Part 2

Physicochemical characterisation of drug candidates

The aim of Part 2 is to provide an overview of physicochemical characterisation of (pro)drug candidates/substances. Physicochemical characterisation of compounds is part of molecular biopharmaceutics and is typically encompassed in preformulation and preclinical development in the pharmaceutical industry. Thus physicochemical properties of (pro) drug candidates and substances are important issues in optimal drug delivery, i.e. the design of optimal formulations containing optimal bioavailable drug substances.

In Chapter 2.1, acid/base properties, solubility, lipophilicity and solid-state properties are described, focusing on theory and methods applied to characterise (pro)drug candidates and substances.

In Chapter 2.2 mechanisms of decomposition of (pro)drug candidates and substances are described. The chapter focuses on describing the most relevant mechanisms affecting drug stability, such as hydrolysis, oxidation and photolytic degradation, and how these mechanisms may be influenced by external factors such as water, oxygen and light.

In Chapter 2.3, the kinetics of decomposition of (pro)drug candidates and substances in aqueous solution, including biofluids, are described. The chapter focuses on describing reaction kinetics of zero, first and second order and how these may be applied in characterising (pro)drug candidates and substances. Furthermore, the chapter examines how kinetic parameters such as rate constant, half lives, and shelf lives are applied to document stability (European Medicines Agency (EMEA), 2006). Finally, the chapter describes how these kinetic parameters may be influenced by various factors such as pH, temperature, ions, buffers and enzymes.

In Chapter 2.4, examples of chemical approaches to improving the bioavailability properties of oral drug candidates are described. The chapter describes Lipinski's rule of five, and how it may be applied in selecting chemical strategies to optimise the bioavailability of (pro)drug candidates and substances (Lipinski *et al*., 2001). Approaches such as salt formation, chemical stabilisation of peptides, and prodrug strategies are described. The chapter includes examples of salts used to optimise bioavailability and pharmaceutical formulation. An overview of intestinal peptidases relevant to stabilising peptide and protein drug candidates is presented, as well as examples of prodrug candidates registered in the EU.

In Chapter 2.5, industrial preformulation is described step by step. The chapter gives an industrial perspective on how drug candidates are evaluated in terms of physicochemical characterisation. The chapter focuses on examining to what extent characterisation takes place in the various stages of drug development.

References

EMEA (2006). *Note for Guidance on Stability Testing: stability testing of new drug substance and products* (CPMP/ICH/2736/99). http://www.emea.europa.eu/pdfs/human/ich/273699en.pdf (accessed 29 April 2009).

Lipinski CA, Lombardo F, Dominy BW, Feeney PJ (2001). Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv Drug Deliv Rev* 46: 3–26.

2.1

Acid/base properties, solubility and distribution of drug candidates

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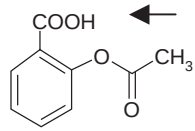
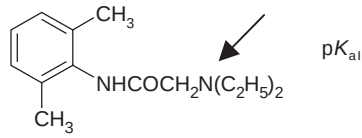
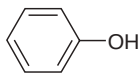
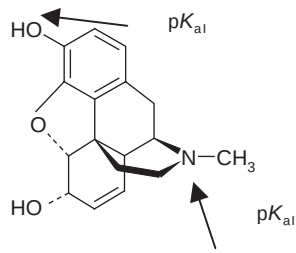
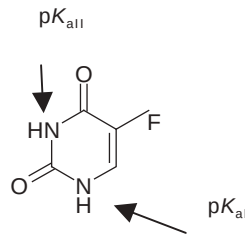
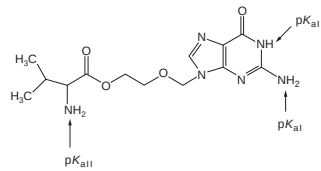
Physicochemical properties of drug candidates, such as acid/base properties, which are characterised by the ionisation/acidity constant (K_a), aqueous solubility (S) and partition (P) and distribution (D) between two immiscible phases (often *n*-octanol and water), as well as solid-state characteristics, are fundamental properties used in the sciences of biopharmaceutical and pharmaceutical chemistry. Furthermore, physicochemical characterisation of drug candidates is essential for industrial preformulation and for the preclinical development selection process of drug candidates. For solute acidic and basic drug candidates, S and D are generally dependent on the concentration of protons $[H^+]$. K_a, S, P, D and $[H^+]$ are often very large or very small numbers. Thus, for convenience, they may be expressed in logarithmic terms by $-\log K_a = pK_a$, $\log S$, $\log P$, $\log D$, and $-\log[H^+] = pH$ respectively. The aim of this chapter is to introduce the theory and some methods that are used to characterise drug candidates with regard to their molecular acid/base properties, molecular solubility, molecular partition and distribution between two immiscible phases, as well as their solid-state characterisation.

2.1.1 Acid/base properties of drug candidates

Lewis describes an acid as an electron pair acceptor and a base as an electron pair donor (Lewis, 1923). Conjugated Lewis acid and base pairs may therefore not necessarily involve proton transfer. However, Brøndsted–Lowry defines an acid as any substance that can ionise in aqueous solution to create a solvated hydrogen ion, i.e. an oxonium ion (H_3O^+). H_3O^+ is often abbreviated H^+. Thus, a Brøndsted–Lowry acid is a proton donor and a Brøndsted–Lowry base a proton acceptor. The ionisation process always involves the two, which are known as conjugated acid–base pairs (Brøndsted, 1923). The pK_a functional groups of selected (pro)drug substances are shown in Table 2.1.1.

Table 2.1.1 pK_a functional groups of selected (pro)drug substances

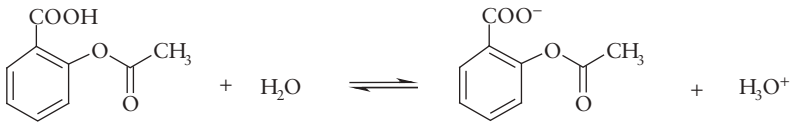
pK_a functional groups	*Structures of selected drug substance*	*pK_a*
RCOOH		
Carboxylic acid	ASA	pK_{aI} = 3.5
R$_2$-NH		
Secondary amine	Lidocaine	pK_{aI} = 7.84
 Phenol		pK$_{aI}$ = 8.17
R$_3$N Tertiary amine	Morphine	pK_{aII} = 9.26
 NH-acidic	 5-Fluorouracil	pK_{aI} = 8.0 pK_{aII} ≈ 13
R-NH$_2$ Primary amine (aciclovir)		pK_{aI} = 2.0
R-NH$_2$ Primary amine (valine)	Valaciclovir	pK_{aII} = 7.2
NH-acidic (amide) 		pK_{aIII} = 9.1

Notes: Arrows in the NH-acidic structure indicate the direction of electron-withdrawing effect, whereas arrows in the drug substance structures point at pK_a functional groups.

In this chapter, the focus is on drug candidates that are Brøndsted–Lowry acids or bases, in which pH-dependent proton transfer takes place. This proton transfer can be determined by the ionisation/acidity constant, K_a, for the ionisation process.

2.1.1.1 Drug candidates with acid properties

For a monoprotic acid, i.e. an acid containing only one replaceable hydrogen atom per molecule such as the drug substance acetyl salicylic acid (ASA), the ionisation process takes place as illustrated in Scheme 2.1.1. Here the proton-donating acidic neutral species of ASA is represented together with water on the left side of the arrows, and the proton-accepting basic ionised species of ASA is represented together with the oxonium ion on the right side of the arrow.

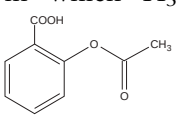


Neutral species of ASA + water Charged species of ASA + oxonium ion

(Scheme 2.1.1)

The equation for calculating the ionisation/acidity constant K_a of the monoprotic ASA in dilute solution is given in Equation 2.1.1:

$$K_a = \frac{[RCOO^-][H^+]}{[RCOOH]} \tag{2.1.1}$$

in which H_3O^+ is shortened to H^+ for monoprotic acids and  to RCOOH, and in which $[RCOO^-]$, [RCOOH] and $[H^+]$ represent concentrations of ionised species, neutral species and protons involved in the process, respectively. If drug substances are not in dilute solution, concentrations should be substituted by activities, which are difficult to measure. Thus, for practical reasons K_a is generally determined in dilute aqueous solution. This is the reason why concentrations rather than activities will be used in the present chapter, assuming that experiments take place in dilute aqueous solution. Therefore, [RCOOH] can be described by Equation 2.1.2:

$$[RCOOH] = \frac{[RCOO^-][H^+]}{K_a} \tag{2.1.2}$$

The total concentration of drug candidate $[R]_T$ can be described as the sum of species, as is apparent from Equation 2.1.3:

$$[R]_T = [RCOOH] + [RCOO^-] \tag{2.1.3}$$

According to Equation 2.1.3 it is possible to calculate the neutral species fraction f_{RCOOH} by Equation 2.1.4:

$$f_{RCOOH} = \frac{[RCOOH]}{[RCOOH] + [RCOO^-]} \tag{2.1.4}$$

So, by combining Equations 2.1.2 and 2.1.3, Equation 2.1.5 arises. Thus, the neutral species fraction f_{RCOOH} of any monoprotic drug candidate that is an acid may be calculated from $[H^+]$ and K_a, i.e. pH and pK_a respectively, using Equation 2.1.5:

$$f_{RCOOH} = \frac{[H^+]}{[H^+] + K_a} \tag{2.1.5}$$

According to Equation 2.1.1, the $-\log K_a$ (pK_a) for monoprotic acids is defined as the pH value at which 50% of the compound is protonated [RCOOH] and 50% is deprotonated $[RCOO^-]$. Thus for monoprotic acids, K_a may be determined once the species distribution at a given pH is known. This is described for ASA and monoprotic acids in general by Equations 2.1.6 and 2.1.7 respectively.

$$pK_a = pH + \log \frac{\left[\text{COOH, O, CH}_3, \text{O}\right]}{\left[\text{COO}^-, \text{O, CH}_3, \text{O}\right]} \tag{2.1.6}$$

$$pK_a = pH + \log \frac{[RCOOH]}{[RCOO^-]} \tag{2.1.7}$$

Equation 2.1.7 is easily transformed into the well-known Henderson–Hasselbalch equation (Hasselbalch, 1916) as shown in Equation 2.1.8:

$$pH = pK_a + \log \frac{[RCOO^-]}{[RCOOH]} \tag{2.1.8}$$

Once the pK_a values are known, the Henderson–Hasselbalch equation may be used to calculate the ratio between ionised and neutral species at a given pH of any drug candidate or excipient that is a monoprotic acid.

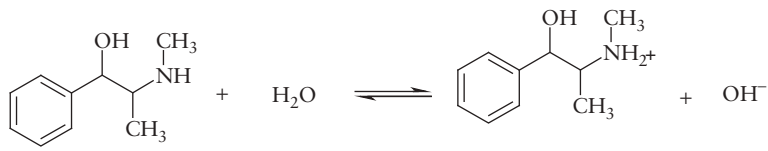
Similarly, the ionised species fraction f_{RCOO^-} for drug candidates that are monoprotic acids may be calculated by Equation 2.1.9.

$$f_{RCOO^-} = \frac{K_a}{[H^+] + K_a} \tag{2.1.9}$$

Thus, an acidic drug candidate, i.e. a monoprotic acid such as ASA, with a pK_a value of approximately 3.5 will primarily be represented by its neutral species in gastric juice at pH 1–3, whereas it will be primarily represented by its ionised species in intestinal fluids at pH 5.5–8 and in blood at pH 7.4. The pH of relevant physiologic media and the absorption area at which the biological media are represented are shown in Table 2.1.2.

2.1.1.2 Drug candidates with base properties

For a monoprotic base such as ephedrine (see the illustration in Scheme 2.1.2), i.e. a base accepting one H^+ per molecule, the ionisation/base constant K_b for the ionisation process in aqueous solution can be described by Equation 2.1.10:



Neutral basic species of ephedrine — Ionised acidic species of ephedrine

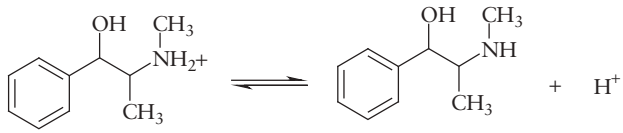
(Scheme 2.1.2)

$$K_b = \frac{[RH^+][OH^-]}{[R]} \tag{2.1.10}$$

However, in preformulation and preclinical development, K_a rather than K_b values are determined. Thereby, K_a values are generally used to describe ionisation properties of both acidic and basic drug candidates. As $pK_a + pK_b = pK_w$, where pK_w is the ionisation product for water, i.e. 14.00 at 25 °C, it easy to transform pK_b into pK_a values and vice

versa. If the pK_b of ephedrine is determined to be 4.35 at 25 °C, it transforms into a pK_a for ephedrine of 9.65.

Thus, the ionisation process of ephedrine may alternatively take place as illustrated in Scheme 2.1.3, in which it is implied that ionisation takes place in dilute aqueous solution:

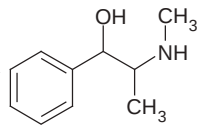


(Scheme 2.1.3)

Charged species of ephedrine Neutral species of ephedrine

The K_a for the monoprotic base ephedrine and for monoprotic bases in general may be calculated by Equation 2.1.11,

$$K_a = \frac{[R_3N][H^+]}{[R_3NH^+]} \tag{2.1.11}$$

in which  is shortened to R_3N. Fractions of neutral and ionised species of drug candidates that are monoprotic bases can be calculated by Equations 2.1.12 and 2.1.13 respectively.

$$f_{R_3N} = \frac{K_a}{[H^+] + K_a} \tag{2.1.12}$$

$$f_{R_3NH^+} = \frac{[H^+]}{[H^+] + K_a} \tag{2.1.13}$$

Thus, a monoprotic base such as ephedrine with a pK_a value of approximately 9.6 may be primarily represented by its ionised species in both gastric juice and intestinal fluid, as well as blood (see Table 2.1.2).

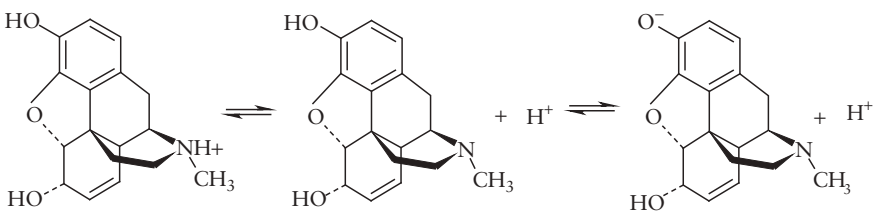
2.1.1.3 Amphiphilic drug candidates

For a drug substance that has two acid/base functionalities, and therefore two pK_a values, such as morphine (p$K_{aI} = 8.17$; p$K_{aII} = 9.26$), the

Table 2.1.2 pH in various biological media together with surface area at which the biological media is represented (Wilson, 1967; Newton and Kluza, 1978; Madara, 1991; Dressman *et al.*, 1993; Gray and Dressman, 1996; Kutchai, 1996; Charman *et al.*, 1997, Dressman *et al.*, 1998; Avdeef, 2001)

Biological fluid	*pH*	*Absorption area (m^2)*
Salivary fluid	6.4	–
Gastric juice	1–2.1 (fasted)	0.11
	5.0 (0–0.1 h after eating)	
Pancreatic juice	7.9–8.4	–
Duodenal fluid	2.4–6.8	60
Jejunal fluid	5.8–6.2 (fasted)	60
	4.5–5.5 (0–1 h after eating)	
Ileal fluid	6.5–8.0	60
Colonic fluid	5.0–8.0	0.25
Blood	7.40 (arterial)	–
	7.39 (venous)	
Cerebrospinal fluid	7.35	–
Urine	5.7 (male)	–
	5.8 (female)	
Lachrymal fluid (tears)	7.4	–
Aqueous humour fluid	7.21	

ionisation process takes place as illustrated in Scheme 2.1.4, in which it is implied that the ionisation takes place in dilute aqueous solution:



Morphine

Acidic positive charged species Ampholytic neutral species Basic negative ionised species

(Scheme 2.1.4)

Morphine is an ampholyte with a basic pK_{aI} of 8.17 and an acidic phenolic pK_{aII} of 9.26.

Once the pH, the pK_a values, [H^+] and the K_a values are known, the various species fractions of the acidic, ampholytic and basic species may be calculated by Equations 2.1.14, 2.1.15 and 2.1.16 respectively.

$$f_{\text{acid}} = \frac{[H^+]^2}{[H^+]^2 + K_{\text{al}}[H^+] + K_{\text{al}}K_{\text{all}}} \tag{2.1.14}$$

$$f_{\text{ampholyte}} = \frac{[H^+]K_{\text{al}}}{[H^+]^2 + K_{\text{al}}[H^+] + K_{\text{al}}K_{\text{all}}} \tag{2.1.15}$$

$$f_{\text{base}} = \frac{K_{\text{al}}K_{\text{all}}}{[H^+]^2 + K_{\text{al}}[H^+] + K_{\text{al}}K_{\text{all}}} \tag{2.1.16}$$

2.1.1.4 pK_a values determined by pH metric titration

For experimental determination of pK_a values of drug candidates, it is necessary to expose the molecule to an environment with a changing pH. This may be investigated by monitoring a specific species-dependent property of the drug candidates, such as species-dependent conductivity, solubility, potential, or absorbance (Albert and Serjeant, 1984). Potentionmetric or pH metric titration is the method most often used to determine pK_a values. Therefore, this method will be described next.

In pH metric titrations, known values of a precisely standardised strong acid (typically HCl) or base (typically KOH) are added to a solution of a proteogenic drug candidate, during which pH is continuously measured with a hydrogen-sensitive electrode. Titrations using acid or base titrants, as described above, are called acidimetric and alkalimetric titrations respectively. The drug candidate being assayed should be dissolved in water or in water:methanol mixtures, i.e. by the Yasuda–Shedlowski technique, which is described in more detail later.

It is possible to measure precise and accurate pK_a values in aqueous dilute solution by pH metric titration under controlled conditions, i.e. in a well-stirred system where the temperature and apparent ionic strength are kept constant and where correction for the content of CO_2, which is dissolved in the base titrant, is performed. The pK_a values are dependent on ionic strength within the solution. Because ionic strength may change during titration, due to variations in species distribution of the drug candidate, the ionic strength within the solution should be kept at a relatively high concentration compared to the ionic strength arising

from the drug candidate, which varies during titration depending on its ionisation state. An ionic strength of approximately 0.16M KCl, which corresponds to an isotonic solution, is therefore often used in preformulation and preclinical evaluation of drug candidates.

Graphical presentation of the pH metric titration is performed well by a Bjerrum difference plot (Bjerrum, 1941). In these plots, titrant volumes are recalculated to the average number of bound protons per drug candidate molecule ($\bar{n}_H$), which is then plotted versus the pH. Such a plot can be obtained by subtracting a titration curve containing no drug candidate (blank titration) from a titration curve with a known drug candidate concentration.

A Bjerrum difference plot for the monoprotic base ephedrine is shown in Figure 2.1.1. The Bjerrum plot of ephedrine shows that each ephedrine molecule loses one proton in the pH range of 8.5 to 11. It is seen that for pH values below approximately 8.5, the average number of bound protons per molecule of ephedrine $\bar{n}_H$ is 1, whereas zero protons are bound to ephedrine for pH values above approximately 10.8.

Furthermore, it is apparent from Figure 2.1.1. that the pK_a of ephedrine is approximately 9.5.

A Bjerrum difference plot may be transformed into plots of pH versus % species distribution, when species distribution is correlated to proton release. Theoretically, % species distribution of a basic drug substance such as ephedrine is easily calculated from the species fraction, as indicated in Equations 2.1.12 and 2.1.13, by multiplying the fractions by 100.

Amine and phenol functionalities of buprenorphine are characterised by pK_a values that are determined to be 8.3 and 9.6 respectively. The ionisation process of buprenorphine is shown in Scheme 2.1.5.

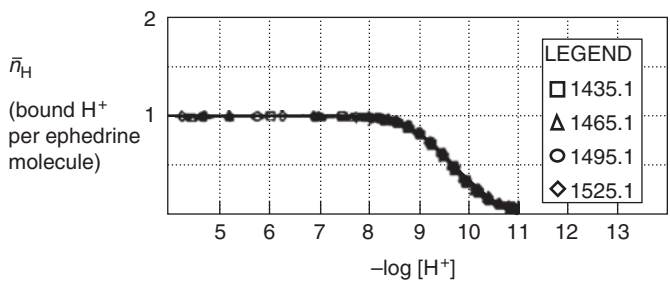
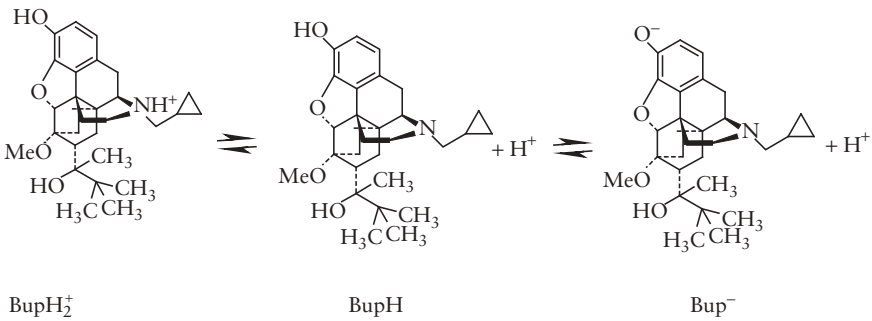


Figure 2.1.1 Bjerrum difference plot of ephedrine. Average number of bound protons per molecule of ephedrine ($\bar{n}_H$) versus pH.



(Scheme 2.1.5)

A species distribution curve of buprenorphine as a function of pH is shown in Figure 2.1.2. Theoretically, % species distribution of the ampholyte buprenorphine can easily be calculated from the species fraction indicated in Equations 2.1.14, 2.1.15 and 2.1.16, by multiplying the fractions by 100. Thus, at a pH lower than 10 the positively charged species of buprenorphine ($BupH_2^+$) is represented. At a pH interval between 6 and 11, the neutral ampholytic species of buprenorphine (BupH) is represented, whereas the negatively charged species of buprenorphine (Bup^-), is represented at pH values higher than 8.

