR, which binds to PXRE, luciferase is expressed and detected.

Induction of CYP isozymes by the test compound can be directly measured.[9,10] After test compound treatment of hepatocytes, enzyme substrates that are specifically metabolized by one isozyme are incubated with the hepatocytes (or microsomes obtained from the treated cells), and the activity of each isozyme is measured using the specific substrates. An increase in the metabolic rate of a substrate indicates that the isozyme was induced by the test compound. Other induction methods biochemically quantitate the actual amount of each isozyme or its mRNA.

35.2.1.3 Aryl Hydrocarbon Receptor Assay

The aryl hydrocarbon receptor (AhR) assay examines AhR-mediated induction of gene expression, which can lead to carcinogenicity.[11] AhR activation is an initial event in adverse effects of dioxins and related compounds that bind to AhR and trigger several toxic biochemical responses, such as induction of CYP1A1 and CYP1A2. These are important in the metabolic activation of many promutagens. CYP1A1 metabolizes polynuclear aromatic hydrocarbons (PAHs) to carcinogenic arene oxides.

One in vitro assay for detecting this induction, called chemical-activated luciferase expression (CALUX), quantifies AhR-mediated reporter gene expression. A rat hepatoma cell line is stably transfected with a luciferase reporter gene, which is under control of dioxin-responsive enhancers. The cells are grown in 96-well plates for 24 hours and then treated with test compound. After 24 hours, the medium is removed, the cells are washed, luciferase is extracted by lysis, and luciferase activity is measured using a luminometer. Increasing luciferase activity indicates increasing AhR induction.

35.2.2 hERG Block Assays

These assays test the blockage of the hERG K^+ ion channel. hERG methods are discussed in Chapter 34.

35.2.3 Mutagenicity/Genotoxicity

Mutagens (genotoxic compounds) change the DNA sequence of genes, or they damage chromosomes. This leads to changes in the gene products (proteins), which usually makes them either less functional or nonfunctional. Most importantly, mutation can cause cancer. Several in vitro and in vivo assays for detection of DNA damage caused by test compounds have been developed. Use of two or more tests in parallel provides the highest sensitivity for detecting the mutagenicity of a compound.[12] These assays detect many, but not all, carcinogens, especially those that work through mechanisms other than DNA damage. Many of the following assays metabolically activate the test compound as an early step in the assay by adding liver S9 and cofactors to the assay matrix with the cells.

35.2.3.1 Micronucleus Assay

This mutagenicity assay detects compounds that damage chromosomes or the cell division apparatus, producing abnormal DNA fragments. During mitosis, the chromosomes do not migrate properly because the centromere is damaged or lacking. The resulting DNA pieces, called *micronuclei*, adhere to membranes and can be observed by microscope.

The assay in single point mode is conducted by incubating cells in culture with test compound at 10 mM, with and without metabolic activation. The treated cells are cultured after treatment. Chromosome damage causes formation of micronuclei. The cells are stained and examined by microscope for the number of cells that contain micronuclei and how many micronuclei are present per cell. If the test compound has a dose dependency in producing micronuclei or if the production is reproducible, the test compound is classified as "positive."[13–15] Advances in cellular imaging technologies have allowed more rapid unattended examination of micronuclei.[16]

35.2.3.2 Chromosomal Aberration Assay

Chromosome aberrations, caused by compounds, can lead to mutations, induce cancer, and cause other genetic diseases. The test compound is added to a mammalian cell culture and incubated for 3 hours, with and without metabolic activation. The medium then is replaced with compound-free medium, and the cells are cultured for 15 hours. Colcemid is added to arrest the cells in metaphase. The cells are fixed and examined by microscope for aberrant chromosomes.

35.2.3.3 Comet Assay

Cells in culture are treated with test compound, with and without metabolic activation, at concentrations up to 10 mM for 6 hours. The cells then are embedded in agarose on a microscope slide and lysed under mildly basic conditions. The cells are subjected to gel electrophoresis. DNA fragments from single- and double-stranded breaks and relaxed chromatin migrate faster in the electric field than does unchanged DNA, making them appear as a comet tail. The DNA is visualized using fluorescence microscopy after staining. The relative fluorescence intensity in the tail (DNA fragments) indicates the frequency of DNA breaks. The method is also called single-cell gel electrophoresis (SCGE) assay. The method is very sensitive to DNA breakage caused by genotoxic compounds.[17–19]

35.2.3.4 Ames Assay

The Ames assay examines the ability of a compound or its metabolite to cause DNA mutations. Both frameshift and base-pair substitutions are detected. Special mutant strains of *Salmonella typhimurium* or *Escherichia coli* bacteria are grown overnight in culture medium. Then the test compound (5 mg per plate), liver S9 fraction, and metabolic cofactors are added and incubated for 1 hour. This solution is mixed with agar and plated. After incubation for 72 hours, the number of colonies is counted. Greater numbers of colonies indicate a greater mutation rate.

The bacterial strains contain base-pair deletions that cause them to be able to grow only in the presence of supplemental histidine (*Salmonella* strains) or tryptophan (*E. coli* strains). If a mutation occurs at the location of the histidine or tryptophan base-pair deletions such that a functional enzyme can be made, the subsequent bacterial generations can grow without the supplemental amino acids. The colonies that are counted are called *revertants*. A strain has been engineered that lacks the genes for DNA excision repair, and another in which the cell wall is more permeable to mutagens. These strains produce a larger number of reversions.[20–25] Similar methods are the SOS Chromotest[26] and the RNR3-lacZ yeast test.[27]

35.2.3.5 Thymidine Kinase Mouse Lymphoma Cell Assay

Like the Ames assay, the mouse lymphoma assay determines test compounds that cause gene mutations by base-pair substitutions and frameshifts, but in mammalian cells. The detection system for this assay is mutation of the thymidine kinase (TK) gene to a nonfunctional form. The normal function of TK is phosphorylation of thymidine to produce thymidine monophosphate (TMP). The TMP concentration controls the rate of DNA synthesis in cells. A thymidine analog trifluorothymidine (TFT) is introduced to cells after treatment with the test compound. Cells that have normal TK die, but cells that have TK mutations are unaffected by the TFT.

Mouse lymphoma cells suspended in culture medium are treated for 4 hours with the test compound (over a range of concentrations), liver S9, and cofactors. After this treatment, cells are centrifuged and washed to remove the test compound, resuspended, and incubated for 2 days. Cells then are seeded into 96-well plates containing TFT. After 14 days, the cell colonies are counted. The number of living colonies is indicative of the mutagenicity of the test compound.[28–30]

35.2.3.6 HPRT Chinese Hamster Ovary Cell Assay

This assay is another mammalian cell mutagenicity assay. The assay is sensitive to mutation of the HPRT gene to a nonfunctional form. HPRT is involved in DNA synthesis. As part of the detection process for this assay, the nucleoside analog 6-thioguanine (6-TG) is introduced to cells after they have been treated with the test compound. Cells that have normal HPRT die, but cells that have HPRT mutations are unaffected by the 6-TG.

Chinese hamster ovary (CHO) cells suspended in culture medium are treated for 4 hours with the test compound (over a range of concentrations), liver S9, and cofactors. After this treatment, cells are centrifuged and washed to remove the test compound, resuspended, and incubated for 3 days. Cells then are seeded into 96-well plates containing 6-TG. After 10 days, the cell colonies are counted. The number of living colonies is indicative of the mutagenicity of the test compound.