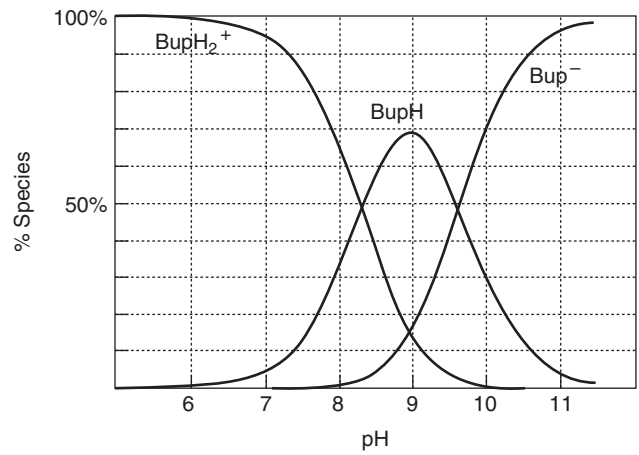


Figure 2.1.2 Species distribution of buprenorphine as a function of pH, protonated positive species ($BupH_2^+$), neutral species (BupH) and negative species (Bup^-) of buprenorphine. Reproduced from *Sirius Technical Application Notes Volume 1* (1994). Riverside: Sirius Analytical Instruments Ltd.

From Figure 2.1.2 it is apparent that at an intestinal fluid pH of 6–9, all three species of buprenorphine will be present. This is convenient for its solubility in intestinal fluid and permeability across the intestinal membrane. This is explained by the pH-partition hypothesis in which it is described that a neutral species has better diffusional permeability across the intestinal membrane than an ionised species, but an ionised species has better solubility in aqueous intestinal fluid than a neutral species. Diffusional permeability is further described in Chapter 3.1 and the pH-partition hypothesis in Section 2.1.3 and by Shore *et al.* (1957) and Neuhoff *et al.* (2005).

For drug candidates with low aqueous solubility, which may play a part during titration, the Yasuda–Shedlowski technique can be used to estimate the pK_a. With this technique, the linear relationship between, for example, methanol content in the titration media (0–60% methanol content) and apparent pK_a values, i.e. p_oK_a values, are determined to estimate true aqueous pK_a values (Shedlovski, 1962; Takács-Novák *et al.*, 1997). The p_oK_a values for ASA and ephedrine are shown in Table 2.1.3. As is apparent from this table, the p_oK_a for ASA increases and the p_oK_a for ephedrine decreases with increasing methanol content in the titration media. The increase in p_oK_a for ASA with increased methanol concentration may be explained by methanol lowering the dielectric constant of the titration media. This disfavours the increased ionisation of acids during alkalimetric titration, resulting in an increased p_oK_a for ASA. The ionisation of ASA is illustrated in Scheme 2.1.1. In contrast, the decreased dielelctric constant of the titration medium with increased methanol content favours increased neutralisation of the base ephedrine

Table 2.1.3 Apparent pK_a (p_oK_a) for ASA and for ephedrine at various methanol concentrations

% Methanol	*p_oK_a ASA*	*p_oK_a ephedrine*
0	4.55[a]	9.46[a]
10	4.68	9.39
20	4.84	9.32
30	5.02	9.11
40	5.21	9.01
50	5.43	8.92
60	5.67	8.81

Note: [a]Estimated aqueous pK_a from linear regression of p_oK_a as function of methanol content.

and thus a decreased p_oK_a during alkalimetric titration. The neutralisation of ephedrine is illustrated in Scheme 2.1.2.

2.1.2 Solubility in aqueous solution

The solubility of drug candidates in aqueous solution is mainly used to investigate its importance for formulating aqueous solutions, e.g. parenteral formulations, and for evaluating the influence of solubility on bioavailability. Consequently, solubility may be investigated in aqueous buffer solutions as well as in various relevant biological media. It is relevant to study drug candidate solubility in biological media as it is observed that generally only dissolved fractions of drug candidates are available for absorption into the blood.

The influence of acid/base and solid-state properties of drug candidates on solubility in aqueous solution is described in this chapter, whereas dissolution methods to describe the solubility rate of drug candidates are described in Chapter 4.1.

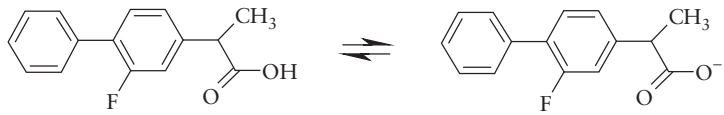
The *European Pharmacopoeia* (Ph Eur, 2009) describes the solubility of drug substances as shown in Table 2.1.4.

Even though ionised species generally dissolve better than their corresponding neutral species, the solubility terms described in Ph Eur do not take species-dependent solubility into consideration. Thus, the solubility is simply determined in aqueous solution without considering what the pH of the aqueous solution may change to during the experiment, i.e. after addition of a drug candidate. The Ph Eur solubility terms are good first estimations of the aqueous solubility of drug candidates and may be used in the initial selection process. This process is used to determine whether the solubility of the drug candidate may be limited for

Table 2.1.4 Descriptive terms of drug substrate solubility according to Ph Eur

Ph Eur descriptive term	*Solubility (mg ml^{-1})*
Very soluble	>1000
Freely soluble	100–1000
Soluble	33–100
Sparingly soluble	10–33
Slightly soluble	1–10
Very slightly soluble	0.1–1
Practically insoluble	<0.1

choice of dosage form and permeability across biological membranes. However, the aqueous solubility of compounds that have acid/base properties may be pH dependent because ionised species are generally more soluble in aqueous solution than neutral species. For monoprotic weak acids such as flurbiprofen (RCOOH), see Scheme 2.1.6, the total apparent solubility of an acid, S_T, at any pH can be described by Equation 2.1.17.



Neutral species of flurbiprofen Ionised species of flurbiprofen

(Scheme 2.1.6)

In Equation 2.1.17, S_{RCOOH} and S_{RCOO^-} refer to the solubility of the neutral and the ionised species respectively.

$$S_T = S_{RCOOH} + S_{RCOO^-} \tag{2.1.17}$$

However, if S_{RCOOH} and S_{RCOO^-} are considered as the respective concentrations in solution, i.e. [RCOOH] and [$RCOO^-$], Equation 2.1.17 corresponds to Equation 2.1.3. Based on the assumption that S_{RCOO^-} is much larger than S_{RCOOH}, and by applying Equations 2.1.3 and 2.1.4, it is apparent that S_T at any pH can be described by Equation 2.1.18.

$$S_T = \frac{[S_{RCOOH}]}{f_{RCOOH}} \tag{2.1.18}$$

In Equation 2.1.18, f_{RCOOH} is the fraction of neutral species, which may be calculated from Equation 2.1.5 once the pH of the aqueous solution and the pK_a of the monoprotic drug candidates are known. Equation 2.1.18 may be described in logarithmic terms by Equation 2.1.19.

$$\log S_T = \log S_{RCOOH} - \log f_{RCOOH} \tag{2.1.19}$$

Thus, for drug candidates that are monoprotic acids, one may determine the solubility of the neutral species, which is also called intrinsic solubility (S_{RCOOH}). The intrinsic solubility of a monoprotic acid is experimentally determined in aqueous solution at 2 pH units below its pK_a, and then S_T at any pH may be estimated by Equation 2.1.19.

2.1.2.1 Solubility–pH profiles

Solubility–pH profiles of drug candidates with acid/base properties may be fully determined experimentally by titration during which the drug substance precipitates (Avdeef, 1996, 2001).

Bjerrum plots of such titrations may be recalculated into solubility–pH profiles where $\log S_T$ values are shown as a function of pH. In Figure 2.1.3 the solubility–pH profile of the monoprotic acid flurbiprofen is illustrated.

From the solubility–pH profile of flurbiprofen, it is apparent that the intrinsic solubility of flurbiprofen may be determined below pH 2 and that the solubility increases with ionised species concentration as described by Equation 2.1.19.

For a monoprotic weak base such as lidocaine, the total apparent solubility S_T can be described by Equation 2.1.20 in which $S_{R_3NH^+}$, and S_{R_3N} refer to the solubility of the neutral and the ionised species respectively.

$$S_T = S_{R_3NH^+} + S_{R_3N} \tag{2.1.20}$$

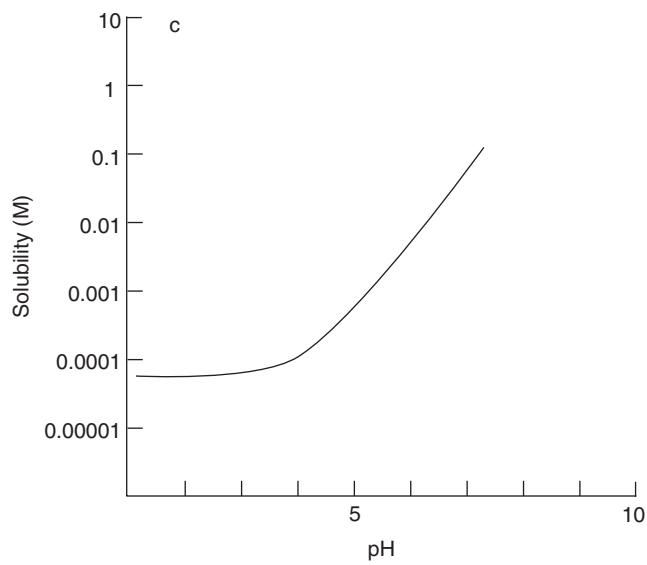
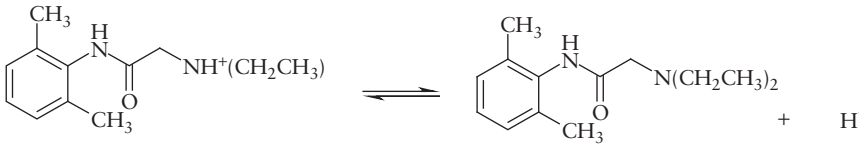


Figure 2.1.3 Solubility–pH profile of flurbiprofen; the pK_a was determined to 4.03 (±0.04) at 25 °C and ionic strength of KCl at 0.15 M. Reproduced from Avdeef A (1998). pH-metric solubility 1. Solubility–pH profiles from Bjerrum plots. Gibbs buffer and pK_a in the solid state. *Pharm Pharmacol Commun* 4: 165–178.

The ionisation scheme for lidocaine is illustrated in Scheme 2.1.7.



(Scheme 2.1.7)

However, based on the assumption that the solubility of the ionised species shows much larger aqueous solubility than its neutral species, the apparent solubility of monoprotic drug candidates that are bases may be determined at any pH by Equation 2.1.21.

$$\log S_T = \log S_{R_3N} - \log f_{R_3N} \tag{2.1.21}$$

In Equation 2.1.21, f_{R_3N} is the fraction of the neutral species. f_{R_3N} may be easily calculated by Equation 2.1.12 once the pH in the aqueous solution and the pK_a of the drug candidate are known. Thus, for drug candidates that are monoprotic bases one may determine the aqueous solubility of its neutral species, i.e. its intrinsic solubility (S_{R_3N}). In practice, this intrinsic solubility is determined in an aqueous solution at 2 pH units above the pK_a value of the drug candidate. In Table 2.1.5 S_T values of lidocaine as well as f_{R_3N} at various pH levels are shown. This is estimated by Equation 2.1.21 by using a lidocaine pK_a of 7.84 at 25 °C and a lidocaine intrinsic solubility of 0.004 g ml^{-1}.

In drug discovery it is common to determine aqueous solubility at a single pH value to calculate solubilities at other pH values by

Table 2.1.5 Lidocaine solubility (S_T)

pH	f_{R_3N}	S_T (g ml^{-1})
5	1.44×10^{-3}	2.778
6	1.42×10^{-2}	0.282
7	1.26×10^{-1}	0.032
8	5.91×10^{-1}	0.007
9	9.35×10^{-1}	0.004
10	9.93×10^{-1}	0.004
11	1.000	0.004

Notes: S_T at various pH values is estimated from Equation 2.1.21 by using S_{R_3N} at 0.004 g ml^{-1} and pK_a of 7.84.

Equation 2.1.21 as described above. However, Artursson and co-workers have shown that such calculated solubility values for at least amine drug substances can differ largely from experimental values. The accuracy of the calculated pH-dependent aqueous solubility of 25 cationic drug substances is summarised by Bergstrøm *et al.* (2004).

The total solubility of an amphoteric drug substance such as buprenorphine may be described by Equation 2.1.22:

$$S_T = S_{ampholyte} + S_{R_3NH^+} + S_{RCOO^-} \quad (2.1.22)$$

in which $S_{ampholyte}$, $S_{R_3NH^+}$ and S_{RCOO^-} are solubilities of neutral, positive and negative species, respectively. Assuming that positive and negative species show infinite solubilities in aqueous solution, the solubility of amphoteric drug candidates may be described by Equation 2.1.23.

$$\log S_T = \log S_{ampholyte} - \log f_{ampholyte} \quad (2.1.23)$$

$\text{Log}f_{ampholyte}$ may be calculated by Equation 2.1.15 once the pH of the aqueous solution and the pK_a values for the amphoteric drug candidate are known.

An experiment-based solubility–pH profile of the diprotic ampholytic drug substance buprenorphine is shown in Figure 2.1.4.

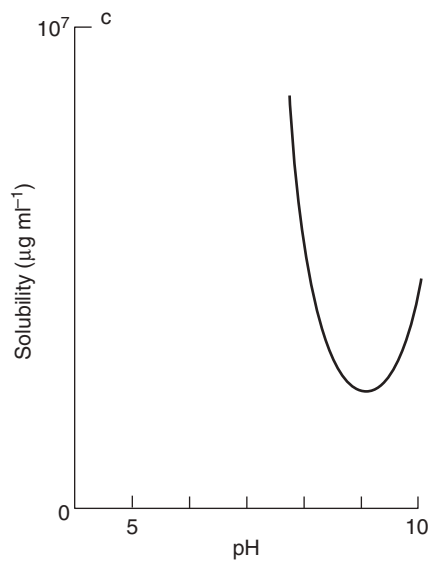


Figure 2.1.4 Solubility–pH profile of buprenorphine. Reproduced from Avdeef A (1998). pH-metric solubility 1. Solubility–pH profiles from Bjerrum plots. Gibbs buffer and p*Ka* in the solid state. *Pharm Pharmacol Commun* 4: 165–178.

From Figures 2.1.2 and 2.1.4 as well as Scheme 2.1.5 it is apparent that the aqueous solubility of buprenorphine is dependent on pH and that the solubility of neutral BupH species is much lower than the solubilities of ionised $BupH_2^+$ and Bup^- species. The profile may be theoretically described by Equation 2.1.23.

Apart from the pH-dependent solubility of drug candidates described above, it is equally important to assess the dissolution, i.e. the solubility rate. This is particularly done for evaluating their oral bioavailability. This is because oral drug candidates should be dissolved during intestinal transit, i.e. within 3–6 h, in order to be absorbed into the blood circulation. The solubility rate is assessed through dissolution studies, which are described in detail in Chapter 4.1.

The influence of drug candidate solubility on bioavailability is considered in the Biopharmaceutics Classification System (BCS), introduced by the US Food and Drug Administration (FDA), in which drug substances are classified as low- or high-solubility drug substances, i.e. one dose is respectively dissolved or not in 250 ml water in the pH range 1–8 (FDA, 2000). The BCS is described in detail in Chapter 4.3.

2.1.3 Partition properties of drug candidates

The partitioning of a solute drug candidate between two immiscible liquids, often *n*–octanol and aqueous buffer, is often referred to as its partition coefficient (P) (Leo *et al.*, 1971). The P of a drug candidate is mainly applied to investigate its formulation properties in two-phase formulation, e.g. emulsions, as well as for evaluating its passive diffusional permeability properties across lipophilic biological membranes which is of importance for its bioavailability, see Lipinski's rule of five in Chapter 2.4. For drug candidates with ionisation properties, P is normally defined as the partitioning of neutral species between *n*-octanol and aqueous buffer. Thus, P is a constant and refers to molecules or species partitioning between two immiscible phases. If volumes of water and organic phases of V_w and V_o respectively are different, this will have an influence on P.

The partition coefficient of monoprotic acids and bases is generally related to their neutral species as described by Equations 2.1.24 and 2.1.25.

$$P = \frac{[\mathrm{RCOOH}]_o}{[\mathrm{RCOOH}]_w}\frac{V_w}{V_o} \tag{2.1.24}$$

$$P = \frac{[\mathrm{R_3N}]_o}{[\mathrm{R_3N}]_w}\frac{V_w}{V_o} \tag{2.1.25}$$

The distribution coefficient, D, of drug candidates is related to P but refers to total species distribution between two immiscible phases, which varies with pH when compounds that can ionise are assessed. $D = P$ in the pH region where a substance is fully un-ionised. However, the distribution of compounds that can ionise is pH dependent because neutral species are more lipophilic than charged ones. Thus, when the drug substance is fully represented by its neutral species, it is maximally partitioning into the lipid/organic phase. Based on the assumption that the neutral species distribute to a much larger degree into the lipid/organic phase than the ionised species, the D of monoprotic acids and bases can be expressed by Equations 2.1.26 and 2.1.27 respectively.

$$D_{\text{acid}} = P \times f_{\text{RCOOH}} \tag{2.1.26}$$

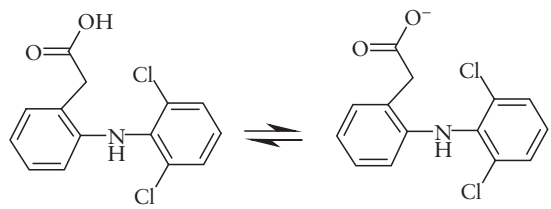
$$D_{\text{base}} = P \times f_{\text{R}_3\text{N}} \tag{2.1.27}$$

Thus, by determining P for the monoprotic drug candidates, D may be estimated by Equations 2.1.26 and 2.1.27 for acids and bases respectively, which by logarithmic terms transforms into Equations 2.1.28 and 2.1.29.

$$\log D_{\text{acid}} = \log P + \log f_{\text{RCOOH}} \tag{2.1.28}$$

$$\log D_{\text{base}} = \log P + \log f_{\text{R}_3\text{N}} \tag{2.1.29}$$

In Scheme 2.1.8 the ionisation process for the acidic drug compound diclofenac is illustrated.



(Scheme 2.1.8)

Neutral species of diclofenac Ionised species of diclofenac

The log$D$–pH profile of diclofenac is shown in Figure 2.1.5, which may be described by Equation 2.1.28 in the pH region 1–9. Above pH 9, the profile is described by log$D$ for its ionic species, which is approximately 0.7. The carboxylic acid functionality of diclofenac has a pK_a of 4.0 (Sirius Technical Application Note 2, 1995).

As illustrated in Figure 2.1.5, the log$D$ of diclofenac decreases with pH in the pH range 4.0–9.0. Thus, diclofenac may penetrate better into a

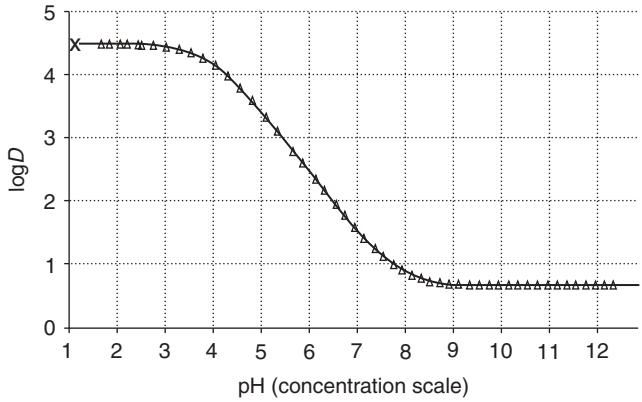
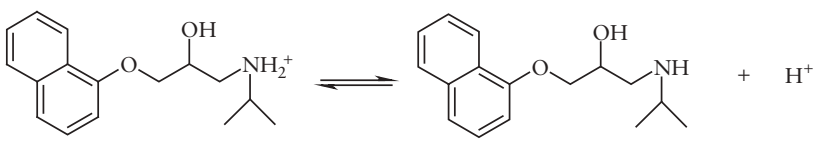


Figure 2.1.5 log*D*–pH profile of the acidic drug substance diclofenac between *n*-octanol and water as function of pH. It is seen that the un-ionised species of diclofenac distributes better to *n*-octanol that the ionised species. Reproduced from *Sirius Technical Application Notes Volume 2* (1995). Riverside: Sirius Analytical Instruments Ltd.

lipophilic environment, such as phospholipids in cell membranes, when delivered from acidic media such as the gastric juice at pH 1–3, than when delivered from intestinal fluid at pH 5.5–8. In general, it is hypothesised that when a pH gradient is present across a lipophilic cell membrane, the neutral species of an acidic drug substance, delivered from acidic gastric or intestinal fluids to the lipophilic cell membrane barrier, arises as its ionised form at an intracellular or blood pH of 7.4. In this way the concentration gradient of the neutral species, across the lipophilic membrane, is maintained, which drives passive diffusion of the neutral species. This hypothesis is called the pH-partition hypothesis.

The ionisation process of the basic drug substance propranolol is shown in Scheme 2.1.9.



(Scheme 2.1.9)

The pK_a of propranolol is determined to be 9.72 and its log*P*, i.e. the partitioning of its fully neutral and fully ionised species between *n*-octanol and water is determined to be 3.41 and 0.48 respectively (Clarke and Cahoon, 1987). An illustration of f_{R_3N} and log*D* at various pH levels is shown in Table 2.1.6.

pH–log*D* profiles may be determined through experiments by first titrating drug candidates in water, followed by titration in non-mixed

Table 2.1.6 Fraction of neutral propranolol f_{R_3N} as well as log*D* of propranolol at various pH values

pH	f_{R_3N}	*logD*
4	0	*0.48
5	0	*0.48
6	0	*0.48
7	0.002	0.69
8	0.019	1.68
9	0.160	2.61
10	0.656	3.22
11	0.950	3.38

Notes: f_{R_3N} is calculated from Equation 2.1.12 and log*D* from Equation 2.1.29, except for *log*D* which is 0.48, i.e log*D* of the fully ionised species.

solvents of *n*-octanol and water. This may be performed by equipment such as the GlpKa meter developed by Sirius Instruments as illustrated next for the drug substance morphine.

The ionisation process of morphine is shown in Scheme 2.1.4, and a Bjerrum difference plot as a result of morphine sulphate titration in aqueous solution is shown by the solid curve in Figure 2.1.6.

The solid curve shows that all protons are bound to morphine at a pH lower than 6 and that morphine loses two protons per molecule in the pH interval 6–11. Thus, morphine has two overlapping pK_a values in this pH range. However, the Bjerrum difference plot produced from

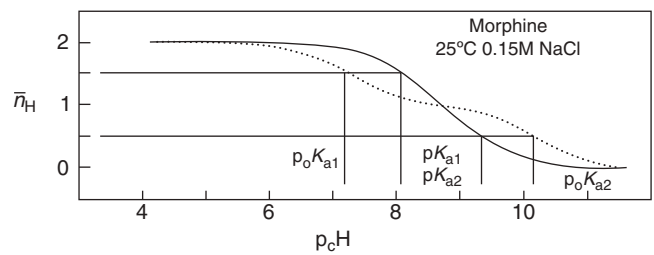


Figure 2.1.6 Bjerrum plot; that is average amount of bound protons n_H as function of $-\log[H^+]$ (p_cH) as a result of alkalimetric titration of morphine sulphate in aqueous solutions (solid line) and in non-miscible mixed solvents of water and *n*-octanol (dotted line) at 25 °C and at ionic strength at 0.15 M KCl. Apparent p_0K_{a1} and p_0K_{a2} determined in non-miscible mixed solvents and pK_{a1} and pK_{a2} determined in aqueous solution. Reproduced from Avdeef A (1996). Assessment of distribution–pH profiles, In: Pliska V, Testa B, van der Waterbeemd, eds. *Lipophilicity in Drug Action and Toxicology*. Weinheim: VCH Publishers Weinheim, 111–139.

titration of morphine sulphate in aqueous solution does not show whether the protons arise from basic or acidic functionalities in morphine. This may be solved by also titrating morphine sulphate in non-mixed solvents such as *n*-octanol and water mixtures, i.e. a pH–log*D* profile determination. As illustrated in Figure 2.1.6, the amine functionality in morphine has a lower pK_a than the phenolic functionality because the first pK_a determined in non-miscible mixed solvent (dotted curve) is lower than when determined in water (solid curve). This indicates that neutral deprotonated amine species of morphine are distributed to the octanol phase and therefore not recognised by the electrode, which results in a lower apparent p$_o K_a$ than when measured in aqueous solution. In contrast, the second pK_a of morphine may arise from the phenol functionality. For phenol the protonated neutral species is distributed to octanol to a much larger degree than its negative ionised species. Thus it follows that the p$_o K_a$ values determined from non-mixed solvents (dotted curve) are larger than when determined in aqueous solution (solid curve).

The Bjerrum plot may be translated into a pH–log*D* profile by Equation 2.1.30.

$$\log D_{\text{ampholyte}} = \log P + f_{\text{ampholyte}} \tag{2.1.30}$$

This is shown for morphine in Figure 2.1.7, where it is seen that log*D* of morphine is highest in the pH interval 7–11, and consequently morphine is optimally delivered to the intestinal lipophilic membrane barrier from an intestinal fluid pH of 7–10.

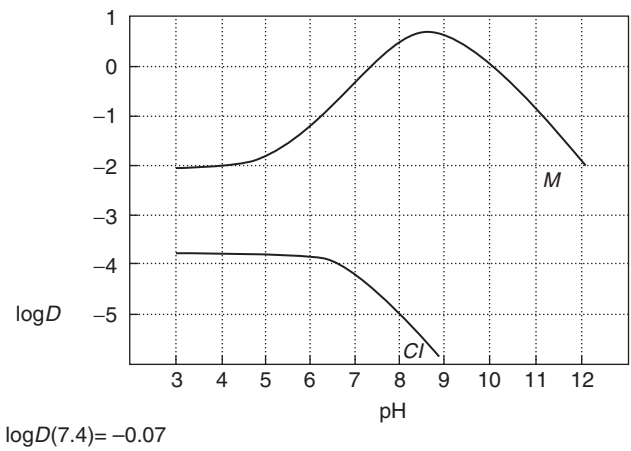


Figure 2.1.7 pH–log*D* profile of morphine hydrochloride performed in *n*-octanol and water. Morphine (M) and chloride (Cl). Reproduced from *Sirius Technical Application Notes Volume 2* (1995). Riverside: Sirius Analytical Instruments Ltd.

2.1.4 Solvation and solid-state limited solubility

Log*P* has also been used as a solvation parameter. Of particular interest is the general solubility equation (GSE) (Equation 2.1.31) developed by Yalkowski and co-workers (Jain and Yalkowski, 2001)

$$\log S = 0.05 - 0.01(T_m - 25) - \log P \qquad (2.1.31)$$

in which S is intrinsic solubility, i.e. the solubility of the neutral species of the drug substance in molar units, and T_m is its melting point used as a solid-state parameter. From Equation 2.1.31 it can be seen that intrinsic solubility is related to both the solvation parameter log*P* and the solid-state parameter T_m.

Bergstrøm and co-workers have applied Equation 2.1.31 to classification of poor-solubility drug substances into solvation-limited and solid-state-limited poor-solubility drug substances (Wassvik *et al.*, 2006; Bergstrøm *et al.*, 2007, Wassvik *et al.*, 2008). For 15 drug substances investigated, nicknamed as 'grease balls', with solubility ranging from 2.9 nM to 1.1 μM and log*P* between 3.5 and 6.8, they found a good correlation between log*P* and log*S*, whereas no correlation was observed between T_m and S (Bergstrøm *et al.*, 2007). Consequently, they suggested the solubility of these 'grease balls' is solvation limited. In another study they investigated 20 poor-solubility drug substances nicknamed 'brick dusts' with S ranging from −1.75 to −4.83 and log*P* close to 2. Good correlation was observed between log*S* and T_m and/or melting enthalpy ΔH, but no correlation between log*P* and log*S*. Consequently the solubility of these 'brick dust' molecules, characterised by features such as rigidity and high aromaticity, were suggested to be solid-state limited (Wassvik *et al.*, 2008).

These observations are highly relevant for selecting and formulating poor-solubility drug candidates. Whereas solvation-limited poor-solubility 'grease ball' candidates represent highly lipophilic species that are unable to form bonds with water molecules, the solid-state-limited poor-solubility 'brick dust' candidates represent stable crystals in which the strong intermolecular bonds within the crystal restrict the solubility of the candidate with water. For 'grease ball' candidates, the solubility may be improved largely by incorporating excipients such as disintegrants, solubility enhancers or lipids in the formulation, whereas this is generally not the case for 'brick dust' candidates. For 'brick dust' candidates, the strong intermolecular bonds should be weakened, for example by changing the shape of the crystal unit cells (polymorphic forms) or crystal habits as described in Section 2.1.5 or by salt or prodrug formation (see Chapter 2.4).

2.1.5 Solid-state characterisation

During selection of drug candidates, crystal forms are characterised by means of investigating their influence on dissolution. This is, as described in Section 2.1.4, especially important for solid-state-limited low-solubility drug candidates where dissolution may be limiting for bioavailability.

Drug candidate crystals may be held together by non-covalent interactions, which influences the melting point of the crystals. A specific crystal consists of repeating unit cells, i.e. it contains the same size and the same number of molecules or ions arranged in the same way, thus creating crystal lattices. Unit cells may be arranged in cubic, hexagonal, trigonal, tetragonal, orthorhombic, monoclinic or triclinic crystal lattices. Cubic, tetragonal, orthorhombic and hexagonal structures are illustrated in Figure 2.1.8.

The form of the unit cells may be characterised in three dimensions. The width, depth and height of the unit cell are represented by b, a and c, respectively (see Figure 2.1.8), and the angles between b and c, a and c, and b and a may then be characterised as α, β and γ respectively.

Cubic crystals are formed as cubes when $a = b = c$ and $\alpha = \beta = \gamma = 90^\circ$ as illustrated in Figure 2.1.8. The tetragonal crystal is a rectangular prism in which $a = b \neq c$ but $\alpha = \beta = \gamma = 90^\circ$ as illustrated in Figure 2.1.8. In orthorhombic unit cells $a \neq b \neq c$ and $\alpha = \beta = \gamma = 90^\circ$, i.e. there are three right-angled axes with different lengths as illustrated in Figure 2.1.8. In the regular hexagonal crystal all sides and angles are equal, i.e. all angles are 120° as seen in Figure 2.1.8.

In the triclinic crystal, $a \neq b \neq c$ and α, β, $\gamma \neq 90^\circ$. In the monoclinic crystal $a \neq b \neq c$ and $\alpha = 90^\circ$, whereas β and $\gamma \neq 90^\circ$. In the trigonal (rhombohedral) crystal, $a = b = c$ and $\alpha = \beta = \gamma \neq 90^\circ$.

Some unit cells may be further described as simple, base-, face- or body-centred unit cells. In simple unit cells, molecules/ions are only represented in the corners. In base-centred unit cells, as well as being

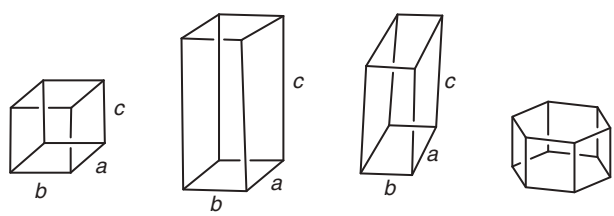


Figure 2.1.8 Lattice structures. From left to right: cubic, tetragonal, orthorhombic and hexagonal.

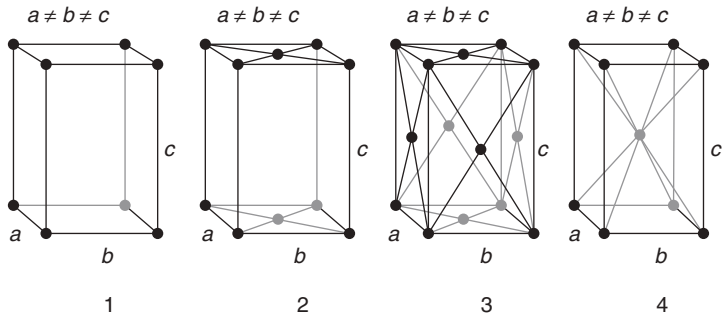


Figure 2.1.9 Simple orthorhombic crystal structures (1), base centred (2); face centred (3); and body centred (4). The width, depth and height are represented by *b*, *a* and *c* respectively, and the angles between *b* and *c*, *a* and *c*, and *b* and *a* are represented respectively by α, β, and γ. From Wikipedia.org http://en.wikipedia.org/wiki/Orthorhombic_crystal_system).

represented in the corners, molecules/ions are also represented in the two base planes of the unit cells. In face-centred unit cells, as well as being represented in the corners, molecules/ions are also centred at each (sur)face. In body-centred unit cells, as well as being represented in each corner, molecules/ions are also centred in the middle (body) of the cells. Simple, base-, face- and body-centred orthorhombic unit cells are depicted in Figure 2.1.9 (2–4).

The shape of the unit cells is generally described by Miller indices, which will not be further described; however, specific drug candidates may crystallise into different polymorphic forms where the molecules/ions arrange themselves in more than one way, either by packing differently in the crystal lattice or by orienting themselves differently in the lattice. These unit cells variations can be characterised by analysing X-ray diffraction patterns of the drug candidate. Properties such as dissolution and melting point are also often different between polymorphic forms, and therefore unit cell variation may also be characterised by analysing their phase transition temperature by differential scanning calorimetry (DSC), to obtain melting point entropies, ΔS, and enthalpies, ΔH. Polymorphic forms may also show different crystal habits, that is, similar unit cells, but different apparent crystal shapes. Apparent crystal shapes may be acicular, prismatic, pyramidal, tabular or lamellar. The different crystal habits may have different dissolution rates. Thus for solid-state-limited low-solubility drug candidates, crystal habits may have an influence on dissolution and thus bioavailability, as well having a possible influence on formulation properties.

Thus, it is important to characterise the various polymorphic forms of a specific drug candidate, including possible crystal habits, with regard to rational pharmaceutical development.

2.1.6 Conclusions

Characterisation of acid/base properties, solubility, lipophilicity and solid-state properties of drug candidates is essential for biopharmaceutical and formulation sciences as well as for industrial formulation and preclinical selection processes. For drug candidates with acid/base properties it is convenient to determine the pK_a to further determine species-dependent solubility and lipophilicity. This is important because species-dependent solubility and lipophilicity may have a major influence on both the formulation and biopharmaceutical properties of drug candidates. Furthermore, they may influence the chemical stability of the drug candidate. Solid-state properties of drug candidates are especially relevant for characterisation of poor-solubility drug candidates because solid-state properties may significantly influence formulation strategies as well the physical and chemical stability of these candidates. The chemical stability of drug candidates is discussed in Chapter 2.1.2.

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2.2

Mechanisms of decomposition of drug candidates

Bente Steffansen, Carsten Uhd Nielsen and Birger Brodin

Characterisation of the stability of drug candidates in aqueous solution is fundamental in the sciences of biopharmaceutical and pharmaceutical chemistry, as well as in industrial preformulation and preclinical development. In industry, stability investigations of drug candidates in aqueous solution generally take place during lead optimisation and late discovery (see Chapter 2.5), whereas stability investigations of drug products generally takes place during pharmaceutical development.

Despite the large structural variety of drug and prodrug candidates, most degradation reactions can be classified as hydrolysis, oxidation, isomerisation, racemisation or polymerisation reactions. These may be initiated by environmental factors such as light, humidity and oxygen. Also, whether the drug candidate is in solid state or aqueous solution may influence its stability. In aqueous solution, factors such as pH, temperature, ionic strength, or the presence of catalysts, e.g. metal ion impurities or buffers, may influence the stability of drug and prodrug candidates. A systematic stability test therefore includes studies of both the mechanism and rate (kinetics) by which drug and prodrug candidates are degraded. The aim of the present chapter is to describe reaction mechanisms of importance for stability of drug candidates and of relevance for drug formulation and bioavailability. The kinetics of stability are described in Chapter 2.3.

2.2.1 Hydrolysis

The most important class of hydrolysis reactions influencing the stability of prodrug and drug candidates is the hydrolysis of carboxyl functional groups. Carboxyl drug and prodrug candidates are carboxylic acid derivatives that are different from carbonyl candidates, which also include aldehydes and ketones and are generally stable towards hydrolysis. The development of prodrugs is often based on the rational introduction of a vulnerable carboxyl functional group to parent drug candidates such

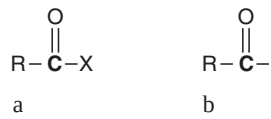


Figure 2.2.1 General molecular structures of a carboxyl compound (a), and an acyl functional group (b). R is an organic unit and X is OR, NR, SR, or Cl, the free bond (–) may be bound to X or R and the bold carbon (**C**) is named as the acyl carbon.

as alcohols, carboxylic acids or amines. Many prodrugs are therefore designed to contain carboxyl functional groups. Thus, it is of great importance to understand the degradation mechanisms of carboxyl candidates. Hydrolysis or water-catalysed reactions of carboxyl candidates take place by acyl transfer reactions. The general molecular structures of carboxyl compounds and acyl groups are shown in Figure 2.2.1a and b , respectively.

Many prodrug and drug candidates are carboxyl compounds such as esters, amides, imides, succimides, lactams, thiol esters, lactones, acid anhydrides or acid chlorides. Table 2.2.1 shows some selected drug substances that are carboxyl compounds.

Hydrolysis reactions are initiated by an electron pair or nucleophilic attack. Electron pairs may be donated by H_2O. This electron pair or nucleophilic attack on the acyl carbon, with a partial positive charge, is illustrated in Figure 2.2.2.

The leaving group HX-, that is HOR′, HNR′, HSR′ arises from hydrolysis of esters, amides, and thiol esters respectively. This acyl transfer reaction, i.e. hydrolysis, is probably the most common reaction of carboxyl compounds and is one of the most frequent degradation routes for drug substances.

Figure 2.2.3 shows such an example, the degradation pathways for acetyl salicylic acid (ASA). The reaction results in the formation of salicylic acid and acetic acid. The kinetics of the degradation of ASA are explained in more detail by Garrett (1957).

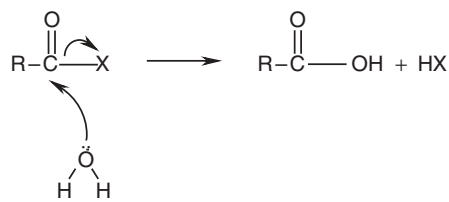
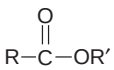
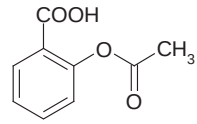
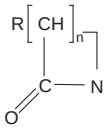
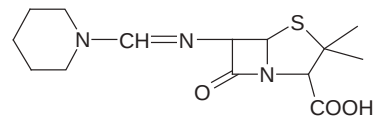
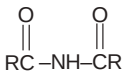
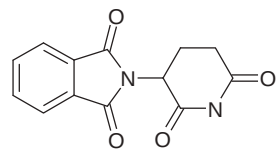
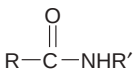
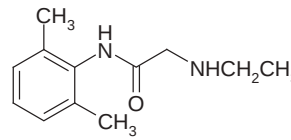


Figure 2.2.2 Hydrolysis, i.e. nucleophilic attack by H_2O on an acyl carbon in a carboxyl compound, resulting in the corresponding carboxylic acid and leaving the group HX. X is e.g. OR, NR or SR, and R is an organic unit.

Table 2.2.1 General structures of selected carboxyl compounds and examples of drug substances that are carboxyl compounds

Hydrolysable carboxyl compounds	*Drug substance examples*
 Ester	 Acetyl salicylic acid (ASA)
 Lactam	 Mecillinam
 Imide	 Thalidomide
 Amide	 Lidocaine

The hydrolysis of amides is another important class of hydrolysis reactions relevant for pharmaceutical stability investigations. Since the electronegativity of nitrogen (3.0) is less than that of oxygen (3.5), the acyl carbon in an amide is, in general, of lower partial positive charge than in an ester. Consequently, amides are often more stable towards hydrolysis than esters. However, if nitrogen is in a terminal position as it

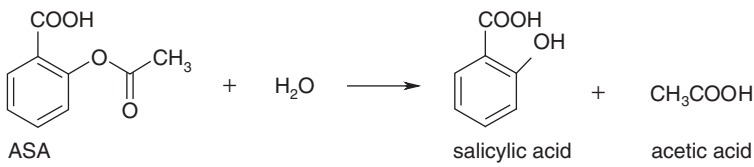


Figure 2.2.3 Hydrolysis of acetyl salicylic acid (ASA) resulting in the formation salicylic acid and acetic acid.

is in the amino acids aspargine (Asn) and glutamine (Gln), the acyl carbon is generally more easily attacked by nucleophiles than in secondary amines. Thus, deamidation of primary amines, such as Asn and Gln, takes place and asparaginic acid (Asp) and glutaminic acid (Glu), respectively are formed. These reactions are generally not very fast in neutral solution, but may be extensively catalysed by acids and bases as well as by intramolecular nucleophilic attack as described below. Thus, deamidation is an important reaction for most drug candidates that are peptides and proteins, even though it is unpredictable for influencing therapeutic activity. Therefore, no influence on therapeutic effects is seen in the case of deamidation reactions in insulin, but in the case of adrenocorticotrophin, deamidation reactions decrease its therapeutic effect (Gráf *et al.*, 1973).

In addition, hydrolysis may be responsible for the release of parent drug substance from an ester prodrug. Prodrugs are described in Chapter 2.4. In Figure 2.2.4 the hydrolysis of the ester prodrug valaciclovir is shown. The hydrolysis is initiated by electron pair attack donated by H_2O, resulting in the release of parent aciclovir together with the amino acid valine (Val). The kinetics of degradation of valaciclovir are described in Chapter 2.3 and in more detail by Thomsen *et al.* (2003a,b).

Hydrolysis may also be catalysed either by stronger nucleophiles than water or by acids due to protonisation of acyl oxygen. Examples of compounds that can catalyse hydrolysis reactions are specific and general acids and bases. Consequently, hydrolysis reactions are pH dependent. The kinetics of pH-dependent hydrolysis are described in Chapter 2.3. Other examples of catalysts are electrophiles and nucleophiles as well as enzymes with esterase or peptidase activity. Examples are shown in Table 2.2.2.

The first step in a hydrolytic acyl transfer reaction of esters in alkaline solution can involve a nucleophile attack by the specific base catalyst on the acyl carbon, i.e. OH^- creates a tetrahedral intermediate as shown in Figure 2.2.5a . This reaction is reversible. However, depending on which of the C-OH or the C-OR′ bonds are strongest, either the

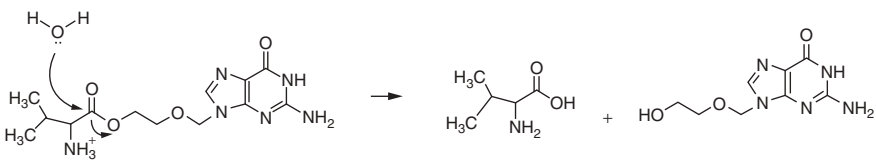


Figure 2.2.4 Hydrolysis of the ester prodrug valaciclovir to the parent aciclovir and the amino acid valine.