35.2.3.7 GADD45a-GFP Genotoxicity Assay

The GADD45a (growth Arrest and DNA damage) gene is a biomarker for genomic stress. It is involved in regulating DNA repair, mitosis delay, and apoptosis. The promoter for GADD45a has been linked to green fluorescent protein (GFP) and transfected into human p53-proficient TK6 cells.[31] This stable cell line (GenM-T01) is used in 96-well format for detection of genotoxins. An assay kit (GreenScreen GC) is available from Gentronix. In initial tests, the performance of the GreenScreen assay exceeded that of other assays in sensitivity (success in detecting carcinogens) and selectivity (success in identifying noncarcinogens). In addition to mutagens, the assay detects aneugens (interfere with chromosome segregation in mitosis), which usually are detected using the micronucleus assay. The presence of p53 in the cell line appears to be important in accurate induction of the cell's response to genotoxins, and p53 is missing in cell lines used for some other in vitro genotoxicity assays. This high-throughput assay could precede current genotoxicity assays to increase efficiency and early detection.

35.2.4 Cytotoxicity

Cytotoxicity is the killing of viable cells by the test compound.[32] This approach surveys many diverse mechanisms by which the compound can impede the normal function of the cell and trigger cell death. Hepatocytes are advantageous for cytotoxicity assays because both the test compound and its metabolites can cause toxicity.

35.2.4.1 MTT Human Hepatotoxicity Assay

This assay detects compounds that are toxic to human hepatocytes in culture. The indicator of healthy function is the reduction of yellow-colored 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to purple-colored formazan by mitochondria. The concentration of formazan is measured by absorption at 570 nm.

Human hepatocytes are plated and cultured for 2 days. The test compound is added and incubated at concentrations of 10 to 1,000 μM for 24 hours. The medium containing the test compound is removed and replaced with medium containing MTT. After 3 hours of incubation, the cells are lysed, formazan is extracted by organic solvent, and the absorption at 550 nm is measured. Absorption below that of the control indicates that the test compound is toxic to the cells. Other dyes used for cytotoxicity assays are 3-(4,5-dimethylthiazol-2-yl)-5-3(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and (2,3)-bis-(2-methoxy-4-nitro-5-sulphenyl)-(2H)-tetrazolium-5-carboxanilide (XTT). Chinese hamster cells also have been used for the MTT assay. These cells lack metabolizing enzymes, so they often are coincubated with liver S9 and cofactors.

35.2.4.2 Lactate Dehydrogenase Assay

The plasma membrane of a dead cell will lyse and release the cell contents into the medium. Lactate dehydrogenase (LDH) is an abundant enzyme that is released. The concentration of LDH is measured using a colorimetric reaction. Cells in culture are treated with the test compound at a range of concentrations for 24 hours. The medium is harvested and tested for LDH activity using a test kit (Roche) that quantitatively produces formazan, which is measured by UV absorption. Assays for other enzymes that are released by membrane lysis include alanine aminotransferase and aspartate aminotransferase.

35.2.4.3 Neutral Red Assay

Neutral red is a dye that is absorbed by hepatocytes and concentrated in lysosomes. It is a marker for healthy cells. Hepatocytes are treated with the test compound, as in other hepatotoxicity assays. Increasing uptake of neutral red is associated with increasing cell survival. This assay is claimed to be simpler and more sensitive than the LDH leakage test.[33]

35.2.5 Teratogenicity: Zebrafish Model

Developmental toxicology has been studied using rodent embryo development. An emerging model is zebrafish development.[34] Zebrafish are small (adults are 1–1.5 inches) and easily maintained. They require only a small amount of test compound for dosing. Embryos are easily handled in 384-well plates. Their size and transparency make them easily examined for specific developmental end points of teratogenicity studies. Abnormalities are readily observed.

35.2.6 Selectivity Screens

In order to avoid disadvantageous activity at another enzyme, ion channel, or receptor in the body, a screen of diverse targets usually is performed on selected advanced leads. This screening usually is performed by a contract laboratory (e.g., MDS Panlabs, Ambit Biosciences, NovaScreen).

35.2.7 Reactivity Screens

These assays examine the reactivity of compounds and metabolites with cellular components.[35]

35.2.7.1 Glutathione Trapping

Glutathione is a ubiquitous compound that traps reactive compounds and prevents damage to vital proteins and nucleic acids. Reactive metabolites are detected by adding glutathione to the in vitro metabolic stability incubation (see Chapter 29) along with microsomes or hepatocytes. The glutathione and *N*-acetylcysteine adducts are extracted and analyzed using liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) techniques to detect adducts by the added molecular weight and then elucidate the structures of the adducts to determine the reactive site of the molecule.[36,37] This approach is useful in discovery, when no radiolabeled compound is available.

35.2.7.2 Covalent Protein Binding

A radiolabeled compound is used in the in vitro metabolic stability incubation (see Chapter 29) along with microsomes or hepatocytes for 1 hour. The protein is separated and analyzed for incorporated (covalently bound) radioactivity. A guideline for assessment is whether >50 pmol equivalents per milligram of the compound has been incorporated in the protein. The assay can be performed in vivo.[38]

35.3 In Vivo Toxicity

In vivo studies are of great importance because they are the ultimate indicator of toxicity. They are especially useful during studies leading up to first-in-human (FIH) phase I dosing and those conducted during phase II to III.

35.3.1 Discovery In Vivo Toxicity

Toxicity studies are performed during discovery for selected lead compounds or as specific issues arise. In vivo toxicity studies supporting drug discovery are intended to examine critical safety issues. A limited number of animals is used, and studies are short duration (e.g., 2 weeks). Many of the same parameters studied in preclinical toxicity studies are examined during discovery, but in less detail and under non-Good Laboratory Practices (GLP) conditions. Preclinical and clinical toxicity studies are discussed in Section 35.3.2. Discovery toxicity studies are sometimes conducted in coordination with in vivo pharmacology dosing studies, so fewer resources are needed. For discovery lead compounds, the purposes of in vivo toxicity studies are as follows:

- Check for any signs of toxicity
- Obtain data for lead prioritization
- Follow up with toxicological examination of other leads if toxicity is observed

35.3.2 Preclinical and Clinical In Vivo Toxicity

In vivo toxicity studies are described here in general terms to provide discovery scientists with an introduction to many of the tests to which clinical candidates are subjected once the discovery project team advances them to development. In vivo toxicity tests are required prior

to FIH dosing and are performed under GLP conditions using highly detailed protocols.[39] A general scheme used in industry for preclinical studies between candidate advancement and phase I trials is as follows:

- Acute (single-dose) toxicity
- Chronic toxicity: 2 to 14 weeks, daily dosing, rodent and nonrodent
- Carcinogenicity: 2 weeks of chronic dosing
- Genotoxicity: In vitro Ames test, in vivo mouse micronucleus test, chromosome aberration
- Safety pharmacology: Monitoring of normal health, behavior, and function using medical examinations and tests for central nervous system, cardiovascular (including radiotelemetry), respiratory, gastrointestinal systems, and kidney. Tests include physical appearance, body weight, food consumption, eye function, electrocardiography, blood chemistry, urine, and organ weight. Many of these studies are defined by the ICH guidelines.[7]

A full microscope histology examination is performed on as many as 50 tissues from dosed animals.