Table 2.2.2 Examples of compounds catalysing hydrolysis reactions. Regarding gastrointestinal peptidases see Table 2.4.3 as well as Steffansen *et al.*, 2005

Hydrolysis catalysts	*Structures of various catalysts*
Specific acid	H_3O^+
Specific base	OH^-
Water	H_2O
General acid (proton donor), i.e. Brønsted acid (see Section 2.1.1)	Examples: several buffers: H_3PO_4, H_2CO_3, CH_3CH_2COOH
General base (proton acceptor), i.e. Brønsted base (see Section 2.1.1)	Examples: several buffer systems: CO_3^- HCO_3^-
Electrophile (electron pair acceptor), i.e. Lewis acid (see Section 2.1.1)	Examples: metal ions such as Na^+, K^+ as well as acyl carbon (cf. Figure 2.2.1)
Nucleophile (electron pair donor), i.e. Lewis base (see Section 2.1.1)	Examples: amines and alcohols R–ÖH R–N̈H$_3$
Enzymes with esterase or peptidase activity	Examples: carboxyesterases, aminopeptidases, carboxypeptidases

corresponding alcohol is formed or the ester is reformed. In the hydrolysis process illustrated in Figure 2.2.5b, the alcohol is formed and the specific base catalyst OH^- is reformed.

It has been suggested that ring-opening hydrolysis of the mecillinam in the pH range 7–9 may be catalysed by the intramolecular non-protonated nucleophilic amidino group. The reaction is also catalysed by specific base catalysis (Bundgaard 1977; Bundgaard and Larsen, 1977). The intramolecular nucleophilic attack by the amidino group is illustrated in Figure 2.2.6.

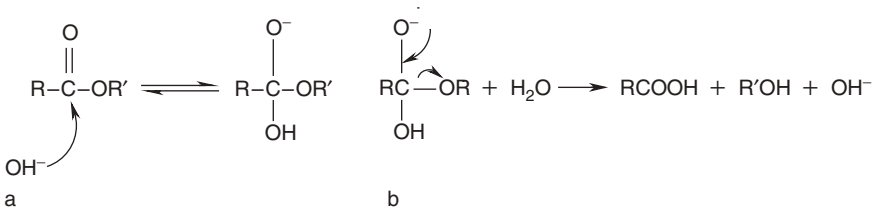


Figure 2.2.5 Hydrolysis of an ester in alkaline solution involving nucleophilic attack by the specific base catalyst OH^- on the acyl carbon with partial positive charge, which results in the reversible formation of the tetrahedral intermediate (a) followed by the breakage of the C-OR′ bond to produce the corresponding carboxylic acid $RCOO^-$ and alcohol R′OH (b).

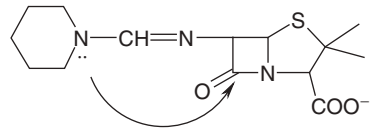


Figure 2.2.6 Intramolecular nucleophilic attack by the amidino group in mecillinam.

Intramolecular nucleophilic attack often takes place in a similar way in peptides enclosing Asn and Gln. This is illustrated in Figure 2.2.7, where an electron pair from the secondary peptide bond attacks the β-acyl carbon, i.e. in the side chain of Asn, to form a pentacyclic imide intermediate that opens up to form either the corresponding Asp-containing peptide or the corresponding iso-Asp-peptide. This reaction explains one of the most important chemical instability reactions of peptides and proteins.

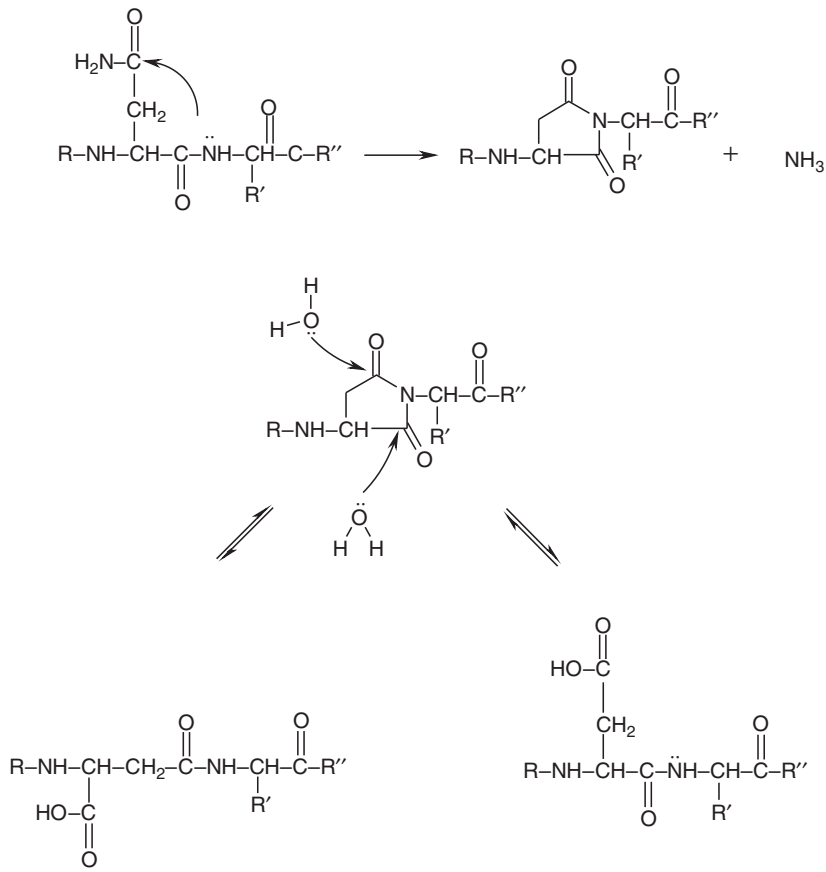


Figure 2.2.7 Deamidation in peptides and proteins enclosing the amino acid asparagine (Asn).

However, carboxyls are catalysed not only by bases or nucleophiles but often also by acids. Protonation of acyl oxygen by acids further polarises the carboxyl group, which attracts nucleophiles within its neighbourhood to attack acyl carbon and thus catalyses the reaction. Steffansen and co-workers (Steffansen *et al.*, 2005) have suggested this reaction takes place in the acid-catalysed ester hydrolysis of the proposed main degradation pathway of the model prodrug Glu(aciclovir)-Sar that, via two parallel reactions, forms parent aciclovir and a suggested cyclic impurity (see Figure 2.2.8).

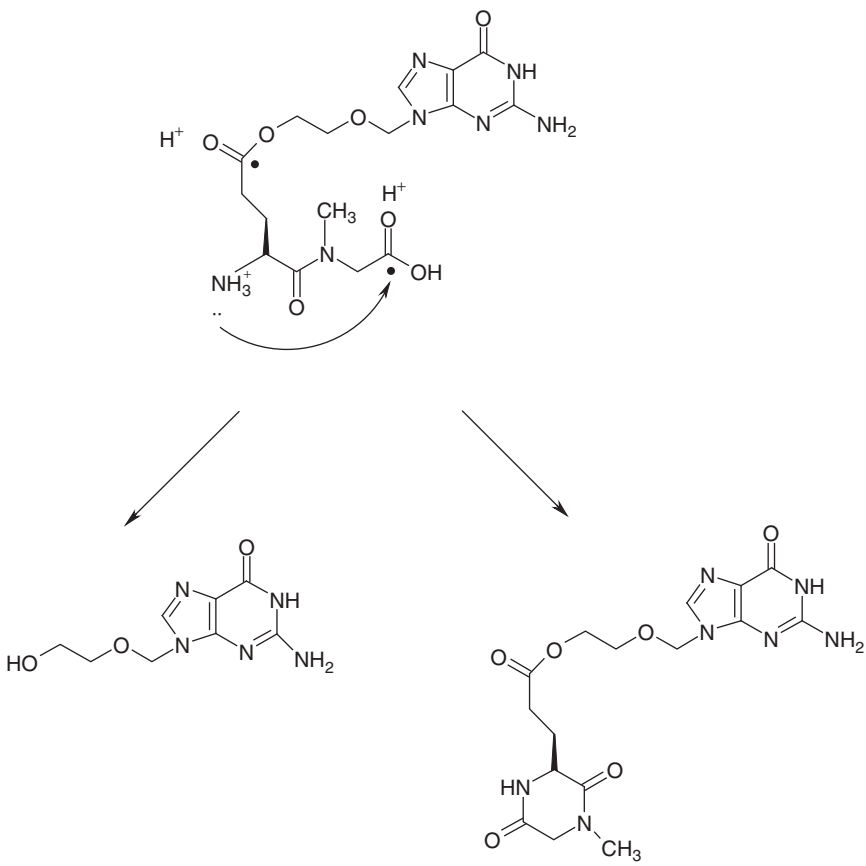


Figure 2.2.8 Specific acid-catalysed hydrolysis of Glu(aciclovir)-Sar to release parent aciclovir and a suggested cyclic impurity. Acyl carbons are marked by •. Redrawn from Thomsen AE, Friedrichesen GM, Sørensen AH, *et al.* (2003a). Prodrugs of purine and pyrimidine analogues for the intestinal di/tri-peptide transporter PEPT1: affinity for PEPT1 in Caco-2 cells, drug release in aqueous media and in vitro metabolism. *J Control Rel* 86: 279–292.

Mechanisms of degradation reactions investigated in aqueous solutions may differ with pH. Thus, even though the parallel specific acid-catalysed hydrolysis reaction of Glu(aciclovir)-Sar takes place at pH <4, the specific acid-catalysed aciclovir formation increases with increasing pH, whereas the specific acid-catalysed formation of the cyclic impurity increase with decreasing pH (Thomsen *et al.*, 2003a,b).

Another example of hydrolysis mechanisms being pH dependent is the specific acid-catalysed hydrolysis of erythromycin 2′-acetate, i.e. at pH ≤4.0, where erythromycin 2′-acetate prodrug is quantitatively and irreversible degraded to its corresponding impurity anhydroerythromycin via dehydration of the initially formed 6.9 hemiketal erythromycin (Atkins *et al.*, 1986; Kurath *et al.*, 1971; Steffansen and Bundgaard, 1989). The reaction scheme for this acid-catalysed conversion of erythromycin 2′-acetate is shown in Figure 2.2.9.

However, erythromycin 2′-acetate is reacting as a true prodrug in the pH range of 7–10, i.e. it is quantitatively hydrolysed by specific base-catalysed reaction to its corresponding drug substance erythromycin. The reaction scheme for this reaction is illustrated in Figure 2.2.10.

In order to identify possible impurities arising from decomposition reactions, it is of utmost importance to investigate the mechanisms behind the hydrolysis reactions of (pro)drug candidates.

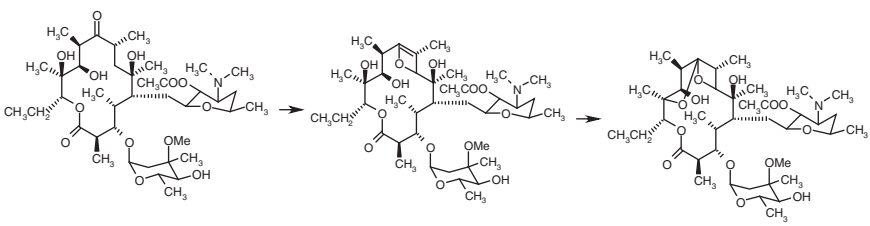


Figure 2.2.9 Acid-catalysed degradation of 2′-acetate erythromycin to impurity 2′-acetate anhydroerythromycin via 2-acetate erythromycin 6.9-hemiketal.

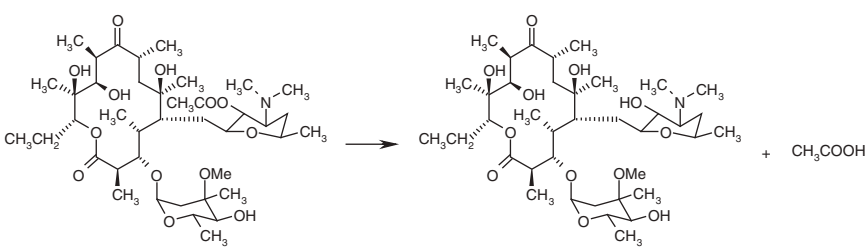


Figure 2.2.10 Hydrolysis of the prodrug 2′-acetate erythromycin at pH 7.4 to the corresponding drug substance erythromycin.

For drug and prodrug substances that decompose due to hydrolysis, it is clearly important to formulate the drug product as a solid dosage form that limits the access of water to the drug substances. However, in humid conditions the drug substance is exposed to a limited amount of water even in a solid dosage form. Therefore, the solid dosage form should be formulated with excipients that do not catalyse hydrolysis, so that the drug substance shows maximal stability. It is therefore of great importance to investigate and understand the hydrolysis reactions of drug candidates even though they are developed by means of a solid dosage form.

2.2.2 Oxidation

Whereas hydrolysis involves a two-electron transfer reaction, oxidation proceeds through one-electron transfer, i.e. free radical transfer reactions. Many drug substances and candidates exist in a reduced form, e.g. alcohols, alkylbenzenes, aldehydes, alkenes, amines, so that the presence of oxygen in the atmosphere may create oxidised degradation products.

Oxidation is complementary to reduction, i.e. oxidation and reduction involve electron release and uptake processes, respectively. These electron transfer processes take place only to some extent with regard to redox processes involving covalent bonds such as in alcohols. Although covalently bound carbon is encompassed by the same numbers of electrons before and after its oxidation process, the oxidation state may change because electrons are regarded as belonging to the most electronegative atom involved in the bond.

Some oxidation reactions are initiated by energy such as light. However, not all photolytic reactions are oxidative in nature (see Section 2.2.3).

An example of non-photolytic oxidation is phenol oxidising to ketone, which can be catalysed in alkaline solution and is thus pH dependent. The base initiates the reaction by making a free radical at the phenol oxygen. The free radical then initiates a chain reaction by which a new free radical is formed. In Figure 2.2.11 (right), the free radical is placed in a para-position to the ketone; however, that is just to provide an illustration since this free radical initiates further, inter- or intramolecular, degradation. Thus base-catalysed oxidation of phenols, which in the case of the amino acid tyrosine (Tyr) is very complex, is, nevertheless, often seen. Since tyrosine is a building block of many protein and peptide drug candidates, this oxidation reaction has great importance for stability investigations of many of these drug candidates. The same type of reaction takes place in morphine and epinephrine (adrenaline) oxidations.

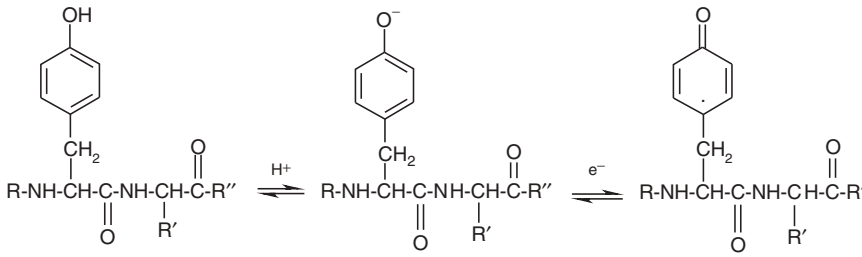


Figure 2.2.11 Redox equilibrium of the amino acid tyrosine.

The oxidation states of carbon may change from –4 in CH_4 to +4 in CCl_4. In oxidation state zero, carbon has four accessible electrons in its outer electron shell.

The oxidation of a primary alcohol, where the alcohol carbon (**C*** in Figure 2.2.12 left) is bound by single bonds to one alcohol (an oxidation state of –1), two hydrogens (each in an oxidation state of +1), and one carbon atom, the five electrons in its outer electron shell are considered to belong to **C***. Consequently, the **C*** atom is in its oxidation state –1. This is illustrated in Figure 2.2.12 left, where electrons considered to belong to the **C*** are marked by a bold **x** and those marked with a regular x are considered to belong to neighbouring atoms. However, when **C*** releases two electrons during oxidation of the compound from phenol to aldehyde, **C*** changes its oxidation state from –1 to +1. The carbonyl carbon in aldehyde is illustrated as bold **C** with the three bold **x**'s in Figure 2.2.12 right. In other words, compared to free carbon in oxidation state zero, which has four electrons in its outer electron shell, alcohol-bound carbon has five and carbonyl carbon involved in aldehyde functionality has three. Consequently its oxidation state changes from –1 to +1.

Apart from in O_2, where oxygen is in an oxidation state of zero, oxygen is normally in its oxidation state of –2, and it is more electronegative (3.5) than carbon (2.5). Hydrogen, on the other hand, is (apart from H_2) normally in an oxidation state of +1 and is less electronegative (2.2) than carbon (2.5). Electrons involved in covalent bonds are considered to

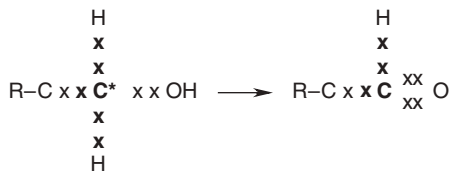


Figure 2.2.12 Oxidation of primary alcohol to aldehyde. For explanation, see text.

belong to the most electronegative atom involved in the bond. Bold **C*** in Figure 2.12 illustrates alcohol-bound carbon in its oxidation state of –1 (left) and bold **C** carbonyl carbon in aldehyde functionality is in its oxidation state of +1 (right). Bold **x** illustrates electrons considered to belong to **C*** or **C**, whereas regular x's are considered to belong to neighbouring atoms. Thus, compared to free carbon, which has four electrons, **C*** involved in the primary alcohol functionality has five (**x**), and carbonyl carbon **C** involved in aldehyde has three (**x**). Thus, bold **C*** goes from having an excess of one electron, in order to reach an oxidation state of –1, to having a deficiency of one electron, in order to reach an oxidation state of +1, when alcohol is oxidised to aldehyde.

Other oxidation reactions are light-induced oxidation processes such as takes place in riboflavin. The two carbons within the second and third ring systems each take up one electron, and riboflavin is thus reduced by two. The neighbouring nitrogen atoms are not involved in the redox process, since nitrogen has six electrons in the outer electron shell throughout the reaction. The oxidation process of riboflavin is illustrated in Figure 2.2.13.

Oxidative reactions may also be catalysed by electron donors such as the metal ions Fe^{3+}, Cu^{2+} or Co^{3+}. Since Fe^{3+} and Cu^{2+} particularly are often present in distilled water, drug candidates that may oxidise should be formulated from ion-free water.

Methods of avoiding oxidation reaction are dependent on the oxidation mechanism. For example if oxygen itself is involved in the process, the method will need to exclude oxygen, for example by exchanging the air in containers for an inert gas or by packaging in hermetically sealed containers. In cases where oxidation is base catalysed, the pH may be

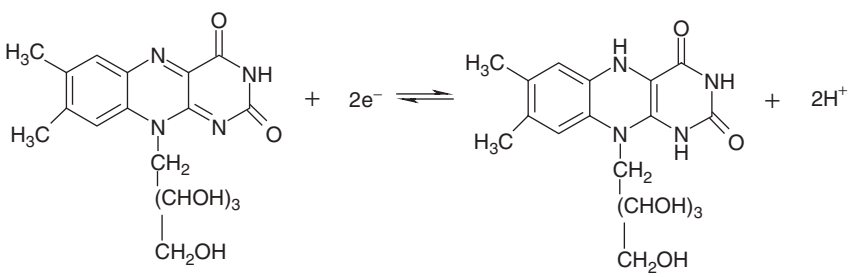


Figure 2.2.13 Redox equilibrium between riboflavin (left) and dihydroriboflavin (right). In the oxidation process, two carbon atoms in the second and third ring systems are reduced. Redrawn from Connors KA, Amidon GL, Stella VJ (1986). *Chemical Stability of Pharmaceuticals. A Handbook for Pharmacists,* 2nd edn. New Jersey: Wiley-Interscience Publication John Wiley and Sons 3–847.

reduced to 3–4. If the oxidation is photolytic, the oxidative process may be minimised by avoiding light. Impurities of metal ions such as Cu^{2+} or Fe^{3+} may catalyse oxidation reactions, but by adding chelating agents such as EDTA, redox processes can be limited. Alternatives are addition of antioxidants (reducing agents) such as sodium thiosulphate and ascorbic acid.

2.2.3 Non-oxidative photolytic degradation

Photolytic reactions may be oxidative in nature as described above. However, they may also be excitative in which a drug candidate develops into an excited state species as a consequence of absorption of light or radiation energy. The absorbed energy can then be lost by radiation, fluorescence, phosphorescence, heat loss or collision with other molecules, i.e. quenching. During this energy loss, chemical decomposition may occur. One example is photolysis-induced *cis–trans* isomerisation where carbon–carbon π-electrons may be excited by light, which allows rotation and thus *cis-trans* isomerisation. One example of such light-induced reaction is the *cis*-conformation of licochalcone A, which isomerises in the presence of light to form impurities of the corresponding *trans*-form. This is illustrated in Figure 2.2.14.

One way to limit photolytic isomerisation is to avoid the wavelengths initiating the excitation reaction. The whole production procedure should be performed in artificial light where compromising wavelengths are avoided. The drug product should be packaged in containers that exclude all light or that filter those wavelengths that catalyse the reaction.

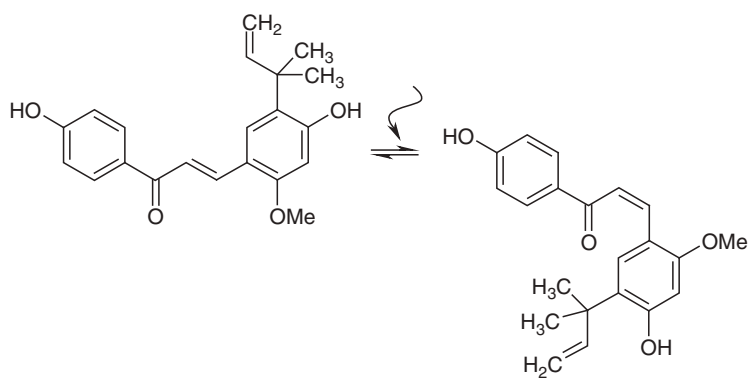


Figure 2.2.14 *cis-trans* isomerisation of licochalcone A.

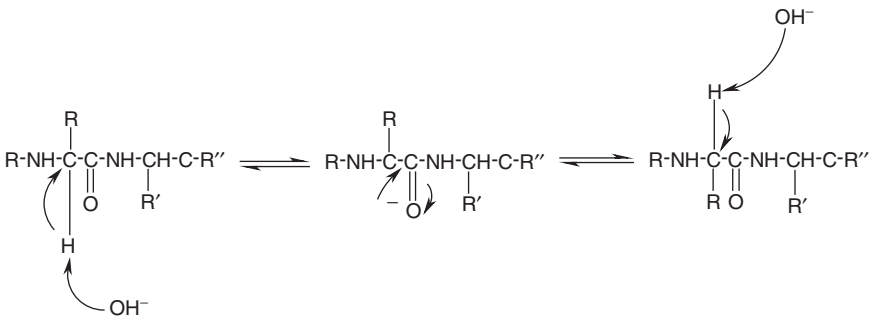


Figure 2.2.15 Racemisation from L- to D-amino acids.

2.2.4 Degradation by racemisation

Possible racemisation may take place in drug candidates that include a chiral centre, i.e. carbon bound to four different groups. There are many examples, and one is amino acids except for glycine, where base-catalysed racemisation of L-amino acids forms impurities of corresponding D-amino acids. This is due to acidic properties of the alpha-hydrogen of amino acids. The acidity of the alpha-hydrogen is increased in amino acids with electronegative side chains, which weakens the carbocation properties of the acyl carbon, see Figure 2.2.1. The racemisation reaction is shown in Figure 2.2.15 and may take place in all drug candidates that are peptides or proteins.

2.2.5 Polymerisation

Polymerisation reactions between identical drug substance molecules may form large and complex degradation products. This is especially seen for several β-lactam antibiotics. The polymersation of β-lactams has been shown to occur via various reactions; however, one example is aminolysis between the free carboxylic acid formed in the ring-opened impurity of ampicillin, ampicilloic acid, and the side-chain amine of another ampicillin molecule as shown in Figure 2.2.16 (Bundgaard and Larsen, 1977).

Polymerisation impurities may be immunogenic, and polymerisations of penicillins, in the form of an aqueous solution, play a major role in penicillin allergy and should therefore be avoided. That is one of the reasons why many penicillins are formulated as solid drugs or as granulations to be dissolved shortly before administration.

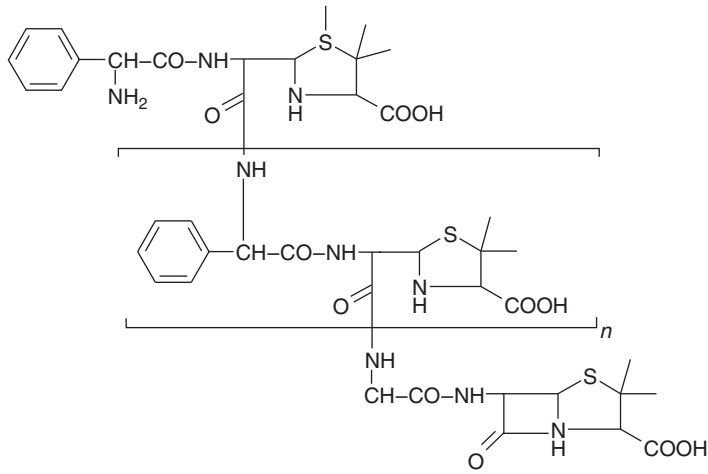


Figure 2.2.16 Polymerisation of ampicillin.

2.2.6 Conclusions

Degradation of prodrug and drug candidates and substances may often be initiated by environmental factors within the atmosphere such as humidity, which may induce hydrolysis; oxygen, which may induce oxidation; or light, which among others may induce *cis-trans* isomerisation. However, it is often possible to identify vulnerable functional groups that are sensitive to one or more of these inducing factors, such as carboxyl, carbonyl, alcohol, chiral centres or double bonds within the molecules. This is important for explaining the degradation mechanisms and describing the kinetics of these reactions, which should be used in the documentation of the stability of a drug substance, i.e. the active pharmaceutical ingredient (API), and the final drug product.

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2.3

Kinetics of decomposition in aqueous solution

Bente Steffansen, Carsten Uhd Nielsen and Birger Brodin

The stability of (pro)drug candidates in an aqueous solution can be described not only mechanistically (see Chapter 2.2) but also kinetically, in terms of both the rate and order of degradation. Such kinetic stability investigations are relevant in molecular biopharmaceutics, as well as in preclinical development, in order to investigate the rate of possible *in vitro* metabolism of drug candidates and/or their release from prodrugs. Investigations may take place in relevant buffer solutions at a specific pH or in artificial biological media with or without specific enzymes, or in genuine biological media, such as intestinal, gastric, lachrymal, salivary and/or other blood fluids. The pH values of a number of potentially relevant biofluids are shown in Table 2.1.2. Furthermore, *in vitro* metabolism or release from prodrugs may be investigated in various tissue homogenates, for example intestinal, hepatic, or nephritic homogenates, or in homogenates of various relevant cell cultures such as enterocytic, hepatocytic, erythrocytic or proximal tubular cell homogenates. Kinetic investigations in biopharmaceutical and preclinical development are often described by degradation orders, rate constants and half lives ($t_{1/2}$), and sometimes, if specific enzymes are identified as rate limiting, by Michaelis–Menten kinetics, and thus by K_m and V_{max} values (see Chapter 3.3).

However, kinetic stability investigations are also an integral part of the documentation of impurities arising from possible decomposition reactions that may take place during storage of the pharmaceutical product as well an integrated part of establishing their shelf life. Shelf-life investigations are normally initiated during preformulation and pharmaceutical formulation development, through the study of such kinetics as reaction order and rate constants of (pro)drug substances in various aqueous solutions at specific pH, temperature and ionic strengths. The criterion generally accepted by authorities in the field is that a maximum of 5% of the formulated (pro)drug substance may be decomposed during storage. For this reason, shelf-life investigations often deal with the time

taken for 5% of the drug substance, i.e. the API (active pharmaceutical ingredient), to decompose, determined as $t_{5\%}$.

Besides determining the half life ($t_{1/2}$) and shelf life ($t_{5\%}$) of drug candidates, it is also important to understand the kinetics and thus the order of the decomposition reactions. Furthermore, one needs to identify and describe the influence of various factors, for example enzymes, pH, solubility, excipient and temperature, on the kinetics and thus on the rate of degradation reactions.

The purpose of this chapter is to present the importance of zero-, first- and second-order kinetics in the description of stability kinetics of (pro)drug candidates and of relevance for formulation and bioavailability. Furthermore, the influence of pH, temperature, buffers and ionic strength on the decomposition kinetics of (pro)drug candidates and thus on the establishment of shelf life will be described. Finally, a description of the influence of biofluid (pH and enzymes) on *in vitro* metabolism and *in vitro* release kinetics of prodrugs will be given.

2.3.1 First-order and pseudo zero-order kinetics of irreversible reactions

The spontaneous decomposition reaction of a drug candidate A, in which the reaction is purely dependent on the decomposition of A to form metabolites or impurities by an irreversible reaction, is illustrated in Scheme 2.3.1. The same kind of reaction may take place in the release of drug candidate (A*) from prodrug PA.

$$\text{(P)A} \longrightarrow \text{impurity/metabolite/A*} \qquad \text{(Scheme 2.3.1)}$$

The rate of such a decomposition reaction v may be described by Equation 2.3.1 in which [A] is the concentration of (P)A, k is the rate constant of its decomposition reaction and $[A]_t$ is the concentration of (P)A at a given time t.

$$v = -\frac{d[A]}{dt} = k[A]_t \qquad (2.3.1)$$

The definition of the overall order of a reaction is defined as the sum of its concentration term exponents in a rate equation (Connors *et al.*, 1986). The sum of concentration term exponents in Equation 2.3.1 is one ($[A]^1$), the reaction being purely dependent on (P)A, and consequently the degradation is of the first order. The rate constant k is thus the first-order rate constant given at time^{-1}. The rate constant k is independent of the

initial concentration $[A]_0$, whereas the numbers of (P)A degraded per time unit (e.g. mol (P)A degraded min^{-1}) change with time. It is easier to understand this relation when Equation 2.3.1 is integrated into Equation 2.3.2 and rearranged to form Equation 2.3.3, since the first-order degradation may be described by a logarithmic function.

$$[A]_t = [A]_0 e^{-kt} \tag{2.3.2}$$

$$\log[A]_t = \log[A]_0 - \frac{k}{2.303} t \tag{2.3.3}$$

From Equation 2.3.3 it can be seen that the degradation of (P)A can be illustrated graphically by a linear first-order plot of $\log[A]_t$ versus t with a slope $-k/2.303$. An example of such a graph is shown in Figure 2.3.1. Equation 2.3.3 may be rearranged to Equations 2.3.4 and 2.3.5 in order to calculate the half life ($t_{1/2}$) of (P)A, which is the time (t) it takes (P)A to reach one half of $[A]_0$, and the shelf life ($t_{5\%}$), which is the time it takes to reach 95% of $[A]_0$ respectively; k_{obs} is the observed rate constant.

$$t_{1/2} = \frac{\ln 2}{k_{obs}} \tag{2.3.4}$$

$$t_{5\%} = \frac{\ln 100/95}{k_{obs}} \tag{2.3.5}$$

In cases where only dissolved (P)A spontaneously decomposes to form an impurity, metabolite or A* according to Scheme 2.3.1, and where (P)A is formulated in a suspension, the accessible amount for degradation, i.e. $[A]_0$, corresponds to the solubility (S) of (P)A. As soon as one molecule of (P)A is decomposed, there is room for another molecule to dissolve from the suspension, and consequently $[A]_0$ is constant and the rate of degradation is then described by Equation 2.3.6. In Equation 2.3.6, the concentration term exponent may be considered as constant, i.e. zero, and the observed pseudo zero-order rate constant $k_{obs} = k[A]_0$ subsequently describes the reaction kinetics.

$$v = -\frac{d[A]}{dt} = k[A]_0 = k_{obs} \tag{2.3.6}$$

Equation 2.3.6 can be integrated to form Equation 2.3.7:

$$[A]_t = [A]_0 - k_{obs} t \tag{2.3.7}$$

From Equation 2.3.7 it can be seen that the degradation of (P)A may be illustrated graphically by a linear plot of $[A]_t$ versus t with a slope of $-k_{obs}$

given as a concentration term per unit time. Thus pseudo zero-order k_{obs} is dependent on $[A]_0$. However, the number of moles decomposed per time unit does not change with time as long as the suspension is maintained. The half life ($t_{1/2}$) and shelf life ($t_{5\%}$) of (P)A may be calculated from Equations 2.3.8 and 2.3.9 respectively.

$$t_{1/2} = \frac{[A]_0}{2k} \tag{2.3.8}$$

$$t_{5\%} = \frac{[A]_0}{20k} \tag{2.3.9}$$

Very few decomposition reactions of (pro)drug candidates are genuine first-order or pseudo zero-order reactions. However, many may be described by second-order and pseudo first-order kinetics as described in Section 2.3.2.

2.3.2 Second-order and pseudo first-order kinetics of irreversible reactions

Second-order reactions occur, for example, if a dissolved (pro)drug substance (P)A reacts at a ratio of 1:1 with another similar dissolved (pro) drug molecule (P)A to form a product by an irreversible reaction according to Scheme 2.3.2. The rate of such decomposition may be described by Equation 2.3.10:

$$\text{(P)A + (P)A} \longrightarrow \text{impurities/metabolites/A*} \qquad \text{(Scheme 2.3.2)}$$

$$v = -\frac{d[A]}{dt} = k[A]^1[A]^1 = k[A]^2 \tag{2.3.10}$$

Two concentration term exponents ($[A]^2$) are involved, and thus the reaction may be described by second-order kinetics. Equation 2.3.10 may be transformed into Equation 2.3.11 and rearranged to form Equation 2.3.12 in which k is the second-order rate constant given as concentration^{-1} time^{-1}.

$$\int_{[A]_0}^{[A]_1} -\frac{d[A]}{[A]^2} = \int_0^t kt \tag{2.3.11}$$

$$\frac{1}{[A]_t} - \frac{1}{[A]_0} = kt \tag{2.3.12}$$

From Equation 2.3.12, it can be seen that the decomposition may be illustrated graphically by a linear plot of $1/[A]_t - 1/[A]_0$ versus t with a slope of $-k$ given as concentration^{-1} time^{-1}. Equation 2.3.12 may be rearranged to give Equations 2.3.13 and 2.3.14 from which $t_{1/2}$ and $t_{5\%}$ may be calculated respectively.

$$t_{1/2} = \frac{1}{k[A]_0} \tag{2.3.13}$$

$$t_{5\%} = \frac{0.052}{[A]_0 k} \tag{2.3.14}$$

An example of such a second-order reaction is the dimerisation of ampicillin, in which two identical ampicillin molecules form ampicillin dimers that further polymerise, as illustrated in Figure 2.2.16 (Bundgaard, 1976).

Second-order reactions may also take place between (P)A and a second substance B, e.g. an excipient, to form impurity or metabolite or A* by an irreversible reaction according to Scheme 2.3.3.

$$\text{(P)A} + \text{B} \longrightarrow \text{impurity/metabolite/A*} \qquad \text{(Scheme 2.3.3)}$$

The rate of degradation v may then be described by Equation 2.3.15, in which k is the second-order rate constant of the reaction.

$$v = -\frac{d[A]}{dt} = -\frac{d[B]}{dt} = -k[A]^1[B]^1 \tag{2.3.15}$$

If $[A]_t = [A]_0 = [P]_t$ and $[B]_t = [B]_0 = [P]_t$, where $[A]_0$ and $[B]_0$ are initial concentrations of (P)A and B respectively, and $[P]_t$ is the product formed at time t, then Equation 2.3.15 may be rearranged to give Equation 2.3.16:

$$\ln\frac{[A]_0 - [P]_t}{[B]_0 - [P]_t} = ([A]_0 - [B]_0)kt + \ln\frac{[A]_0}{[B]_0} \tag{2.3.16}$$

The decomposition may be illustrated graphically by a linear plot of $\ln [([A]_0 - [P]_t)/([B]_0 - [P]_t]$ versus t with slope $([A]_0 - [B]_0)k$.

Many second-order reactions may be kinetically simplified to pseudo first-order reactions. An example of this is the irreversible second-order hydrolysis of the ester prodrug valaciclovir to form parent aciclovir and valine, which is shown in Figure 2.2.4 (Thomsen *et al.*, 2003a,b). In

general terms, if a (pro)drug candidate (P)A reacts irreversibly with e.g. water (B) at a ratio of 1:1 to produce drug substance A* (Scheme 2.3.3), the rate of degradation v can be described by the Equation 2.3.16 in which k is the second-order rate constant, and the sum of concentration term exponents ($[A]^1 + [B]^1$) is two.

However, in the case of [B] >> [A], the kinetics of the reaction may be described by Equation 2.3.17, which may be rearranged to form Equations 2.3.18 and 2.3.19. The apparent sum of concentration term exponents is then reduced from two in Equation 2.3.17 to one in Equations 2.3.18 and 2.3.19, namely from ($[A]^1 + [B]^1$) to ($[A]^1$), and consequently the apparent kinetics are termed pseudo first-order, in which the observed pseudo first-order rate constant ($k_{obs} = k[A]$) is given as unit time^{-1}.

$$-\frac{d[A]}{dt} = k_{obs}[A] \text{ and } k_{obs} = k[B] \tag{2.3.17}$$

$$[A]_t = [A]_0 e^{-k_{obs}t} \tag{2.3.18}$$

$$\log[A]_t = \log[A]_0 - \frac{k_{obs}}{2.303}t \tag{2.3.19}$$

From Equation 2.3.19 it can be seen that the degradation of (P)A can be illustrated graphically by a linear first-order plot of $\log[A]_t$ versus t.

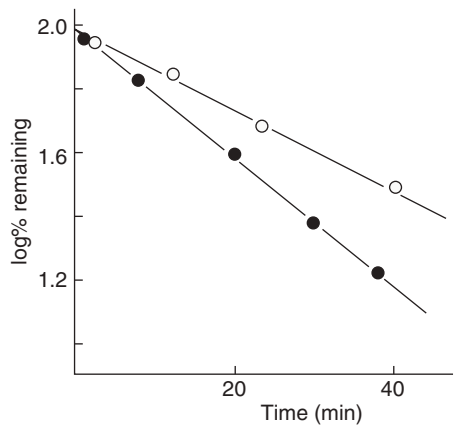


Figure 2.3.1 The log of % remaining erythromycin 2′-acetate prodrug versus time, representing a pseudo first-order plot of erythromycin 2′-acetate degradation in an aqueous buffer solution at pH 4.00 (•) and pH 7.4 (o) at 37 ± 2 °C. Reproduced from Steffansen B, Bundgaard H (1989). Erythromycin prodrugs: kinetics of hydrolysis of erythromycin and various erythromycin 2′-esters in aqueous solution and human plasma. *Int J Pharm* 56: 159–168.

Table 2.3.1 Time points at which % remaining erythromycin 2′-acetate prodrug was analysed in an aqueous buffer solution pH 7.4 at 37 ± 2 °C

Time (min)	*% Remaining erythromycin 2′-acetate prodrug*	*Log % remaining erythromycin 2′-acetate prodrug*
0	100	2.00
3	95.5	1.98
15	75.8	1.88
26	50.1	1.70
41	31.6	1.50

Another example of irreversible pseudo first-order hydrolysis besides that of valaciclovir is the hydrolysis of erythromycin 2′-acetate prodrug (PA). An example of a first-order plot is shown in Figure 2.3.1 in which a plot of overall % degradation of PA in aqueous buffer solution of pH 4.00 (filled circle) and pH 7.40 (open circle) at 37 ± 2 °C is shown (Steffansen and Bundgaard, 1989). The slope of such graphs is represented by $-k_{obs}/2.303$.

The data illustrated in Figure 2.3.1 are given in Table 2.3.1. Using these data, k_{obs} may be calculated by linear regression (time versus logk_{obs}) as 0.0053 min^{-1}. When one has calculated k_{obs} of the decomposition reaction, its corresponding half life ($t_{1/2}$) and shelf life ($t_{5\%}$) may be calculated using Equations 2.3.4 and 2.3.5, to be approximately 129 min and 10 min respectively.

2.3.3 Pseudo first-order kinetics of reversible reactions

First-order reactions may also be reversible (Scheme 2.3.4), in which two first-order rate constants are involved, one that describes the rate of the forward reaction (k_{+1}) and a second that describes the rate of the reverse reaction (k_{-1}). At equilibrium the amount going forward must equal the amount going in reverse.

$$(P)A \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} B \qquad \text{(Scheme 2.3.4)}$$

The rate, $v = d[A]_0/dt$, of such reversible decomposition for which the forward and reverse reactions are of the first order, e.g. the *cis-trans*

isomerisation of licochalcone A (see Figure 2.2.14) may be described by Equation 2.3.20.

$$-\frac{d[A]_0}{dt} = k_{+1}[A] - k_{-1}[B] \tag{2.3.20}$$

The integrated form of Equation 2.3.20 is Equation 2.3.21, which may be rearranged into Equation 2.3.22, where $[A]_0$ represents the initial concentration of the drug candidate, $[A]_t$ the concentration at time t and $[A]_{eq}$ the equilibrium concentration.

$$\log\frac{[A]_0 - [A]_{eq}}{[A]_t - [A]_{eq}} = \frac{(k_{+1} + k_{-1})}{2.303}t \tag{2.3.21}$$

$$\log([A]_0 - [A]_{eq}) = -\frac{k_{+1} - k_{-1}}{2.303}t + \log([A]_t - [A]_{eq}) \tag{2.3.22}$$

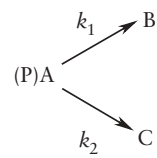
A linear plot of $\log([A]_0 - [A]_{eq})$ versus t has a slope of $-(k_{+1} + k_{-1})/2.303$ and intercept $\log([A]_t - [A]_{eq})$. By rearranging Equation 2.3.22 into Equations 2.3.23 and 2.3.24, the shelf life and half life respectively of drug candidates decomposed by reversible first-order reactions may be calculated:

$$t_{5\%} = \frac{\ln 100/95}{k_{+1} + k_{-1}} \tag{2.3.23}$$

$$t_{1/2} = \frac{\ln 2}{k_{+1} + k_{-1}} \tag{2.3.24}$$

2.3.3.1 Parallel irreversible pseudo first-order kinetics

Decomposition of drug candidates may also proceed via two or more parallel pseudo first-order reactions in which two pseudo first-order rate constants are involved. In Scheme 2.3.5, k_1 is the rate constant for the drug candidate, (P)A, decomposing to B, and k_2 is the rate constant for (P)A decomposing to C. This is the case for the acid-catalysed hydrolysis of the model prodrug Glu(aciclovir)-Sar which decomposes into aciclovir (A*) as well as a suggested cyclised impurity (Thomsen *et al.*, 2003a,b). The proposed degradation pathway for Glu(aciclovir)-Sar is shown in Figure 2.2.8.



(Scheme 2.3.5)

The rate of such parallel reactions may be described by Equation 2.3.25.

$$-\frac{\mathrm{d[A]}}{\mathrm{d}t} = k_1 + k_2[\mathrm{A}]_t \tag{2.3.25}$$

When measuring the disappearance of (P)A, the observed rate constant is the sum of the rate constants for the parallel decompositions, i.e. $k_{\mathrm{obs}} = k_1 + k_2$ and Equation 2.3.25 equals Equation 2.3.19. Thus $t_{1/2}$ and $t_{5\%}$ for the disappearance of A may be calculated by Equations 2.3.4 and 2.3.5 respectively.