Pharmacokinetic studies that are performed and correlated with toxicology studies are called *toxicokinetics*. Toxicokinetic studies allow determination of the following:

- No effect level (NOEL): Highest dose or exposure that produces no toxicity
- No adverse effect level (NOAEL): Highest dose or exposure that produces manageable toxicity
- Therapeutic index (margin of safety): NOEL or NOAEL/efficacious dose or exposure

A candidate is terminated if severe toxicity is observed or if the therapeutic index is too narrow.

The purposes of preclinical studies are to predict patient hazards, ensure a wide therapeutic index, plan clinical phase I studies and dosing regimens, determine what organs are affected, determine which toxicity markers to look for in humans, determine any toxic metabolites, and examine drug responses that cannot be studied in humans.[40]

More detailed animal toxicity tests are performed during the phase I to III time period and include the following:

- Toxicity: 3 to 12 months, rodent and nonrodent (e.g., dog, monkey)
- Reproductive health: Mating behavior, estrous cycles, sperm, fertility
- Embryonic development: Survival, normal fetus and offspring growth, health, and responses (rodent, nonrodent)
- Oncology: 2 years, rat and mouse
- Immunotoxicity (immunosuppression or enhancement)[41]
- Toxicokinetics

35.3.3 Biomarkers of In Vivo Toxic Responses

In vivo toxicity studies have relied heavily on phenotypic response using histological examinations and microscopic examination of tissues prepared from dosed animals. New technologies have demonstrated the ability to observe toxic responses at the biochemical level. These techniques profile small-molecule biochemical intermediates, proteins, and mRNA as biomarkers of toxicity. This is an emerging and developing field. If successful, this approach will increase the number of compounds that can be evaluated and reduce the time required for toxicity studies. These emerging technologies are expected to have a major impact on toxicity studies in the future.

35.3.3.1 Toxicometabonomics

It has been shown that toxicity can, in some cases, be detected earlier using spectroscopic analysis of body fluids.[42,43] Toxic activity, such as inhibition of an enzyme by the drug or a drug metabolite, will cause an imbalance in the normal biochemical intermediates in the organism. The concentrations of intermediates in the pathway of the inhibited enzyme increase or decrease. The study of endogenous biochemical intermediates is called *metabonomics*.

Animals are dosed with the test compound once or daily for several weeks. Urine or blood samples are collected and analyzed. The change in endogenous compound concentration is detected using LC/MS or NMR techniques. Hundreds of components are present in these samples, and sophisticated analysis methods are required. Detection of the affected endogenous compounds compares the spectra and chromatograms of samples from treated individuals to samples obtained before treatment. One challenge is to determine if changes are due to normal biological fluctuations or due to effects of the test compound. In many cases, biochemical changes are detectable in this manner before behavioral or morphological signs of toxic response are observed. The actual intermediates that change with dosing indicate which pathway is affected.

35.3.3.2 Toxicoproteomics

In a similar manner to metabonomics, the balance of proteins in a biological system can change in response to the administration of a drug. Some of these changes are advantageous and consistent with the pharmacological goals of affecting the disease. However, other protein pattern changes, are indicative of a toxic response. The study of the protein ensemble of a cell or an organism is called *proteomics*.[44] These studies use two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and matrix assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometric analysis of protein mixtures from the sample.

35.3.3.3 Toxicogenomics

Genomics is another indicator of toxic response. The mRNA in the cells is profiled to monitor gene expression in response to compound dosing.[45–48] This technique is also called *transcriptional profiling*, and application of this technique has been termed *toxicogenomics*. cDNA and oligonucleotide microarrays are used to profile the thousands of mRNAs that might be modified by drug administration. Strong correlations among histopathology, clinical chemistry, and gene expression profiles have been reported.[45]

Problems

(Answers can be found in Appendix I at the end of the book.)

1. On what are the currently most successful in silico tools for toxicity prediction based?
2. What are in silico toxicity tools useful for?
3. What does the PXR assay test?
4. What method other than PXR can be used for this?
5. Which of the following compounds damage DNA?: (a) enzyme inducers, (b) cytotoxic, (c) mutagenic, (d) potentially carcinogenic.
6. In the following table, link the assay with the observed end point:

Assay	DNA fragments move faster in gel electrophoresis than normal DNA	Abnormally divided DNA is observed by microscope	Reversion mutations allow colonies to grow without histidine	Unusually shaped chromosomes	Normal mammalian cells mutate so TMP does not kill them
Micronucleus					
Chromosomal aberration					
Comet					
Ames					
TK mouse lymphoma					

7. The LDH assay works by which of the following mechanisms?: (a) uptake by healthy cells is detected colorimetrically, (b) LDH is taken up into the cells and reacts in the mitochondria to form a detectable product, (c) lysis of unhealthy cells releases enzymes that are detected with a biochemical assay.
8. Teratogenicity can be determined using what?
9. What compound is used to trap reactive metabolites?
10. What toxicity studies are always performed prior to human phase I dosing?
11. What newer "-omics" methods have much potential for future toxicity studies? What do these assays measure?

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Integrity and Purity Methods

Overview

- *High-throughput integrity and purity methods commonly use high-performance liquid chromatography/ultraviolet detection/mass spectrometry (LC/UV/MS).*
- *UV response indicates the relative percentage of each sample component.*
- *MS indicates consistency with the compound's molecular weight or potential identity of impurities.*
- *Follow-up of inconsistent samples that are active can provide additional project leads.*

36.1 Criteria for Integrity and Purity Assays

Compounds that were synthesized within the last few years and were subjected to independent analytical review typically have high confidence with regard to their identity and purity. In contrast, compounds that are older may not have been rigorously confirmed or may have undergone improper handling or storage. In this case, a check of structural identity and purity will provide greater assurance for structure–activity relationships (SAR).[1–3]

The common practice in synthetic chemistry is to follow reaction steps for new compounds using structure-specific analytical techniques, such as mass spectrometry (MS) for molecular weight (MW) confirmation and NMR for more detailed characterization. High-performance liquid chromatography (HPLC) is used for purity estimation. Often these resources are available in a walk-up open-access format for convenience and flexibility that fits the workflow of chemists. Many organizations have a central analytical department that independently verifies that the spectra are consistent with the putative synthetic product. This is a wise strategy. Unbiased confirmation of the identity and purity of a compound that will be registered in the company's valuable compound collection is worthwhile.

The techniques used by medicinal chemists and analytical departments for this process are relatively detailed and time consuming. When projects are dealing with the thousands of high-throughput screening (HTS) hits that come from a screen, thousands of compounds that might be purchased from a vendor, hundreds of compounds that might come from an alliance collaborator, or hundreds of compounds that might come from a library similarity search, a faster and less resource-consuming assay method is necessary. Tradeoffs must be made to efficiently assay these samples. Turnaround time and resources expended per compound usually must be kept as low as possible. This dictates the need for faster methods and results in reduced resolution. For example, a typical high-resolution HPLC analysis of purity may use an analysis time of 30 to 60 minutes, whereas a higher-throughput integrity and purity assay may use an analysis time of 3 to 5 minutes. Techniques are combined for efficiency, such as integrating HPLC and MS to obtain purity and integrity data from one analytical run. Such compromises reduce the HPLC resolution for separation of impurities and the

spectroscopic identity check is reduced to molecular weight from the molecule ion. These are still acceptable levels of data for the discovery project team to proceed with increased confidence for decision making.