However, it may be desirable to determine the separate values of k_1 and k_2, since such rate constants may give important information about, for example, the rate of impurity formation. Separate values for k_1 or k_2 may be determined by measuring the products B and C. The equation describing the production of B is given by Equation 2.3.26:

$$\frac{\mathrm{d[B]}}{\mathrm{d}t} = k_1[\mathrm{A}]_t \tag{2.3.26}$$

By integrating Equation 2.3.26, in which $[\mathrm{A}]t = k_1/k_{\mathrm{obs}}[\mathrm{A}]_0 \mathrm{e}^{-k_{\mathrm{obs}}t}$ one gets Equation 2.3.27:

$$\log([\mathrm{B}]_\infty - [\mathrm{B}]_t) = \log(k_1/k_{\mathrm{obs}}[\mathrm{A}]_0) - k_{\mathrm{obs}}t/2.303 \tag{2.3.27}$$

Equation 2.3.27 describes a plot of $\log([\mathrm{B}]_\infty - [\mathrm{B}]_t)$ versus t, which is linear with a slope of $-k_{\mathrm{obs}}t/2.303$ and an intercept $\log(k_1/k_{\mathrm{obs}}\ [\mathrm{A}]_0)$, from which k_1 may be calculated according to Equation 2.3.28.

$$k_1 = [\mathrm{B}]_\infty k_{\mathrm{obs}}/[\mathrm{A}]_0 \tag{2.3.28}$$

2.3.4 Pseudo first-order reactions of irreversible consecutive reactions

Irreversible consecutive reactions may proceed if drug candidate (P)A degrades to impurity B, which then further degrades to impurity C by respective first-order rate constants k_1 and k_2, as illustrated in Scheme 2.3.6.

$$\text{(P)A} \xrightarrow{k_1} \text{B} \xrightarrow{k_2} \text{C} \qquad \text{(Scheme 2.3.6)}$$

The rate of decomposition may be described by Equations 2.3.29 to 2.3.31.

$$\frac{d[A]}{dt} = -k_1[A] \tag{2.3.29}$$

$$\frac{d[B]}{dt} = -k_2[B] + k_1[A] \tag{2.3.30}$$

$$\frac{d[C]}{dt} = k_2[B] \tag{2.3.31}$$

Equations 2.3.29 to 2.3.31 integrate to give Equations 2.3.32 to 2.3.34 respectively.

$$[A]_t = [A]_0 e^{-kt} \tag{2.3.32}$$

$$[B]_t = [A]_0 \frac{k_1}{k_2 - k_1}(e^{-k_1 t} - e^{-k_2 t}) \tag{2.3.33}$$

$$[C]_t = [A]_0 - [A]_t - [B]_t \tag{2.3.34}$$

The half life and shelf life describing the pseudo first-order disappearance of (P)A may be calculated from Equations 2.3.4 and 2.3.14 respectively. An example of an irreversible consecutive reaction is that of the decomposition of 2′-acetate-erythromycin prodrug (PA) in acidic solution to 2′-acetate anhydroerythromycin via 2-acetate erythromycin 6.9-hemiketal, as illustrated in Figure 2.2.9.

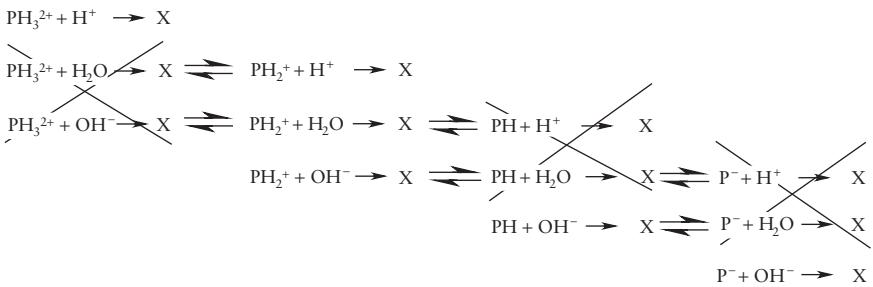
2.3.5 Influence of pH on decomposition rate

The stability of (pro)drug candidates in aqueous solution is often dependent on pH, and as a consequence the process is catalysed by concentrations of specific acids (H^+) and/or bases (OH^-). Information regarding the influence of pH on the stability of drug candidates may be obtained by systematic determination of observed pseudo first-order rate constants k_{obs} versus pH, while maintaining other factors such as temperature and ionic strength at constant values. If the pH is preserved by buffer solutions during an investigation of specific acid- and base-catalysed degradation of drug candidates, it is then necessary to investigate separately the influence of possible buffer concentrations on k_{obs} (see Section 2.3.2.5).

Obtained pseudo first-order k_{obs} values from pH rate experiments may be illustrated as a pH–rate profile, i.e. by depicting $\log k_{obs}$ versus pH. A pH rate profile may be very helpful in the investigation of decomposition mechanisms, but it may also be very helpful in determining shelf life and evaluating possible pH-dependent metabolism. A pH profile may have any number of characteristic features, depending on the specific

acid/base-catalysed processes involved, as well as the possible species distribution arising from acidic and/or basic functionalities of the (pro) drug candidate.

An example of pH-dependent decomposition is the hydrolysis of the ester prodrug valaciclovir (P) as it forms the drug substance aciclovir and the amino acid pro-moiety valine. Valaciclovir has three pK_a values, namely 2.0, 7.2 and 9.1, associated with two primary amine functionalities and one NH-acidic functionality, cf. Table 2.1.1. Consequently, 12 potential reactions may be involved in the hydrolysis of valaciclovir, as illustrated in Scheme 2.3.7, where PH_3^{2+} is the triprotonised, PH_2^+ the diprotonised, PH the monoprotonised, and P^- the fully deprotonised prodrug species.



(Scheme 2.3.7)

The pH–rate profile of valaciclovir is denoted by bold squares in Figure 2.3.2. By comparing the pH–rate profile with possible reactions it is suggested that (1) below pK_{aI} the profile is described by a specific acid-catalysed reaction of the triprotonised species, (2) at pK_{aI} < pH < PK_{aII} it is described by specific acid, water-catalysed (spontaneous) and specific base-catalysed hydrolysis of the diprotonised species, (3) at pK_{aII} < pH < pK_{aIII} it is described by specific base-catalysed hydrolysis of the monoprotonised species, and (4) at pH > pK_{aIII} it is described by specific base-catalysed hydrolysis of the fully deprotonised species.

Those reactions believed not to occur are marked by crosses in Scheme 2.3.7. Thus the proposed rate equation is given in Equation 2.3.35 (Thomsen *et al.*, 2003a,b).

$$k_{obs} = k'_{H^+}\cdot[H^+]\cdot f_{PH_3^{2+}} + k_{H^+}\cdot[H^+]\cdot f_{PH_2^+} + k_o f_{PH_2^+} + k_{OH^-}\cdot[OH^-]\cdot f_{PH_2^+} + k'_{OH^-}[OH^-]\cdot f_{PH} + k''_{OH^-}\cdot[OH^-]\cdot f_{P^-} \quad (2.3.35)$$

In Equation 2.3.35 fPH_3^{2+} is the species fraction of triprotonised, fPH_2^+ the species fraction of diprotonised, fPH the species fraction of

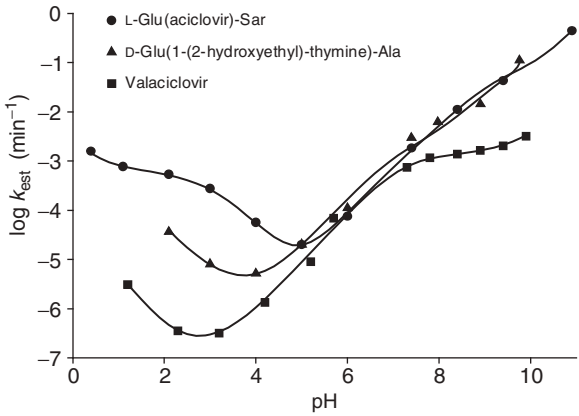


Figure 2.3.2 The pH–rate profile of logk_{obs} (min^{-1}) *versus* pH for valaciclovir (bold squares), L-Glu(aciclovir)-Sar (bold circles) and D-Glu[1-(2-hydroxyethyl)thymine]Ala (bold triangles) at 37 °C. The solid valaciclovir curve was obtained by fitting experimental data to Equation 2.3.34, using experimentally determined p*K*a values. Reproduced from Thomsen AE, Friedrichesen GM, Sørensen AH, *et al.* (2003a). Prodrugs of purine and pyrimidine analogues for the intestinal di/tri-peptide transporter PEPT1: affinity for PEPT1 in Caco-2 cells, drug release in aqueous media and in vitro metabolism. *J Control Rel* 86: 279–292.

monoprotonised and fP^- the species fraction of fully deprotonised prodrug P. Species fractions may be calculated as described in Section 2.1.1.

The profiles of pH rates are a very useful tool in pharmaceutical development. Using such profiles the pharmaceutical developer may gain an overview of the influence of pH on the decomposition kinetics of (pro) drug candidates. This is important not only in terms of determining and documenting the shelf life of (pro)drugs, but also in preclinical development, where it can help to evaluate possible acid/base-catalysed metabolism and elimination issues. One example is the possible influence of various environmental pH values *in vivo*, i.e. possible acid/base-catalysed metabolism of orally administered (pro)drug candidates during their gastrointestinal passage (see the pH of various biological fluids – Table 2.1.2). However, one should bear in mind that metabolism and elimination may often be enzyme-catalysed processes; these points are further described in Section 2.3.9.

2.3.6 Influence of temperature on decomposition rate

The decomposition rate in an aqueous solution of many (pro)drug candidates is dependent on temperature. For many small molecular drug substances such as ASA, the influence of temperature on degradation rate

may be described by the Arrhenius equation (Equation 2.3.36) that in logarithmic terms becomes Equations 2.3.37 and 2.3.38.

$$k_{\text{obs}} = \text{A}e^{-E_a/RT} \tag{2.3.36}$$

$$\ln k_{\text{obs}} = \ln\text{A} - \frac{E_a}{RT} \tag{2.3.37}$$

$$\log k_{\text{obs}} = \log\text{A} - \frac{E_a}{2.303RT} \tag{2.3.38}$$

In Arrhenius Equations 2.3.36–2.3.38, k_{obs} is the observed rate constant; E_a is the activation energy, which usually ranges from 50 to 150 kJ mol^{-1} and is the energy barrier to be overcome if a reaction is to take place when reactants collide; R is the gas constant, i.e. 8.303 J K^{-1} mol^{-1}; A is the frequency factor indicating collision numbers per unit time per mole, which is assumed to be independent of temperature for any given reaction. T is the temperature given in K; 2.303 is a constant that relates the log scale to the ln scale. Thus, from Equation 2.3.38, it can be seen that a plot of log$k$ versus $1/T$ should be linear with a slope of $-E_a/2.303R$ and an intercept of log$A$. The Arrhenius plot for water-catalysed hydrolysis, termed 'spontaneous' hydrolysis is shown for acetyl salicylic acid (ASA) in Figure 2.3.3 and is cited from Garrett, 1957.

For many small molecular drug substances and candidates, an advanced form of the Arrhenius equation, Equation 2.3.39, is applied in order to estimate shelf life at room temperature from temperature-accelerated studies performed at temperatures that are typically between 40 °C and 80 °C. In the EMEA note for guidance on stability testing, long-term, intermediate and accelerated stability testing temperatures of 25 °C, 30 °C and 40 °C respectively are suggested (EMEA, 2006).

$$\log\frac{k_2}{k_1} = -\frac{E_a}{2.303R}\left[\frac{1}{T_1} - \frac{1}{T_2}\right] \tag{2.3.39}$$

Thus, by isolating k_1 from Equation 2.3.39, one can calculate its value by inserting k_2 values determined from accelerated conditions, using Equation 2.3.40.

$$\log k_2 = -\frac{E_a}{2.303R}\left[\frac{1}{T_1} - \frac{1}{T_2}\right] + \log k_1 \tag{2.3.40}$$

The Arrhenius equation holds true as long as no change in reaction mechanism is observed with temperature. However, a change in reaction mechanism may typically be observed by a reaction order change and/or

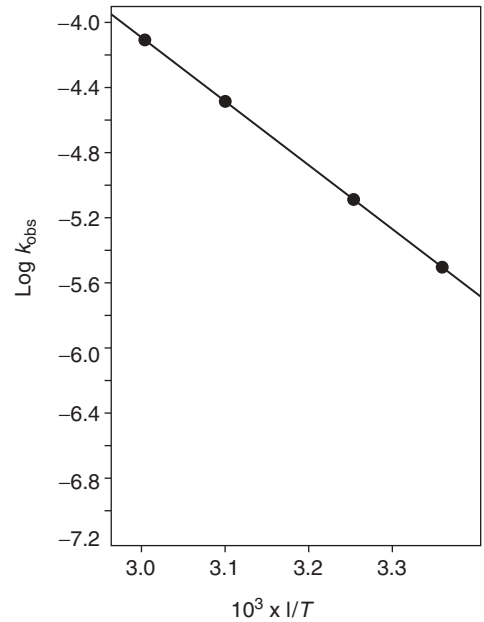


Figure 2.3.3 Arrhenius plot for so-called 'spontaneous' hydrolysis of acetyl salicylic acid (ASA). k_{obs} is given in s^{-1}. Reproduced from Garrett ER (1957). The kinetics of solvolysis of acyl esters of salicyclic acid. *J Am Chem Soc* 79: 3401–3408.

impurity changes with temperature. Examples of this are some macromolecular drug candidates such as proteins, in which the decomposition rate at room temperature may be, for example, a deamidation reaction as shown in Figure 2.2.7. However at temperatures such as 60 °C, for example, denaturation reactions such as a change in tertiary structure may take place. In such cases the decomposition rate may not be accurately described by the Arrhenius equation.

2.3.7 Influence of ionic strength on decomposition rate

Biological media contain electrolyte at a concentration of approximately 0.16M, and therefore there is considerable relevance in preclinical development in investigating the influence of ionic strength on hydrolysis. The same holds true for the pharmaceutical development of parenteral drugs as well as that of eye drops where such electrolytes such as NaCl are added in order to achieve an isotonic solution. The influence of electrolyte concentration on k_{obs} in dilute solutions, i.e. ionic strengths less than

0.01 mol kg^{-1}, may be described by the Brønsted–Bjerrum Equation 2.3.41:

$$\log k = \log k_0 + 1.036 A z_A z_B I^{1/2} \qquad (2.3.41)$$

For ionic strengths between 0.01 and 0.1 mol kg^{-1}, a modified form of the Brønsted–Bjerrum equation, Equation 2.3.42 may be applied:

$$\log k = \log k_0 + 1.036 A z_A z_B \left[\frac{I^{1/2}}{1 - I^{1/2}}\right] \qquad (2.3.42)$$

In the Brønsted–Bjerrum equation, z_A and z_B are the charges of the reacting molecules, I is the ionic strength of the solution and A is a constant dependent on the dielectric constant of the solution, i.e. 0.509 in water at 298 K. The influence of ionic strength (I) on the hydrolysis rate is often determined at varying KCl concentrations but constant pH, temperature and buffer concentration.

An example is the observed decrease in the rate of ester hydrolysis and thus a k_{obs} decrease from 2.17×10^{-3} to 0.11×10^{-3} min^{-1}, with I 0.05–0.5 M by KCl, of the zwitterionic species of the model prodrug Glu(OBzl)-Sar when investigated in 0.02 M phosphate buffer, pH 7.4 at 37°C (Lepist *et al.*, 2000).

It can be seen from Equation 2.3.42 that the influence of I on k_{obs} is charge dependent. Thus when I increases, k_{obs} for OH^--catalysed hydrolysis in molecules carrying a negative charge or positive charge may increase or decrease respectively. In the present case, k_{obs} decreases with I and consequently it is suggested that the amine positive charge in the zwitterionic species of Glu(OBzl)-Sar plays a larger role than that of the carboxylic negative charge. This point is illustrated by Figure 2.3.4 where negative Cl^- electrolytes are attracted by the protonised amine. This negative cloud of chloride ions inhibits the nucleophil attack by the

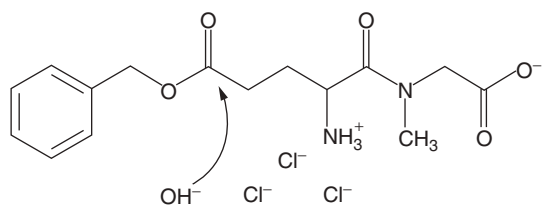


Figure 2.3.4 Nucleophil attack by specific base catalyst OH^- on the acyl carbon of Glu(OBzl)-Sar can be inhibited by Cl^-. Data from Lepist E-I, Kusk T, Larsen DH, *et al.* (2000). Stability and *in vitro* metabolism of dipeptide model prodrugs with affinity for the oligopeptide transporter. *Eur J Pharm Sci* 11: 43–50.

negative specific base catalyst OH^- on the acyl carbon of Glu(OBzl)-Sar and consequently decreases its hydrolysis rate (Lepist *et al.*, 2000).

2.3.8 Influence of buffers on decomposition rate

General acid/base catalysis may, for example, occur from buffers used to maintain constant pH within an aqueous solution. Buffers may be present in biological media, two examples being phosphate and ammonia, the major urinary buffers. The main buffer in blood is carbonate, while other buffer systems in biological media rely on phosphate and lactate. The influence of these general acids and bases on the hydrolysis rate is therefore of relevance for preclinical development where metabolism and elimination studies may be performed *in vitro*. Buffers are also used in pharmaceutical formulations, and their influence on the shelf life of a drug is an important and relevant point to study. Buffer catalysis may be described by buffer catalysis constants (k_{cat}), which may be calculated from slopes of k versus buffer concentration. Such studies are made at constant temperature, ionic strength and pH. The influence of buffers on k may be described mathematically using Equation 2.3.43.

Buffer catalysis may also be observed in pH–rate profile experiments. If that is the case, extrapolated y intercepts from buffer catalysis studies, i.e. k at zero buffer concentration (k_{zero}), should be depicted as k_{obs} values in the pH profile.

$$k = k_{zero} + k_{cat}[\text{buffer concentration}] \qquad (2.3.43)$$

Table 2.3.2 shows k_{cat} for various buffers that have an influence on the hydrolysis rate of the model prodrug Glu(OBzl)-Sar.

Based on the data presented in Table 2.3.2, it seems that OH^--catalysed ester hydrolysis of Glu(OBzl)-Sar, i.e. at pH >5, is especially sensitive to buffer catalysis. Of those buffers investigated, carbonate has the largest k_{cat}. However, from the present study it is not possible to elucidate which species of single buffers are catalysing the hydrolysis of Glu(OBzl)-Sar, since the buffers are applied at pH values in the region of pK_a values, and consequently at least two buffer species are present. Nevertheless, one might postulate that the k_{cat} for PO_4^{3-} species is larger than the k_{cat} for HPO_4^{2-} species since the k_{cat} for a total phosphate buffer increases with pH as does PO_4^{3-}, while HPO_4^{2-} decreases. Moreover, one might propose that the k_{cat} for HCO_3^- is larger than that for the k_{cat} of CO_3^{2-}, since the k_{cat} for total carbonate decreases with pH while CO_3^{2-} increases and HCO_3^- decreases.

Table 2.3.2 Buffer catalyst constants for a number of buffers inducing catalysis of Glu(OBzl)-Sar hydrolysis in an aqueous solution at constant pH investigated at $I = 0.5$ and at 37 °C; data from Lepist E-I, Kusk T, Larsen DH, *et al.* (2000). Stability and *in vitro* metabolism of dipeptide model prodrugs with affinity for the oligopeptide transporter. *Eur J Pharm Sci* 11: 43–50

Buffer	*pH*	k_{cat} $(M^{-1} min^{-1})$
Phosphate	2.0	7.01×10^{-4}
Phosphate	3.0	5.47×10^{-4}
Acetate	4.0	8.67×10^{-5}
Acetate	5.0	6.89×10^{-5}
Phosphate	6.0	1.30×10^{-3}
Phosphate	7.4	2.61×10^{-2}
Phosphate	8.0	8.25×10^{-2}
Carbonate	9.0	0.49
Carbonate	9.5	0.42

Even though buffers are present in most biological media, and for that matter tissues, the overwhelming reason for drug metabolism remains enzyme-catalysed degradation, which will be briefly described in the following Section 2.3.9.

2.3.9 Influence of enzymes on decomposition rate

The release of drug substances from prodrugs as well as the metabolism of drug substances may be catalysed by acids or bases, or indeed be spontaneous, i.e. water catalysed. However, abundant *in vivo* catalysts are also digestive and metabolising enzymes. The kinetics of enzyme-catalysed reactions are characterised by the saturable Michaelis–Menten kinetics which can be described mathematically by Equation 3.3.3. Since enzyme-catalysed degradation of (pro)drug substances is described by saturable kinetics, it may be applied to evaluate possible drug–drug, food–drug and excipient–drug interaction arising from these compounds being substrates for specific enzymes. Examples of identified intestinal pancreatic, brush border or cytosolic peptidases involved in the metabolism of peptide drug candidates, as well as the release of drug substances from some amide and ester prodrugs are given in Table 2.4.3, and the importance of metabolism investigation during discovery and development is discussed in Chapter 4.2.

In preclinical development and molecular biopharmaceutics, the rate of *in vitro* metabolism of drug candidates may be investigated by

determining the half life of first- or zero-order candidate metabolism in various dilute tissue homogenates such as hepatic, nephritic and intestinal preparations, or in biological fluids such as gastric, intestinal, urine, blood plasma, lacrymal or cerebrospinal fluid. However, by using tissue homogenates and biological fluids one is faced with the issue of ethics and the disadvantages connected with this field. For this reason, *in vitro* metabolism may be evaluated in suspensions of various cell culture homogenates. In some cases, membrane transporters such as P-glycoprotein (P-gp) are believed to deliver drug substance substrates to sites of metabolising enzymes such as CYP450s, which may be elegantly studied in whole tissues. There may, however, also be experimental disadvantages in using whole tissues, tissue homogenates, biological fluids or suspensions of cell culture homogenates, for instance if more than one enzyme is involved in the metabolic processes under investigation. Although *in vitro–in vivo* correlation may be performed on metabolic half lives, there are weaknesses associated with such studies, for instance a lack of Michaelis–Menten parameter characterisation, i.e. V_{max} and K_m, and thus compromised understanding of those enzymes involved. Moreover, if biological materials arising from experimental animals, especially different species, are used, one needs to address the issue of variation between experimental animal and human enzymes which may compromise any *in vitro–in vivo* correlations.

An additional experimental problem is that enzymes are easily deactivated during tissue preparation and it is therefore important to characterise enzyme activity of known substrates by Michaelis–Menten parameters. Enzyme-catalysed *in vitro* metabolism may also be characterised in artificial gastric, pancreatic or intestinal fluids as suggested in the *US Pharmacopoeia*. Thus, specific drug candidate–enzyme interaction kinetics should be investigated in solutions of known specific enzymes and characterised by the Michaelis–Menten parameters, i.e. K_m and V_{max}, whereas the overall rate of *in vitro* metabolism may be studied in various specific tissue or cell homogenates as well as in specific biological or artificial fluids. The American FDA has released *Guidance for Industry, Drug Metabolism/Drug Interaction Studies in the Drug Development Process* (FDA, 1997).

2.3.10 Conclusions

The kinetics of stability and *in vitro* metabolism of drug candidates, as well as the *in vitro* release of drug candidates from prodrugs, is often characterised in the molecular biopharmaceutical and formulation

sciences, as well as during industrial pharmaceutical and preclinical development. During pharmaceutical development and in the formulation sciences the kinetics of (pro)drug candidate stability may be characterised, mainly in order to determine parameters of impurity and shelf life. During preclinical development and in the biopharmaceutical sciences, *in vitro* metabolism of drug candidates and *in vitro* release of drug candidates from prodrugs may be characterised in order to evaluate and elucidate the influence of possible catalysts, such as acids, bases and enzymes, on ADME and on pharmacokinetic parameters.

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Thomsen AE, Friedrichsen GM, Sørensen AH, *et al.* (2003a). Prodrugs of purine and pyrimidine analogues for the intestinal di/tripeptide transporter PEPT1: affinity for hPEPT1 in Caco 2 cells drug release in aqueous media and in vitro metabolism. *J Control Release* 86: 279–292.

Thomsen AE, Friedrichsen GM, Sørensen AH, *et al.* (2003b). Erratum to Prodrugs of purine and pyrimidine analogues for the intestinal di/tripeptide transporter PEPT1: affinity for hPEPT1 in Caco 2 cells drug release in aqueous media and in vitro metabolism. *J Control Release* 88: 343.

2.4

Chemical approaches to improving bioavailability properties of oral drug candidates

Kristina Luthman and Bente Steffansen

Potential oral drug candidates are generally identified in high-throughput screening campaigns focused on their *in vitro* pharmacological activity. However, the importance of bioavailability properties is also appreciated and therefore usually included in the selection process, for example based on parameters described by 'Lipinski's rule of five' (Lipinski *et al.*, 2001). Nevertheless, there is still a great need for optimising bioavailability properties of drug candidates not only to include the Lipinski parameters but also to take into account biological factors such as substrate properties directed towards membrane transporters and metabolising enzymes as suggested by the FDA (US Department of Health and Human Services, FDA, Center for Drug Evaluation and Research (CDER) and Center for Biologics Evaluation and Research (CBER), 2006); this is also discussed in Parts 3 and 4. In this optimisation process, chemical approaches such as salt formation, bioisosteric replacement, and prodrug formation may be applied. The aim of this chapter is to describe and exemplify such chemical approaches used to improve the bioavailability properties of drug candidates.

2.4.1 Lipinski's rule of five

Lipinski's rule of five is based on an analysis of key physicochemical properties of drug substances in the World Drug Index (http://www.daylight.com/dayhtml/doc/theory/index.html).

The rule is based on four physicochemical parameters that are globally associated with solubility and permeability, and it states that drug substances are most likely to have good bioavailability when the following four parameters are fulfilled: the number of hydrogen bond (H-bond) donors < 5, the number of H-bond acceptors < 10, the molecular weight (MW) is < 500, and the logarithm of the calculated octanol/water

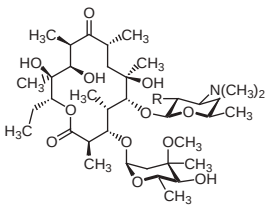
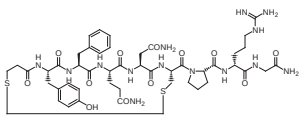
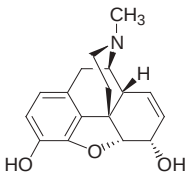
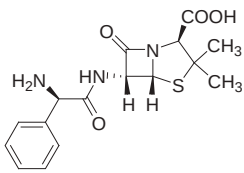
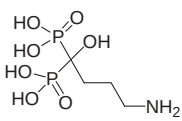
partition coefficient, log$P < 5$, (Lipinski *et al.*, 2001). Since the cut-off values for each of the four parameters were five or a multiple of five, the rule was simply named the 'rule of five'. Hydrogen bond acceptors and donors in the rule of five refer to hydrogen bonds formed between a hydrogen atom covalently bound to an electronegative atom such as N or O, and another electronegative atom, normally N or O. The hydrogen atom that is covalently bound to e.g. N or O is called the *hydrogen bond donor* and the electronegative atom (N or O) receiving the hydrogen bond is called the *hydrogen bond acceptor*. Log*P* in the rule of five refers to the logarithm of the partition coefficient *P* of the un-ionised species as described in Section 2.1.3. Hydrogen-bond donors and acceptors as well as log*P* for some selected drug substances are shown in Table 2.4.1.

Lipinski's rule of five is thus a simple and inexpensive tool to apply in the industrial 'early drug discovery phase', where it is the rule of thumb for medicinal chemists. All drug substances shown in Table 2.4.1, except for ivermectin, are registered as oral drug formulations. As seen from the table, several of these drug substances do not fulfil all Lipinski's rules; however, the rule states that if two or more parameters are not within the limits the compound is expected to show poor absorption properties. To improve the predictability using the rule of five, Veber *et al.* have suggested additional parameters to be included such as polar surface area (PSA) (<140 Å^2), the sum of H-bond donors and acceptors (< 12) and the number of rotatable bonds (< 10) (Veber *et al.*, 2002). However, other important physicochemical parameters such as the degree of ionisation in relevant biological media (pK_a values) are not considered in the rule of five. The biopharmaceutical and preclinical scientists will also identify a number of apparent limitations to the rule, which include biological properties such as substrate properties towards metabolising enzymes and membrane transporters. These properties are not taken into consideration in the rule of five even though, as described in Parts 3 and 4, they may have a major influence on the bioavailability. Furthermore, the rule, which is based on physicochemical parameters that are globally associated with solubility, does not discriminate between solubility and dissolution. Thus it should not be used uncritically in the selection of oral drug candidates.

2.4.2 Salt formation

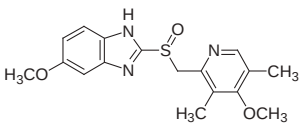
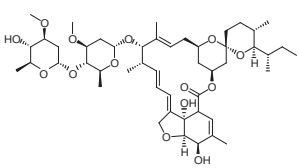
For some drug candidates, it is not the solubility but the dissolution, i.e. the solubility rate (see Chapter 4.1), that is limiting for bioavailability. Dissolution properties may sometimes be improved by salt formation simply by the stronger tendency of salts to dissociate in water. If the acid

Table 2.4.1 Oral bioavailability (OB) (%) of selected drug substances according to the Danish Medicine Catalogue (www.medicin.dk)

Drug/prodrug molecule	*OB (%)*	*MW*	*H-bond acceptors*	*H-bond donors*	*logP* *$logD_{7.4}$
	Varies	714.92 (814.96 prodrug)	14 (16 prodrug)	5 (4 prodrug)	*1.98
R = OH: erythromycin R = $OCOCH_2CH_2COOH$: succinate ester prodrug					
	0.1	1041.2	26	17	−3.72
Desmopressin					
	20–60	285.7	4	1	0.90
Morphine					
	40	317.34	7	3	−2.00
Ampicillin					
	0.7	313.43	8	7	−2.77
Alendronate					

(*continued*)

Table 2.4.1 (*Continued*)

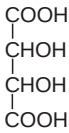
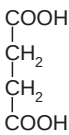
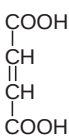
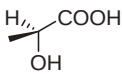
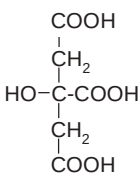
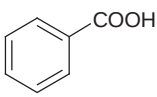
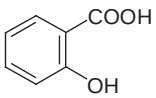
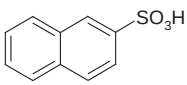
Drug/prodrug molecule	*OB (%)*	*MW*	*H-bond acceptors*	*H-bond donors*	*logP* **logD$_{7.4}$*
 Omeprazole	20–40	313.42	6	1	*2.38
 Ivermectin	Not available	875.1	14	3	6.70

Notes: MW, molecular weight; H-bond acceptors, hydrogen bond acceptors; H-bond donors, hydrogen bond donors; log*P*, logarithm of the distribution of the neutral drug substance between octanol and water; log$D_{7.4}$ logarithm of the observed distribution of the drug substance between octanol and water at pH7.4.

or base used to form the salt is strong, the salt can be expected to completely dissociate in aqueous solution and thus increase the dissolution. Drug candidates that are acids, such as carboxylic acids, may often be crystallised as, for example, sodium (pK_a 16.0 for sodium) or potassium (pK_a 14.8) salts. Sodium hydroxide and potassium hydroxide are strong bases, and their respective cations Na^+ and K^+ are thus Lewis acids (see Section 2.1.1 and Table 2.2.2). They will normally form salts with drug candidates that completely dissociate in aqueous solution. The tendency to dissociate depends in part on the strength of the Lewis acid, i.e. dissociation decreases with decreasing pK_a. Thus for salts of the same drug candidate, the tendency to dissociate is larger for its lithium salt (pK_a 13.8) than for its calcium salt (pK_a 12.9) > magnesium salt (pK_a 11.4) > diethanolamine salt (pK_a 9.7)> zinc salt (pK_a 9.0) > choline salt (pK_a 8.9) > aluminium salt (pK_a 5.0).

Furthermore, salt formation may affect the micro-pH within and close to the formulation, controlled by the strong salt-forming acid or base which, independently of pH in the gastrointestinal juice or dissolution medium, may increase the apparent dissolution. Table 2.4.2 shows the chemical structures of the acids most frequently used in salt formation of basic drug candidates.

Table 2.4.2 Acids used in salt formation of drug substances that are bases

Acid structure used for salt formation	*Name of acid and pK_a*	*Salt formed*
	Tartaric acid, pK_a 3.00	Tartrate
	Succinic acid, pK_{aI} 4.2, pK_{aII} 5.6	Succinate
	Maleic acid, pK_{aI} 1.92, pK_{aII} 6.23	Maleate
HCl	Hydrochloric acid, pK_a –6.10	Hydrochloride
H_3PO_4	Phosphoric acid, pK_{aI} 2.15, pK_{aII} 7.20, pK_{aIII} 12.38	Phosphate
H_2SO_4	Sulphuric acid, pK_{aI} –3.00, pK_{aII} 1.96	Sulphate
	Lactic acid, pK_a 3.10	Lactate
	Citric acid, pK_{aI} 3.13, pK_{aII} 4.76, pK_{aIII} 6.40	Citrate
	Benzoic acid, pK_a 4.20	Benzoate
	Salicylic acid, pK_a 3.00	Salicylate
CH_3SO_3H	Methanesulphonic acid (mesylic acid), pK_a –1.20	Mesylate
	Naphthalenesulphonic acid (napsylic acid), pK_a 0.17	Napsylate
CH_3COOH	Acetic acid, pK_a 4.76	Acetate

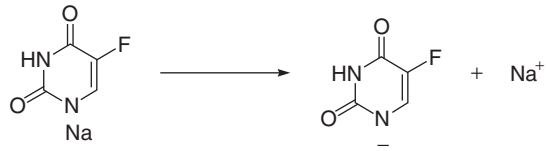


Figure 2.4.1 Dissociation of the sodium salt of 5-fluorouracil in aqueous solution.

When salts of drug candidates become too soluble and hygroscopic, this can lead to instability of both the solid formulation and the drug candidate, as some of the substance will dissolve in its own absorbed moisture. This may not only disintegrate the tablet or capsule formulation during storage but also initiate hydrolytic degradation of the drug substance within the formulation (see Section 2.2.1). In this case a weaker salt-forming acid or base should be applied.

Salts of drug substances may also be used in liquid formulations when it is necessary to keep the substance in solution but desirable to avoid buffer excipients that are not suitable in injection liquids if the pH will deviate too much from the physiological pH of 7.4. An example is the iv (intravenous) aqueous injection solution of the anticancer drug 5-fluorouracil (5-FU). The dissociation scheme of the sodium salt of the NH acidic 5-FU in aqueous solution is shown in Figure 2.4.1.

The aqueous solubility of 5-FU (pK_a 8.0) is approximately 11 mg ml^{-1} (neutral species), whereas the sodium salt of 5-FU forms aqueous solutions in concentrations above 50 mg ml^{-1} at pH 9. At this pH the salt dissociates into the negatively charged species of 5-FU and thereby increases its solubility. The 50 mg ml^{-1} concentration is more convenient to use than the 11 mg/ml solutions, due to the smaller injection volumes needed. Although pH 9 deviates considerably from pH 7.4 in the blood (see Table 2.1.2), it is necessary to keep 5-FU in solution when administered iv; therefore the sodium salt of 5-FU is applied in formulations used for iv injection. Drug solubility can also be enhanced using prodrug strategies (see Section 2.4.4) or for solvation-limited 'grease ball' candidates (see Section 2.1.4) by using solubilisers as ingredients in the pharmaceutical formulation.

2.4.3 Bioisosteric replacement

Stabilisation of metabolically sensitive functional groups such as esters or amides can be obtained by introducing bioisosteric replacements (Patani and LaVoie, 1996; Chen and Wang, 2003). Bioisosteric moieties should be designed to show similar size, shape, electron distribution, lipophilicity/

hydrophilicity, pK_a, and hydrogen-bonding capacity as the group being replaced. However, they do not necessarily overlap atom upon atom, but show similar biological properties.

This section will focus on the stabilisation of peptides against hydrolytic cleavage by protease enzymes. The sensitive parts of the peptides are the amide bonds (peptide bonds) that are connecting amino acids. Peptides are endogenous compounds involved in many important physiological processes, and as peptides have been associated with certain disease states, peptide receptors and processing enzymes have become interesting pharmacological targets for drug-discovery efforts. Peptides are often found to be less useful as drugs in themselves due to their low oral bioavailability. This has mainly been associated with their inefficient penetration of the intestinal membrane, and their rapid degradation by endogenous peptidases both in the gastrointestinal tract and in blood. Intestinal peptidases are summarised in Table 2.4.3.

Although extensive efforts have been put into the areas of formulation and delivery of peptides, it is generally considered that oral delivery of peptides still represents a significant challenge. One example of an orally administered peptide drug is ciclosporin (Sandimmun®), a cyclic undecapeptide used as an immunosuppressant. It is administered as oral solutions or soft capsules. In these formulations ciclosporin is formulated with oils such as ricinus and maize oil together with ethanol, propylenglycol and DL-α-tocopherol. Alternatively, ciclosporin is formulated as an infusion concentrate in which it is dissolved in oils. Ciclosporin is a substrate to the efflux transporter P-glycoprotein (P-gp) (see Chapter 3.6) and may also be a substrate for other transporters as well as for the metabolising cytochrome P-450 enzymes. Consequently, drug–drug interactions between ciclosporin and a wide range of drug substances that interact with P-gp and or P-450 are observed, which may partly explain its highly variable bioavailability. Ciclosporin contains D-amino acids which, together with the cyclic structure, stabilise the compound against protease degradation.

Another interesting example of an orally administered peptide drug is desmopressin (see Table 2.4.1), a synthetic replacement for antidiuretic hormone prescribed for diabetes insipidus. Although it only shows 0.1% oral bioavailability, it can be administered as tablets, and the extreme potency of desmopressin makes even very low plasma concentrations highly efficacious. Desmopressin is stabilised against metabolism by several important structural features including an N-terminal deamination and a C-terminal amidation, as well as the presence of a D-amino acid and an intramolecular disulphide bond.

Table 2.4.3 Enzyme types, enzyme commission number, common name and preferred cleavage site, for human intestinal pancreatic, brush border or cytosolic peptidases. Data cited from Bai *et al.* 1992; Woodley 1994; Andersen 2006; Steffansen *et al.*, 2005; http://www.ebi.ac.uk/thornton-srv/databases/enzymes/; http://www.ebi.ac.uk/intenz/index.jsp; http://www.expasy.org/; http://www.genome.ad.jp/; http://www.brenda-enzymes.info/

Type of peptidases	*Enzyme Commission Number and common name*	*Preferential cleavage site*
Pancreas endopeptidase	EC 3.4.21.4, trypsin	Arg-Xaa, Lys-Xaa
Pancreas endopeptidase	EC 3.4.21.1, chymotrypsin	Tyr-Xaa, Trp-Xaa, Phe-Xaa, Leu-Xaa, Met-Xaa
Pancreas endopeptidase	EC 4.21.36, elastase	Ala-Xaa
Pancreas endopeptidase	EC 3.4.21.70, protease E	Ala-Xaa
Pancreas exopeptidase	EC 3.4.17.2, carboxypeptidase A	Release of Val Leu Ile Phe Tyr Trp C-terminal amino acid
Pancreas exopeptidase	EC 3.4.17.2, carboxypeptidase B	Release of Lys Arg C-terminal amino acid
Brush border exopeptidase	EC 3.4.11.7, aminopeptidase A or glutamyl aminopeptidase	Release of Glu Asp N-terminal amino acid
Brush border exopeptidase	EC 3.4.11.2, aminopeptidase N or alanyl aminopeptidase	Release of N-terminal amino acid
Brush border exopeptidase	EC 3.4.11.9, aminopeptidase P, Pro-X aminopeptidase	Release of Pro N-terminal amino acid
Brush border exopeptidase	EC 3.4.11.16, aminopeptidase W, Trp-X aminopeptidase	Release of Trp Tyr Phe N-terminal amino acid
Brush border endopeptidase	EC 3.4.21.9, enteropeptidase	Activates trypsinogen by selective cleavage of Leu^{6}-Ile^{7} bond
Brush border endopeptidase	EC 3.24.11, neprilosin, endopeptidase 24.11	Between hydrophobicamino acid
Brush border endopeptidase	EC 3.4.24.18, endopeptidase 2, meprin A	Not described
Brush border oligopeptidase	EC 3.4.15.1, angiotensin-converting enzyme, peptidyl-dipeptidase A	Release of C-terminal dipeptide
Brush border oligopeptidase	EC 3.4.14.5, dipeptidyl peptidase IV	Release of N-terminal dipeptide
Cytosolic peptidase	EC 3.4.13.3, carnosinase, X-His peptidase	Xaa-His
Cytosolic peptidase	EC 3.4.13.18, cytosolic non-specific peptidase	Preferentially hydrophobic dipeptides

Another way to improve the plasma half life and decrease the protein binding and renal clearance of peptide drugs has been the formation of macromolecular polyethylene glycol (PEG) conjugates of peptides (Roberts *et al.*, 2002; Veronese and Pasut, 2005).

One strategy to improve the oral bioavailability of peptide-based drug candidates is to introduce structural modifications that lead to decreased metabolism, or increased absorption, i.e. increased intestinal permeability. Backbone-modified peptides, often called pseudopeptides, in which sensitive amide bonds have been replaced with bioisosteric moieties, should be designed to be metabolically stable but also have to retain their pharmacological activity

2.4.3.1 Peptidomimetic strategies

Peptide backbone mimetics belong to the so-called type I peptidomimetics which historically were the first peptidomimetics (Ripka and Rich, 1998). This group includes amide bond isosteres and mimetics of secondary structures. Replacement of peptide bonds with an isosteric moiety often leads to pseudopeptides possessing increased stability towards metabolic enzyme-catalysed hydrolysis (M), but also other ADME properties in addition to metabolism may be affected by pseudopeptidic strategies

Important considerations in the selection of an amide bond replacement have been its ability to exhibit geometrical, conformational, electrostatic and hydrogen-bonding properties that are similar to the amide bond itself. An amide bond replacement can be introduced into a peptide by synthesis of a dipeptide analogue of the general structure Xxx-Ψ[A-B]-Yyy. Next, some examples of important bioisosteric amide-bond replacements and their use in bioactive peptides will be discussed.

2.4.3.2 Amide-based bioisosteres

The synthetically most accessible means to stabilise a peptide against enzyme-catalysed degradation is probably the exchange of L-amino acids for D-amino acids or to introduce N-methylations, i.e. methyl groups on the amide nitrogen atom (see Figure 2.4.2) (Hodgson and Sanderson, 2004). However, such modifications may induce severe conformational constraints and thereby decrease the recognition and binding properties of the pseudopeptide in relation to its pharmacological target.

The retro-inverso peptides xxx-Ψ[NHCO]-yyy (see Figure 2.4.2) (Goodman and Chorev, 1979; Chorev and Goodman, 1993) not only

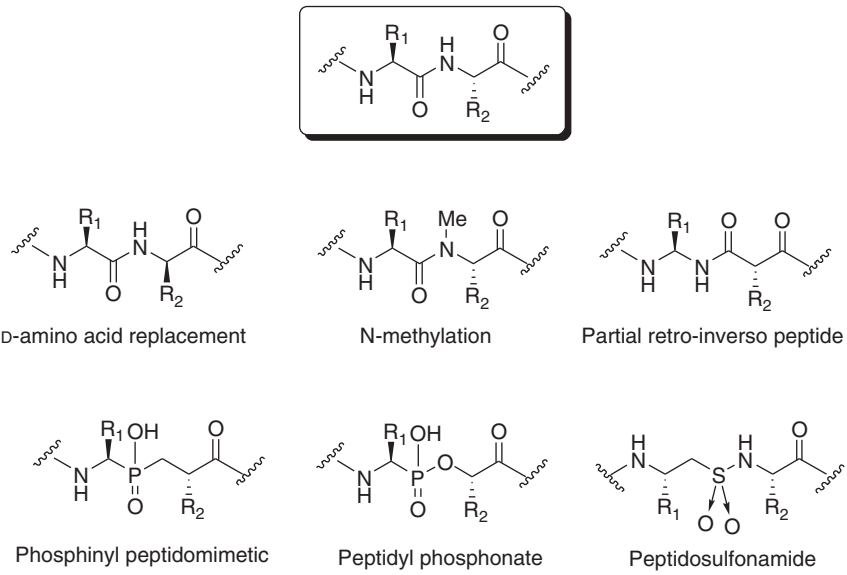


Figure 2.4.2 Structure of some important amide bond isosteres.

reverse the direction of the peptide bonds but also have the L-amino acids exchanged for D-amino acids. Incorporation of a retro-inverse isostere results in a pseudopeptide with a similar topology to the native peptide; however, it is no longer sensitive to peptidase degradation.