These tradeoffs are a common part of a strategy of "*appropriate methods.*" In appropriate methods, the analytical method is streamlined such that it meets the needs of the discovery project team at the current stage of their work and does not consume unnecessary resources and time delays in obtaining too much detail for the question being asked. Integrity and purity profiling addresses the question of whether the compound appears to have the identity that is shown in the corporate database and if its purity is high enough to ensure that the observed activity derives from the putative compound. Resources are not available for more in-depth analysis, and the answer must come quickly to stay in synchronization with the discovery time line. The key activity is prioritization of many compounds for the next level of research experiments. Analytical detail can be assured at later discovery stages using lower-throughput techniques with higher analytical figures of merit for the fewer compounds that are studied in great detail (Table 36.1). Drug discovery currently is strongly guided by business strategies, such as *risk management*. Integrity and purity assays help to reduce the risk at earlier drug discovery stages with appropriate investment of resources.

TABLE 36.1 ► Comparison of High-Throughput and Low-Throughput Integrity and Purity Methods

	High throughput	Low throughput
Purpose	Rapid screen to avoid identity and purity mistakes	Detailed data to provide assurance at later stages
Samples/wk	100–1,000	10–100
HPLC column	2 × 20 mm, 3- to 5-μM particles	4.6 × 150 mm, 3- to 5-μM particles
HPLC run time (min); function	1–3; screen	30–120; resolve all components
Detector	UV relative area percent	UV, ELSD, CLND high-accuracy quantitation
Mass spectrometer	Single-stage quadrupole, ion trap, or time-of-flight MS	Single-stage MS to MS/MS; accurate mass analysis
Other spectroscopy	None	NMR

36.2 Samples for Integrity and Purity Profiling

When considering which samples to profile, it is important to remember the question to be answered. If the question is: "What is the quality of the solid sample from the compound repository?", then it is appropriate to obtain the sample in a vial and analyze it. However, if the question is: "What is the quality of the HTS hit?", then it must be remembered that HTS is performed with plates of compounds in solution that have been stored for a period of time. For this question, it is appropriate to obtain a small aliquot (2–5 μL) of the solution used for the HTS run. It is this solution that is linked to the HTS activity hit. This sample may differ from the solid that is in the compound repository because of handling and storage.

A high-throughput integrity and purity screen also allows the rapid profiling of compounds that come into laboratories from outside sources. Thus, at low expense potentially costly and time-consuming mistakes from the use of an inaccurate compound can be avoided.

36.3 Requirements of Integrity and Purity Profiling Methods

A wide array of analytical technologies potentially could be incorporated into integrity and purity methods. Typically, these exhibit the common analytical tradeoffs of selectivity, sensitivity, speed, and cost. Thus, different techniques can be chosen to match the needs of the assay to answer the particular research question at a specific place in the discovery time line. In late discovery, there is a need for *in-depth profiling* of a few selected compounds. With these few compounds and the importance of specific information for decision making, it is appropriate to use techniques with slower speed, such as extended HPLC separations with longer columns, slower gradients, and smaller particle size for high-resolution separation of sample components. Other examples are MS/MS product ion spectra for detailed fragmentation analysis, multiple NMR experiments (proton, carbon, two-dimensional), x-ray crystallography, and elemental analysis. These techniques provide unambiguous confirmation of identity, regiochemistry, stereochemistry, and quantitative measurement of purity on which critical late-stage experiments can be based for go/no-go decisions. Unfortunately, this approach is not appropriate for early discovery HTS integrity profiling. It requires considerable scientist's time, materials, instrumentation, and time line to accomplish. A *high-throughput profiling* approach is necessary for earlier studies.

In early discovery, only a small amount of each compound is available for property profiling. Only submilligram levels of HTS hits are available from HTS plates, and only milligrams are available as solids from the compound repository. This necessitates sensitive methods, such as HPLC, UV detection, and MS.

Integrity profiling methods also must provide data that are correlated to structure. NMR is highly correlated to detailed structural moieties, but NMR interpretation is too time consuming for integrity screening. MS provides a confirmation of MW, which is closely associated with structure. MS also provides initial structural information for impurities. Degradants and reaction by-products usually differ greatly in MW from the primary component.

Selectivity can be provided for a method by using a separation technique, such as HPLC. MS adds selectivity by distinguishing compounds by MW, even if they co-elute. Fast analytical methods are required to provide rapid turnaround. This allows discovery teams to make major decisions rapidly. Biological assay data usually are available within days or weeks, so integrity and purity data also should be provided in this time frame to make a meaningful contribution.

Integrity and purity methods for early discovery need speed so that they can handle a large number of samples, on the order of 1,000 samples per week. The large number of samples also demands that costs be kept low. The figure of $250,000 per scientist per year is commonly used in the industry. An analyst must process about 125 compounds per day in order to keep the analysis cost down to $10 per sample.

36.4 Integrity and Purity Method Advice

The integrity and purity assay may seem to be a simple assay; however, several aspects can ensure a reliable and efficient analysis. These are discussed in terms of the assay's steps: sample preparation, component separation, detection, quantitation, and confirmation. It is useful to begin by considering all of the available techniques that might be used for this method (Table 36.2). The appropriateness of these techniques is discussed below (Section 36.4.1–36.4.5).

TABLE 36.2 ► Various Techniques that may be Incorporated into Integrity and Purity Assays

Technique	Throughput (samples/h)	Analytical detail	Appropriate for HT profile	Appropriate for in-depth profile
Flow injection	60	L	M	L
Fast HPLC	20	M	H	L
High-resolution HPLC	2	H	L	H
NMR	5	H	L	H
MS	20	M	H	L
MS/MS	5	H	L	H
IR	5	M	L	M
UV	20	M	H	M
ELSD	20	H	M	H
CLND	20	H	M	H

Used with permission from [13].
The characteristics of the techniques make them appropriate for methods to address the needs of different parts of the discovery timeline.
H, High; L, low; M, moderate.

36.4.1 Sample Preparation

Accurately weighed and labeled samples can be readily handled for high throughput in well plates. For low-throughout analysis, vials are adequate. Sample handling and solvent addition using laboratory robots are very efficient.

It is important to completely dissolve the sample in order to assure accurate purity quantitation and to observe all of the sample components. The solubilities of different sample components can differ, and the main component may not, at first, be completely soluble. Dimethylsulfoxide (DMSO) is often used as a dilution solvent because of its "universality," but not all compounds are soluble. Freeze–thaw cycles tend to cause a compound to recrystallize to a stable polymorph, and this polymorph may not readily redissolve.[4] Some polar compounds (salts) have low DMSO solubility. In these cases, addition of a small volume of a second miscible solvent of differing polarity or use of a solvent mixture for the original dilution can assist dissolution. Precipitates cannot always be seen by visual inspection. A careful examination, under conditions where fine particulates can be observed, is warranted. Another issue with using DMSO is its strong UV absorption, which produces an intense peak at the void volume. This peak may obscure compounds that are not well retained on the HPLC column.

36.4.2 Component Separation

Some laboratories use flow injection analysis (FIA), in which the sample is injected directly into the detector. This is commonly done for a quick check at open-access facilities. FIA is very fast, requiring less than 1 minute per sample. However, there is no component separation, and the method is not adequate for purity estimation. In addition, the dilution solvent causes a strong signal, termed *solvent front*, which can interfere with component detection. In most samples, multiple components are present, even if they are at trace levels. The putative compound usually is the main component; however, many times the impurities are the main components. Integrity and purity assays are best accomplished using HPLC separation.

HPLC can separate the sample components from the solvent front that would interfere in FIA. High-resolution HPLC assays require up to 1 hour per sample. This would be appropriate for late discovery release of batches for toxicology studies. For early discovery studies in high throughput, this would consume too many resources. One issue for selection of a "generic" method for all samples is that compounds vary in their chromatographic characteristics. HPLC conditions that work for a wide range of compound polarities must be selected. Typically, reversed-phase HPLC is used with a wide mobile phase polarity gradient. For example, the gradient might start with 100% aqueous buffer and proceed to 100% acetonitrile. In recent years the technique of "fast HPLC" has been widely implemented, where the gradient is completed in a short time (1–2 minutes), the small-particle stationary phase is used (3–5 μm), and the mobile phase is at high flow rate (e.g., 1 mL/min).[5] These conditions may not provide sufficient chromatographic resolution and an acceptable tradeoff of resolution and speed must be found.

Recently, instrument manufacturers have introduced higher-resolution HPLC systems that use particle sizes of around 1.7 μm. If higher flow rates of 1 mL/min are used, very high pressures are generated that are beyond the capability of standard HPLC instruments. One company has termed the new technique *ultraperformance liquid chromatography*. Very high resolutions can be achieved with gradient times of about 1 to 1.5 minutes for high throughput, plus 0.5 to 1 min for re-equilibration. Supercritical fluid chromatography has been used to achieve higher resolution and increased throughput in some laboratories,[6] but it is not a common technique.

There are pitfalls to any HPLC separation method for which the analyst should be vigilant. A sample component may co-elute with another component and remain undetected. These components can be deconvoluted using MS if they have different MWs. Polar components may be buried under the solvent front. Very lipophilic components may not elute during the gradient and require an extended hold of the solvent ratio at the top of the gradient to assure elution. Enantiomers require chiral methods to be independently quantitated.

If MS is used for identity checking, the mobile phase must be compatible with the MS. Commonly, water, acetonitrile, and methanol are used as solvents. Common modifiers are ammonium acetate and formic acid. If a chemiluminsecent nitrogen detector (CLND) is used, acetonitrile cannot be used as a solvent. The mobile phase must be completely volatile if an evaporative light scattering detector (ELSD) or CLND is used.

36.4.3 Quantitation

A technique for detecting and measuring each component is necessary for purity estimation. A "universal" detector would be useful, but no detector responds to every compound with the same response on a molar basis. The most common detector is UV, which is sensitive and cost efficient. Most compounds studied in discovery absorb in the UV range. It is important to consider the UV wavelength that is used. The wavelength 254 nm typically is associated with aromatic groups and is commonly used. Some compounds do not contain an aromatic group; thus, the 214- to 220-nm region is often used for a broader compound response. Unfortunately, DMSO also absorbs in this region and makes an intense solvent front peak. Diode array UV detectors can be set to scan over a broader portion of the UV spectrum for more universal compound detection. The analyst should keep in mind that the diversity of UV spectra and molar absorptivities at a given UV wavelength can cause great differences in the quantitation of purity, depending on the wavelength chosen. Minor impurities at one wavelength may appear to be much greater at another wavelength. Despite these limitations, UV detection usually is sufficient for most integrity and purity profiling

needs. It helps provide information that reduces the risk of wasted time and resources on inaccurate or impure compounds.

The ELSD and CLND appear to have somewhat greater universality than the UV. The ELSD nebulizes the HPLC effluent and evaporates the volatile solvents to condensed particles that are detected by light scattering.[7] The CLND vaporizes the HPLC effluent.[8,9] Components are pyrolized at high temperature, and nitrogen oxides produce light, which is detected. CLND requires relatively higher maintenance than other detectors, and its response is proportional to the number of nitrogen atoms in the molecule. This poses a problem with quantitation of unknown impurities.

Purity estimation typically is done using the relative response of each component. It is assumed that each component has an equal molar response. This approach results in some inaccuracy compared to quantitation using standards for each component. However, it is approved by the Food and Drug Administration for the purpose of quantitating unknown impurities in clinical batch release for development and manufacturing. Quantitation can be more accurate using ELSD with internal standards and CLND for known compounds. This is most useful for later discovery work. Despite these precautions, some components, such as trifluoroacetic acid, inorganic salts, silica, plastic extracts, and volatile solvents, can remain undetected.[9]

36.4.4 Identity Characterization

Another technique needed in the analysis is one that produces data that are related to structure and can be used to confirm the compound identity. For high-throughput profiling, the technique must produce signals at the nanogram compound level and be easily interpreted. Newer probes have allowed NMR to work with flowing aqueous streams. Eventually, the throughput will be consistent with higher throughput and sensitivity needs. However, NMR spectra continue to be time consuming for interpretation.

Single-stage MS is often used for this application. It is sensitive and interfaces readily to HPLC. The mass-to-charge ratio (m/z) of the molecule ion is rapidly interpreted in terms of the MW of the compound. Electrospray ionization (ESI) is often used. Both positive and negative ions are readily provided by ESI. Amines typically produce positive ions, and acids typically form negative ions. Occasionally, atmospheric pressure chemical ionization (APCI) and atmospheric pressure photo ionization (APPI) are used to produce ions for compounds that are not sensitive by ESI. Ion sources that combine two ionization methods, such as ESI and APCI, have begun to be commercially available and broaden the opportunities for producing ions from sample components in a single HPLC run.[10] All of these techniques usually produce ions that are easy to interpret as $(M+H)^+$ or $(M-H)^-$ ions. MS instruments can alternate between positive and negative ion analysis in a single HPLC run, thus efficiently providing a broad detection of diverse sample components.

In examining the mass spectra, less experienced scientists should be aware of potential interpretation mistakes. A compound may form adduct ions, which are produced when an ion from the mobile phase attaches to the analyte molecule and produces ions such as $(M+NH_4)^+$, $(M+Na)^+$, $(M+H+CH_3CN)^+$, $(M+HCOO)^-$, or a dimmer molecule ion $(2M+H)^+$. A labile ion may fragment to lose a molecule of water $(M+H-H_2O)^+$. The presence of a chlorine or bromine atom in the molecule can confuse interpretation because of the high abundance of $M+2$ ions from naturally abundant stable isotopes. It is not sufficient to plot an ion chromatogram for the $(M+H)^+$ or $(M-H)^-$ ions of a compound to confirm its presence or absence. The spectra must be examined to determine that they are consistent with the putative structure and to assign the MW of an unknown impurity.

Several types of mass spectrometer are equally useful for integrity profiling: quadrupole, ion trap, and time of flight (TOF). TOF provides the possibility of highly accurate mass analysis when it is used with a mass calibration standard. Some groups prefer the greater confidence provided by accurate mass, but it may be more time-consuming to maintain. Quadrupole analyzers are the least expensive systems. Two MS manufacturers offer options for interfacing more than one HPLC column stream (2–8) to a single MS. The interface sequentially shifts each stream for analysis every fraction of 1 second. With one HPLC stream and a 5-minute analysis, 240 samples can be analyzed in 20 hours; with 4 HPLC streams, 960 samples can be analyzed in 20 hours.[11] Although some MS vendors provide software for an automated check of spectra, it is advisable for an experienced analyst to review the assignments, which can be time consuming.

NMR remains the best approach for high confidence in identity assignment. Proton NMR now can be coupled to HPLC for analysis of submilligram amounts of samples. However, this requires examination by a trained analyst for confident assignment and is more expensive than MS. Late-stage studies require the detail provided by one-dimensional and two-dimensional NMR analysis.

36.5 Follow-up on Negative Identity Results

In certain circumstances, it may be beneficial to follow up and try to identify compounds that were not confirmed using a standard method. One useful case is when an HTS hit is found not to be the expected compound but is present in high purity. Despite not being what it was supposed to be, it still is an active compound and may be a unique and valuable pharmacophore. It may be worth some additional effort to use more detailed analytical techniques, such as MS/MS and NMR, to elucidate the structure. When multiple components are present, they are sometimes isolated for individual activity testing. Modern fraction collection systems are used in combinatorial chemistry groups and are an effective approach. The structure of the active isolate can be elucidated to provide an active lead. However, this approach may be too time consuming for some companies.

Another follow-up opportunity presents when the compound's characteristics do not allow separation or detection using the standard method. Follow-up can use different chromatographic conditions or detection methods. Examining the structure can indicate a useful alternate method.

36.6 Example Method

Five microliters of a 10 mg/mL stock solution of test compound in DMSO is transferred to a well in a 96-well plate. The sample is diluted further with DMSO (or 50% acetonitrile/50% isopropanol) to a concentration of 500 ng/μL using a laboratory multiprobe robot. Each well is examined for any undissolved material. A small volume of polar solvent is added, or the plate is sonicated if some material remains undissolved. A 2- to 5 μL aliquot is injected onto the HPLC column. Separation utilizes an Aquasil column (2 ×50 mm, 5-μM particles) at 40°C and a flow rate of 0.8 mL/min. This column has a mixed stationary phase consisting of bonded C18, which retains lipophilic compounds, and bonded ethanol, which retains polar compounds, for use with a broad diversity of compound polarities. The mobile phase gradient is as follows: 100% mobile phase A (95% 10 mM ammonium acetate/5% acetonitrile)/0% mobile phase B (5% 10 mM ammonium acetate/95% acetonitrile) to 0% A/100% B in 2.5 minutes, hold for 1.5 minutes, and re-equilibrate for 1.5 minute. The mobile phase eluent flows into a UV diode array detector that is scanned from 190 to 600 nm to detect diverse components. Purity is estimated from the relative area under the

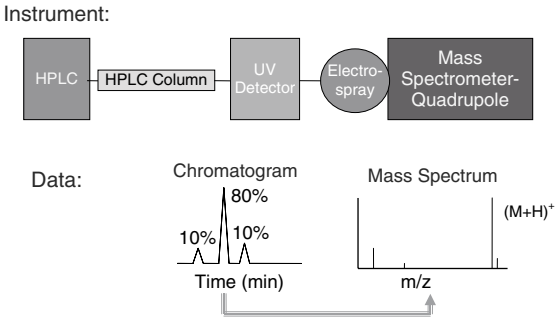

Figure 36.1 ► Schematic diagram of LC/UV/MS instrumentation in integrity and purity profiling.

chromatographic peaks of each component at 214 nm. A single-stage quadrupole MS with ESI obtains both positive and negative ion spectra each 1.0 second, while scanning from m/z 100 to 1,000. Analysis automation and postacquisition data processing are performed using the mass spectrometer's quantitation software. A schematic of the instrumentation is shown in Figure 36.1.

36.7 Method Case Studies

Kyranos et al.[12] profiled the identity and purity of compounds from high-throughput synthetic chemistry libraries. They found LC/MS to be more successful than FIA/MS or FIA/NMR for samples containing multiple components. APCI was found to be more universal than ESI but produced more fragment ions. "Fast HPLC" conditions were very reliable and had high throughput. The ELSD detector had consistent absolute molar purity quantitation if the compounds were known, and CLND had good absolute molar purity accuracy ($\pm 5\%$) for compounds containing nitrogen. Multiplexed parallel HPLC with a single MS provided very high throughput but was limited to a minimum HPLC peak width for accurate quantitation.

Hsu et al.[7] studied methods for monitoring drug discovery compounds. They found that not all compounds have UV chromophores and that molar absorptivities can vary widely. They found that the ELSD responded better to the weight percent of each component and thus is better for absolute quantitation, especially if an internal standard is used. ELSD did not produce a solvent front that can interfere with quantitation of early eluting components in UV. ELSD only produced signals for compounds that did not solidify in the drift tube. They found that UV was better for generic relative purity estimation and ELSD was better for absolute purity quantitation.

For cases needing absolute purity and yield quantitation, Taylor et al.[8] demonstrated that CLND is a useful tool for universal quantitation. A very consistent molar response for nitrogen-containing compounds, the majority of drug-like compounds, was observed. CLND response was independent of the mobile phase. An internal standard is recommended for accurate quantitation.

Yan et al.[9] found that many "invisible" impurities occur in combinatorial chemistry-derived library compounds, and these are not observed with common UV or MS detection schemes. Such compounds include trifluoroacetic acid, plastic extracts, inorganic salts, catalysts, silica, and resin washout.

Fang et al.[11] described the application of a high-capacity autosampler with eight parallel reversed-phase HPLC columns that are interfaced to eight UV detectors and a single TOF mass spectrometer using a multiplexed ion source. A cycle time of 3.5 minutes produced analysis throughputs of 3,200 samples per day.

Gallagher et al.[10] applied a combined ESI/APCI ion source and alternated both ion polarity (+/−) and ionization methods within a single HPLC run. They found that ESI provided useful spectra for about 80% of discovery compounds. APCI was useful for an additional 10% of the compounds. Combination of all these modes increased throughput at a low cost.

Ventura et al.[6] applied supercritical fluid chromatography with MS and reduced the analytical cycle time to one third that of LC/MS. SFC also exhibited enhanced resolution compared to HPLC. By using an APCI interface with SFC, throughputs of 400 samples per 24 hours were obtained.

Kerns et al.[13] combined integrity and purity analysis with Log D estimation in the same run. Log D was estimated from the same HPLC retention time data (see Section 23.2.2) that was used for integrity and purity profiling. Thus, multiple data types were obtained from one analysis.

Problems

(Answers can be found in Appendix I at the end of the book.)

1. Why is it useful to use a short (1–5 minutes) HPLC analysis as part of an integrity and purity assay?
2. Why is NMR not used for higher-throughput integrity and purity assays?
3. Why is it necessary to completely dissolve the sample for integrity and purity analysis?
4. How can newer HPLC columns with small particles enhance the assay?
5. What is a drawback of HPLC detectors for purity analysis?
6. What is a drawback of mass spectrometer interfaces for identity analysis?

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Pharmacokinetic Methods

Overview

- *In vivo pharmacokinetic (PK) parameter assessment is highly important for project teams.*
- *PK experiments have been accelerated using cassette dosing.*
- *PK quantitation ("bioanalysis") is accelerated by sample pooling and liquid chromatography/mass spectometry/mass spectrometry.*
- *Uptake into tissues can be very useful, as for brain or tumor targets.*

Considerable resources are dedicated to pharmacokinetics (PK) studies in drug discovery. Advancement criteria in drug discovery and development usually include guidelines for the major PK parameters. PK behavior represents a composite of the underlying physicochemical and biochemical properties of the compound in the dynamic living system. Individual PK parameters can be used as guides for diagnosing the properties of the compound (see Chapter 38) in order to improve performance by structural modification.

Discovery methods for animal PK studies utilize all of the techniques that are applied for human clinical PK study samples.[1] Good Laboratory Practice (GLP) and Good Manufacturing Practice (GMP) are not required for discovery PK studies.

37.1 PK Dosing

37.1.1 Single-Compound Dosing

Pharmacokinetic studies in drug discovery typically dose two to four animals with a test compound in a generic formulation (see Chapter 41). Doses vary, depending on the project, but a common dose level is 10 mg/kg oral (PO) and 1 mg/kg intravenous (IV). PO dosing typically is performed gavage administration of a compound solution or suspension directly into the stomach. Intraperitoneal (IP) dosing and subcutaneous (SC) dosing (see Chapter 41) also are used. Dosing one compound into an animal is sometimes called *discrete dosing*.

37.1.2 Cassette Dosing

The large number of compounds studied in drug discovery has stimulated interest in accelerated methods that increase the throughput of pharmacokinetic analysis. In one approach, called *cassette dosing* or *N-in-one*, several compounds (often 4-10) are mixed together in the same dosing solution ("cocktail") and coadministered.[2] Each compound is subjected to

the barriers of the living system and exhibits concentrations in plasma and tissues concomitant with its properties. The concentration of each compound is independently measured in the plasma sample by means of unique liquid chromatography/mass spectometry/mass spectrometry (LC/MS/MS) signals (unique molecular weight and product ions). With this strategy, if five compounds are co-dosed and coanalyzed in one LC/MS/MS analysis, the throughput is increased by about five-fold. The major concern with this strategy is that compounds may interact, that is, they compete for the same proteins involved in membrane transport, metabolism, and elimination (see Chapter 15). Thus, their PK parameters can be changed by inhibition from another compound in the same dose. Interaction can be minimized by lowering the dose of each compound, which lowers the concentration of individual compounds at the proteins at which interaction may occur, thus reducing the risk of interaction. Another approach for reducing interaction is to include a compound of known PK parameters (previously measured) in the cassette mixture. If its PK parameters are changed in the cassette, the investigators know that at least one compound in the cassette causes drug–drug interactions. In the case of demonstrated interaction, the compounds in that set can be restudied individually to assure accurate data. In an industry-wide benchmarking study, the majority of the respondents (64%) believe that cassette dosing can be used to rank order compounds, provided proper controls are used, but <10% use cassette dosing as their primary PK screening strategy.[3] The technique remains controversial, and shortcomings have been discussed.[4]

Cassette dosing has several advantages. Data can be generated for more compounds using the same resources (scientist's time, instrumentation). Fewer animals are used for more compassionate use. Data can be provided to teams faster than if they had to wait for each discrete dosing study to be performed in sequence.

37.2 PK Sampling and Sample Preparation

Blood samples are collected manually at specific time points after dosing (0.03 to 24 hours), treated with anticoagulant, and centrifuged to remove the blood cells and produce plasma. Automated sampling instruments are commercially available to collect samples unattended at specified time points (Table 37.1). Such systems can collect blood, bile, urine, and feces samples from awake and freely moving animals.

TABLE 37.1 ► Commercial Suppliers of Products for PK Studies

Product name	Company	Web site
Culex	BASi	*www.culex.net*
DR-II Automated Sampling System	Protech International	*www.protechinternational.com*
AccuSampler	DiLab	*www.dilab.com*
WinNonLin	Pharsight	*www.pharsight.com*

Samples are prepared for instrumental analysis by addition of two or more volumes of organic solvent (e.g., acetonitrile) and agitation. This treatment precipitates much of the plasma protein material so that instrumental analysis interference is greatly reduced. This technique is often called *acetonitrile crash*. The solution is centrifuged and the supernatant analyzed. Alternatively, plasma samples are extracted using solid-phase extraction. A measured volume of plasma sample is applied to a porous solid phase cartridge and

washed with aqueous phase. The cartridge is eluted with organic phase, and the eluent is instrumentally analyzed. Solid-phase extraction cartridges are commercially available in 96-well formats for automated processing.

Another analytical strategy to accelerate analysis is the cassette-accelerated rapid rat screen (CARRS) approach.[5] Two animals are dosed with a compound, and samples are collected from 0.5 to 6 hours. The samples from the two animals from the same time point are pooled into one mixture. This allows analysis of only a single sample per time point per compound. The method provides all of the PK parameters but requires only half the sample preparation and LC/MS/MS analyses of standard approaches. A disadvantage of the approach is that there is no information on the variability in PK parameters between the animals, which can vary greatly.

Samples from discrete dosing experiments are sometimes mixed for processing, commonly termed *pooling*. This allows for less sample preparation and LC/MS/MS analyses. It is necessary to pool only compounds of different molecular weight.

37.3 Instrumental Analysis

The adoption of LC/MS/MS quantitation for PK studies in the early 1990s greatly accelerated the speed and sensitivity of the analyses. A low-energy ion source (atmospheric pressure chemical ionization or electrospray) produces molecule ions for the analyte (e.g., MH^+, $(M-H)^-$). The first MS stage is set to selectively pass only the mass-to-charge ratio (m/z) value for the molecule ion. The second MS stage contains an inert gas (e.g., argon) with which the analyte molecules collide and become vibrationally excited. This energy is dissipated by fragmentation of the molecule ion into specific product (fragment) ions. The third MS stage is set to selectively pass only the m/z value of one of these specific product ions. Thus, the LC/MS/MS system provides three stages of separation (high-performance liquid chromatography [HPLC], MS for molecule ions, and MS for the product ion) for a highly specific technique that has a high signal-to-noise ratio (S/N).

When a cassette of compounds in the same sample is analyzed (from either cassette dosing [see Section 37.1.2] or pooling [see Section 37.2]), different molecule and product ions are used for specific analysis of each compound in the mixture. Compounds also usually are partially or fully separated by the HPLC system prior to entering the mass spectrometer. The instrument can be set to sequence between multiple parent–product ion pairs for selective quantitation of each compound.

Usually, rapid HPLC chromatography is used for PK. An analytical cycle time (injection to injection) of 2.5 to 3 minutes is common. Cycle times have been reduced to as low as 1.25 minutes.[6] New HPLC techniques, such as ultraperformance liquid chromatography, have accelerated the chromatographic separation further by use of small particle size, which allows reduction of cycle time to about 1.5 min.[7] Automation and software calculations assist in data handling and analysis.

Other instrumental techniques allow accelerated LC/MS/MS analysis. In the LC/MS/MS multiple HPLC interface method (called *MUX* by one vendor), the interface is set to sequence between multiple HPLC inputs (two or four), thus allowing simultaneous parallel HPLC separations and quantitation using just one mass spectrometer.[8] In the staggered input method, injections into multiple HPLCs are staggered in time and the effluent is switched to the MS just in time for the analyte peak to elute and be quantitated.[9]

Matrix suppression is one difficulty of LC/MS/MS analysis of plasma samples. Sample preparation often does not remove all of the plasma components. When some of these components (e.g., phospholipids) elute from the HPLC, they can suppress ionization of the analyte compound that co-elutes from the HPLC. This results in reduction and variability in

the signal from the test compound. Various approaches have been developed to successfully deal with this problem.[10]

The concentration–time data are analyzed using software (e.g., WinNonLin). These rapidly fit the data to mathematical PK models and calculate the standard PK parameters.

37.4 Example Pharmacokinetic Data

An example of hypothetical pharmacokinetic data from an experiment is given in Table 37.2 and plotted in Figure 37.1. In this experiment, the compound was individually dosed PO at 10 mg/kg and IV at 1 mg/kg. Samples were collected over 24 hours, starting at 2 minutes. PK parameters were calculated using WinNonLin software. The values of C_0, C_{max}, and t_{max} are illustrated in Figure 37.2. The PK parameters and the equations used to calculate clearance (Cl), V_d, and %F are given in Table 37.3.

TABLE 37.2 ► Hypothetical Data from a Single-Compound PK Experiment

Time (h)	IV (ng/mL)	PO (ng/mL)
0.033	970	Not sampled
0.25	420	550
0.5	250	600
1	110	310
2	30	100
4	1.9	20
6	0	6.3
8	0	1.8
24	0	0

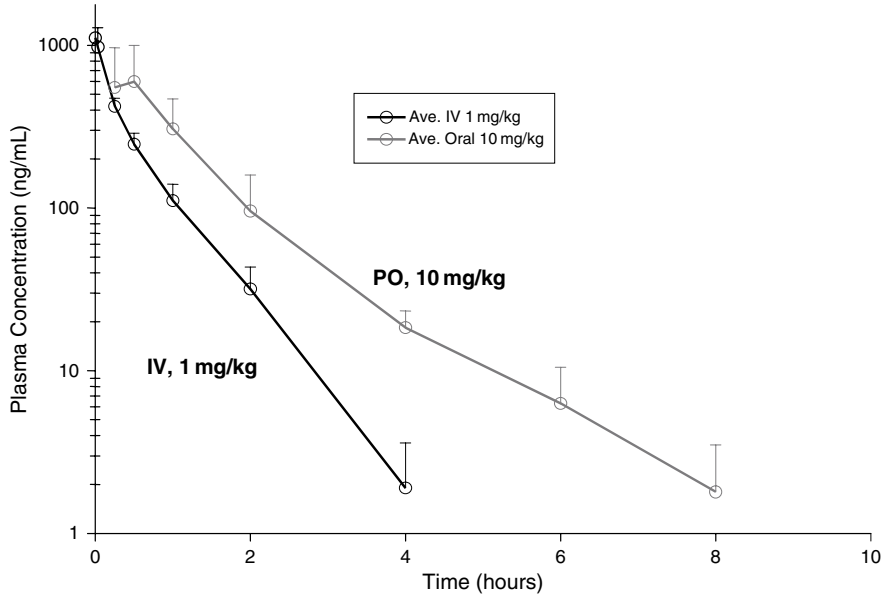

Figure 37.1 ► Hypothetical data from a pharmacokinetic experiment (see Table 37.2).

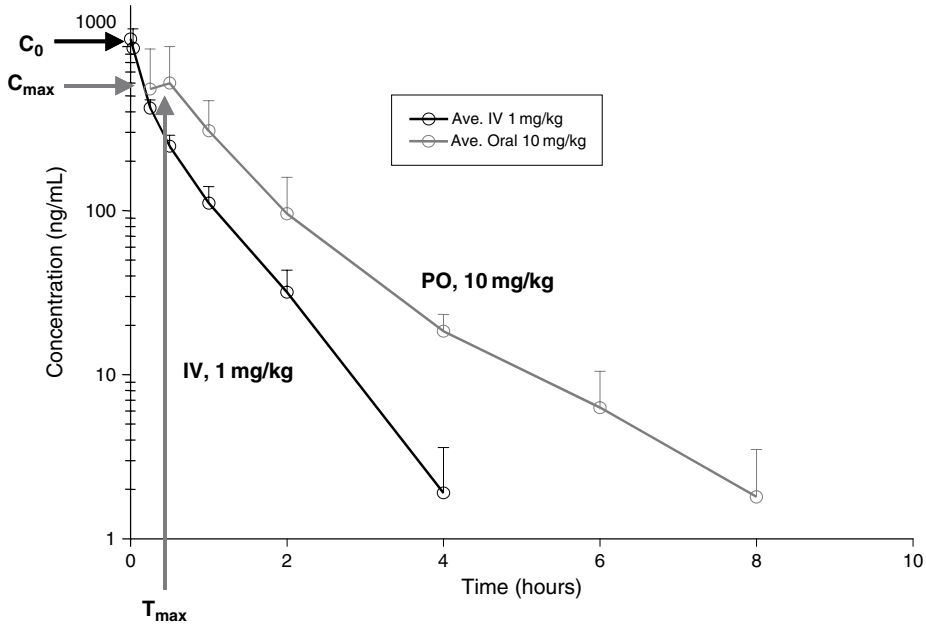

Figure 37.2 ► Determination of C_0, C_{max}, and t_{max} from PK data.

TABLE 37.3 ► PK Data Processed Using WinNonLin Software to Calculate C_0 and AUC, from Which Other PK Parameters are Calculated

	IV (1 mg/kg)	PO (10 mg/kg)
C_o (ng/mL)	1000	
$t_{½}$ (h)	0.5	1
AUC (h*ng/mL)	420	840
t_{max} (h)		0.5
C_{max} (ng/mL)		600
CL (ml/min/kg)	40	
V_d (L/kg)	1	
% Bioavailability		20%

$CL = Dose/AUC = (1\ mg/kg)/(420\ h^*ng/ml)^* 10^6\ ng/mg^* 1\ h/60\ min \sim 40\ mL/min/kg$
$V_d = Dose/C_0 = (1\ mg/kg)/(1000\ ng/mL)^* 10^6\ ng/mg^* 10^{-3}\ L/mL = 1L/kg$
$\%\ Bioavailability = AUC_{PO}/AUC_{IV}{}^* Dose_{IV}/Dose_{PO}{}^*100\% = 840/420^*1/10^*100\% = 20\%$

37.5 Tissue Uptake

Tissue uptake is necessary in order for the compound to reach most therapeutic targets. Blood–organ barriers limit penetration into some tissues. For example, CNS drugs must penetrate into brain tissue through the blood–brain barrier (see Chapter 10). The penetration of cancer drugs into tumors may be hindered, compared to other tissues, by reduced blood flow and tumor morphology.

Performing these studies involves sampling of the tissue at selected time points. Blood should be purged from the tissue prior to analysis so that compound levels really derive from the tissue and not from the blood. Tissue samples are first homogenized and then analyzed in a manner similar to plasma. The data typically are analyzed by calculating the ratio of (a) tissue concentration to plasma concentration at a particular time point, (b) AUC_{tissue} to AUC_{plasma}, or (c) $C_{max,tissue}$ to $C_{max,plasma}$.

Problems

(Answers can be found in Appendix I at the end of the book.)

1. How does cassette dosing assist discovery PK studies?
2. What is the criticism of cassette dosing? How has this potential problem been addressed?
3. How does an automated sampling instrument help PK studies?
4. How does the CARRS approach differ from cassette dosing?
5. What is matrix suppression?
6. Why would a project team want tissue uptake data?
7. Calculate the PK parameters in the open cells below using the data provided:

cpd	Dose [IV, PO] (mg/kg)	AUC_{PO} (ng • h/mL)	C_0 (ng/mL)	AUC_{IV} (ng • h/mL)	Cl (mL/min/kg)	V_d (L/kg)	%F
1	1, 10	2,000	1,000	4,000			
2	1, 10	2,000	2,000	1,000			
3	5, 10	200	1,000	8,000			
4	1, 10	500	1,000	200			
5	5, 30	305	2,000	1,900			

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