A recent example of the use of a small retro-inverso peptide, to improve its ADME properties, is the Tat antagonist R.I.CK-Tat9 (N-acetyl-ckrrrqrrkkrNH_2), which has been used either alone or in combinations with, for example, saquinavir, in *in vitro* studies for anti-HIV activities. The compounds were used as multifunctional PEG-based bioconjugates, and their increased anti-HIV activity was suggested to emanate from an enhanced intracellular uptake and a synergistic inhibitory effect of saquinavir and the Tat peptide (Wan *et al.*, 2006). The strongly basic Tat peptide sequence is known to have cell-penetrating properties and has been used as a vehicle for transport of cargo molecules across plasma membranes.

Replacements of amide bonds by bioisosteric groups mimicking the transition state (TS) of the hydrolysis of the amide has been widely used in the development of protease inhibitors. Examples of possible TS mimetics are the phosphine xxx-Ψ[PO(OH)]-yyy, phosphonate xxx-(Ψ[PO_2(OH)])-yyy and sulfonamide xxx-Ψ[SO_2NH]-yyy, shown in Figure 2.4.2, and the dihydroxyethylene and hydroxyethylene isosteres shown in Figure 2.4.3. Pseudopeptides containing phosphinyl-based TS mimetics have found

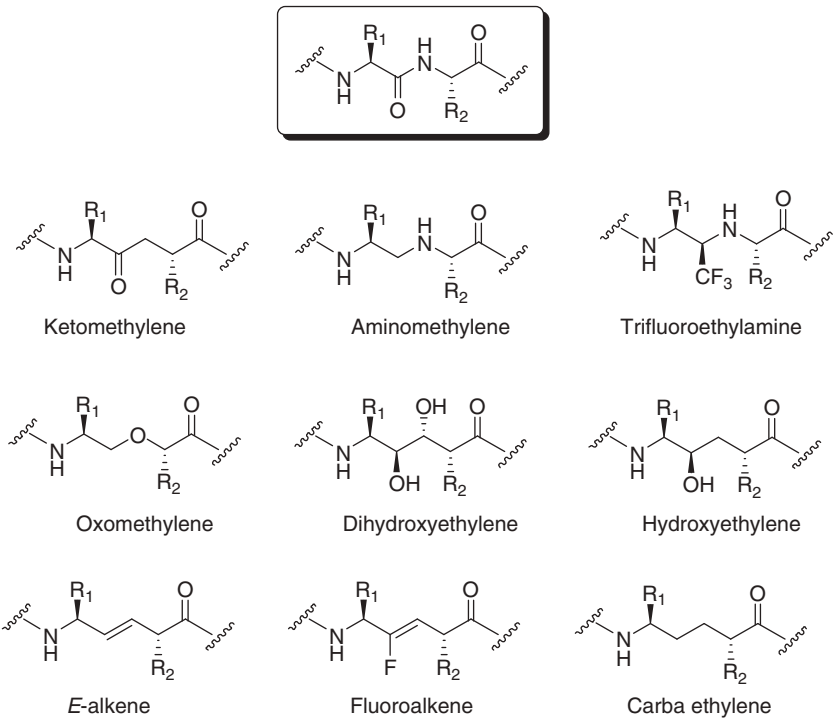


Figure 2.4.3 Amide bond bioisosteres.

applications as stable zinc, serine and aspartate protease inhibitors. The corresponding phosphonates have also shown potent inhibitory activity toward aspartate proteases. Hydroxyethylene moieties are incorporated in all marketed HIV-protease inhibitors, e.g. indinavir, to mimic the TS for the hydrolysis reaction.

Peptidosulfonamide analogues of Leu-enkephalin and TAP have been shown to display improved stability towards pepsin and trypsin degradation (De Bont *et al*., 1999). Another important property of this type of bioisostere is the increased acidity of the sulfonamide N-H compared to the amide. This will lead to stronger hydrogen-bonding interactions as have been shown for some oligopeptidosulfonamide 'tweezer' molecules used as synthetic receptors (Loewik *et al*., 1996).

Some other popular peptide bond surrogates, which have found application in the development of metabolically stable pseudopeptides, are shown in Figure 2.4.3.

The inherent properties of the amide bond have been studied using mimetics in which either the NH or the carbonyl moieties have been removed. The ketomethylene isostere xxx-(Ψ[$COCH_2$])-yyy, which is

not recognised by peptidases and is consequently metabolically stable, has been shown to possess interesting substrate properties towards the absorptive peptide transporter PEPT1. Absorptive transporters and their importance for drug delivery are further described in Chapter 3.5. The hydrogen-bonding interaction of ketomethylene isosteres with PEPT1 is due to the presence of the carbonyl group in the isosteric moiety. For example, PheΨ[$COCH_2$]Gly, PheΨ[$COCH_2$]Asp, ValΨ[$COCH_2$]Gly and ValΨ[$COCH_2$]Asp mimetics have been shown to be excellent substrates for PEPT1 and consequently suggested as pro-moieties for low-permeability drug candidates (Vå benø *et al.*, 2004a,b). Such a prodrug delivery approach is further described below in Section 2.4.4. A hydrogen-bonding interaction with PEPT1 via the carbonyl group in the original peptide is believed to be crucial, and in the ketomethylene isosteres that interaction is still intact. In these mimetics, the increased conformational flexibility of the ketomethylene moiety is compatible with PEPT1 interaction.

The aminomethylene mimetic xxx-(Ψ[CH_2NH]-yyy is a reduced amide in which the increased basicity of the secondary amine will confer dramatic changes in electrostatic properties compared to the parent amide. In some studies an increased cell permeability of pseudopeptides has been shown, in addition to increased stability toward hydrolytic enzymes; however, in other studies the cell permeability has been considerably decreased. Thus, to improve the amide bond-mimicking properties of the aminomethylene isostere, the basicity of the secondary amine has to be decreased. Introducing a trifluoromethyl group in the α-position to the amine (xxx-Ψ[$CH(CF_3)NH$]-yyy) has been shown to more accurately mimic the hydrogen-bonding properties of the amide (Sani *et al.*, 2007). This replacement has been successfully used in highly pharmacologically potent and selective cathepsin K inhibitors. According to X-ray crystallographic studies of complexes between the pseudopeptides and the pharmacological target (cathepsin K), an important hydrogen bond in the complex is intact (Black *et al.*, 2005).

The *E*-alkene (xxx-Ψ[CH=CH]-yyy) is a well-known bioisosteric unit; it has an almost identical geometry to the amide bond (see Figure 2.4.4). It was first used by Hann *et al.* as an amide bond replacement in Leu-enkephalin, where it was shown that the *E*-alkene was tolerated as a Tyr-Gly but not a Gly-Gly replacement (Hann *et al.*, 1980, 1982).

One important consideration related to the use of *E*-alkene isosteres is that any backbone hydrogen-bonding interaction will be disrupted. This feature has recently been used advantageously when incorporated into the Aβ Alzheimer amyloid peptide. Replacing only one out of 39

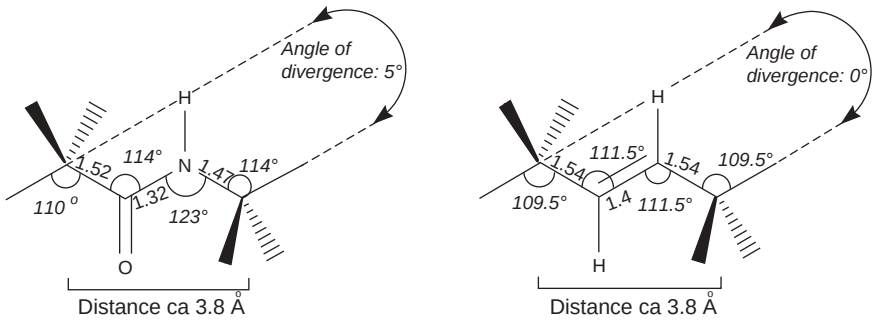


Figure 2.4.4 Bond lengths and bond angles of amides and *E*-alkenes are almost the same. Adapted from Hann MM, Sammes PG, Kennewell PD, Taylor JB (1980). On double bond isosteres of the peptide bond; an enkephalin analog. *J Chem Soc Chem Commun* 234–235.

amide bonds by an *E*-alkene mimetic prevented the transformation of Aβ into amyloid fibrils (Fu *et al.*, 2005).

Using the *E*-alkene isostere as starting point, other dipeptidomimetics can be designed and synthesised which show improved electrostatic and/or hydrogen-bonding properties. These new mimetics can also be designed to introduce different lipophilic or stereochemical effects in comparison with the parent structure. Such changes might be advantageous for transport, receptor binding, or membrane interaction. One such example is the more electrostatically favourable fluoroalkene mimetic (xxx-Ψ[CF=CH]-yyy), which has been used to study conformational preferences of peptides and also for studies of ligand/receptor interactions. The first example of the use of fluoro-olefin peptide isosteres was the introduction of Phe(Ψ[CF=CH])Gly in substance P (Allmendinger *et al.*, 1990). This compound showed almost the same activity as substance P itself, but exhibited increased chemical stability and receptor-binding affinity compared to the *E*-alkene analogue. In a recent study, *Z*- and *E*-fluoroalkene dipeptide mimetics were used to investigate the conformational *cis*- and *trans*-amide bond preferences for oligopeptide transport via PEPT1. It was shown that the *E*-fluoroalkene and *E*-alkene isosteres possessed higher affinities for PEPT1 compared to the *Z*-isomers (Niida *et al.*, 2006).

The aim of bioisosteric replacement of peptide bonds is to stabilise peptidic drug candidates against metabolism and to increase permeability without compromising its pharmacological activity. This is, as described, a quite complex strategy that needs to be developed for each specific case. Another strategy for improving the bioavailability of drug candidates is the prodrug approach, which will be briefly described in the following section.

2.4.4 Prodrug formation

Low solubility and low permeability properties of drug candidates can sometimes be circumvented by prodrug formation. In Table 2.4.4 examples of European-registered prodrugs and their corresponding parent drug substances are shown.

One prodrug strategy that is often applied to improve the solubility of drug candidates is to introduce a pro-moiety containing phosphate, sulphate or succinate groups, which can ionise in relevant biological media. An example is the succinate prodrug of chloramphenicol for iv administration (see Table 2.4.4). A carboxylic acid group in the succinate pro-moiety, with a pK_a value far below physiological pH, will be ionised at pH 7.4 and thereby increase the aqueous solubility of chloramphenicol-succinate ester compared to the parent chloramphenicol. Chloramphenicol itself is an alcohol with a pK_a value far above pH 7.4, and consequently it is present as neutral species, with lower aqueous solubility, than the corresponding ionised succinate-ester prodrug.

Table 2.4.4 Examples of European registered prodrugs and parent drug substances, taken from Steffansen B, Thomsen AE, Frokjaer S (2003) Prodrugs. In: van de Waterbeemd H, Lennernäs H, Artursson P, eds. *Methods and Principles in Medicinal Chemistry, Estimation of Permeability, and Oral Absorption*. Weinheim: Wiley VCH, 532–546

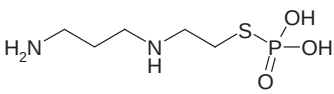
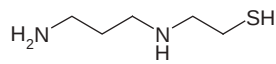
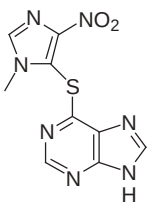
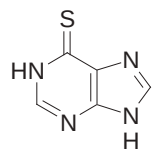
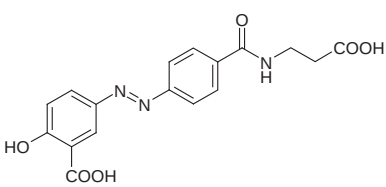
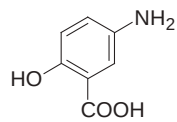
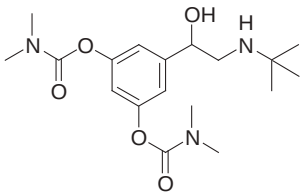
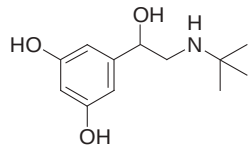
Prodrug	*Drug*
Amifostine iv administration 	Dephosphorylated aminofostine 
Azathioprine 	6-Mercaptopurine 
Balsalazide 	Mesalazine 

Table 2.4.4 (*Continued*)

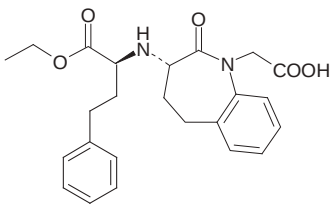
Bambuterol



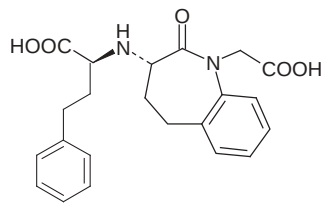
Terbutaline



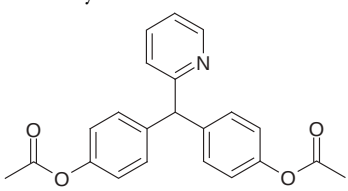
Benazepril



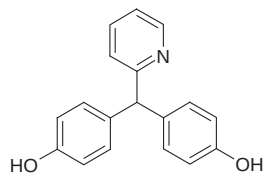
Benazeprilat



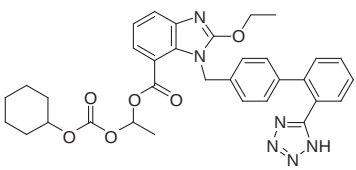
Bisacodyl



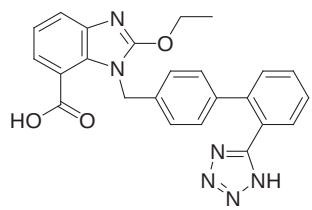
bis-(p-Hydroxyphenyl)-pyridyl-2-methane (BHPH)



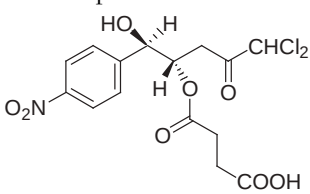
Candesartan cilexetil



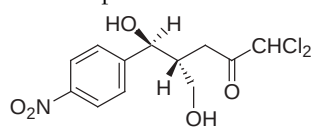
Candesartan



Chloramphenicol succinate



Chloramphenicol



(*continued*)

Table 2.4.4 (*Continued*)

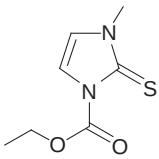
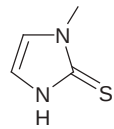
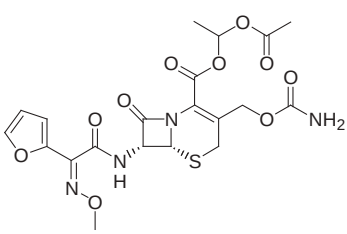
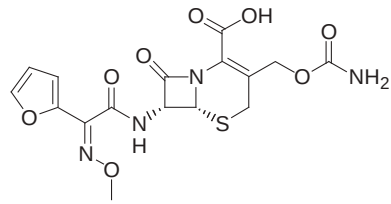
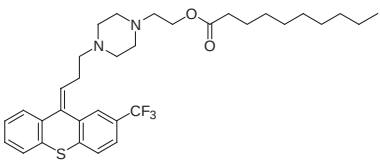
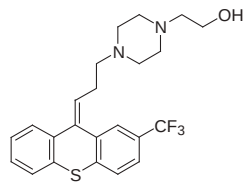
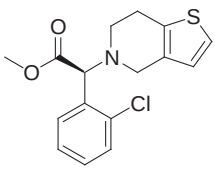
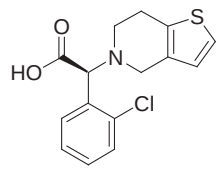
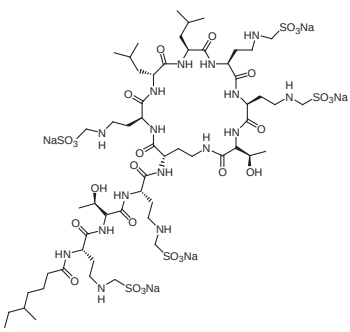
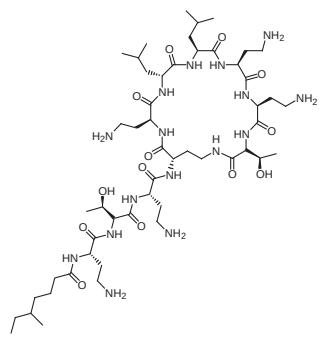
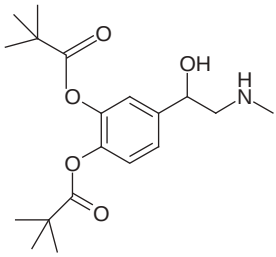
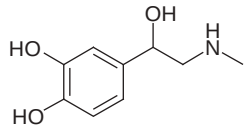
Prodrug	*Drug*
Carbimazole 	Methimazole 
Cefuroxime axetil 	Cefuroxime 
(*Z*)-flupentixol decanoate 	(*Z*)-flupentixol 
Clopidogrel 	The corresponding acid 
Colistimethate 	Colistin (polymyxin E) 

Table 2.4.4 (*Continued*)

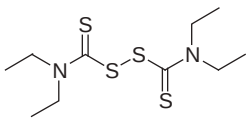
Dipivefrine



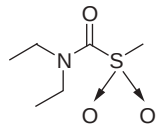
Epinephrine (adrenaline)



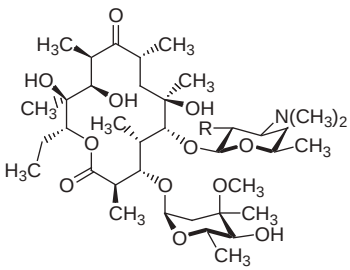
Disulfiram



S-Methyl-*N*,*N*-diethylthiocarbamate sulfone

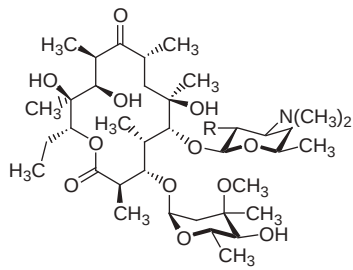


Erythromycin ethylsuccinate



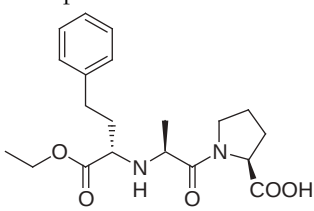
R=$OCOCH_2CH_2COOEt$

Erythromycin

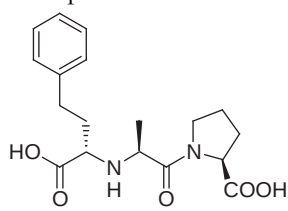


R=OH

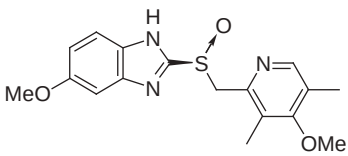
Enalapril



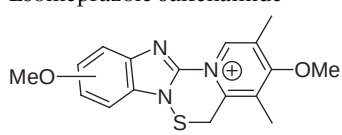
Enalaprilat



Esomeprazole



Esomeprazole sulfenamide



S-enantiomer of omeprazole

(*continued*)

Table 2.4.4 (*Continued*)

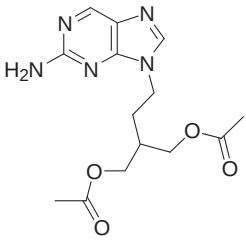
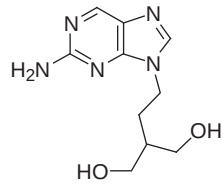
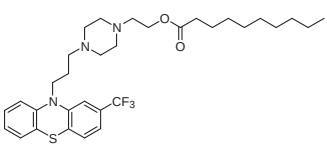
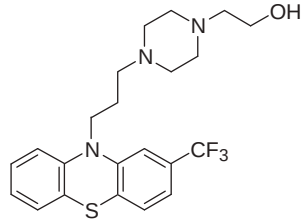
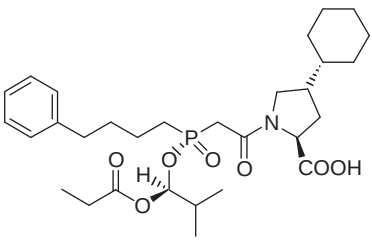
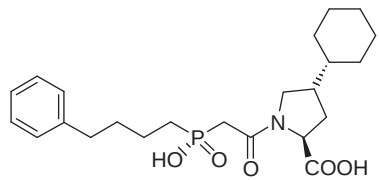
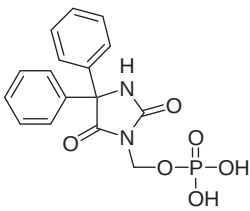
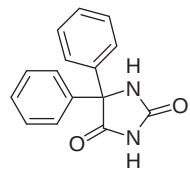
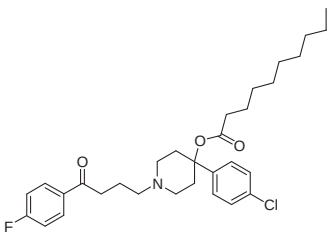
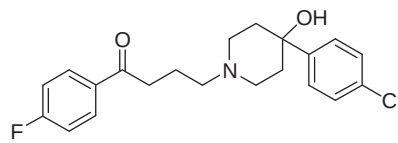
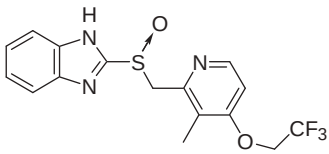
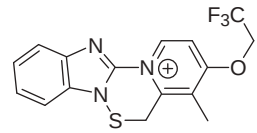
Prodrug	*Drug*
Famciclovir 	Penciclovir 
Fluphenazine decanoate 	Fluphenazine 
Fosinopril 	Fosinoprilat 
Fosphenytoin 	Phenytoin 
Haloperidol decanoate 	Haloperidol 

Table 2.4.4 (*Continued*)

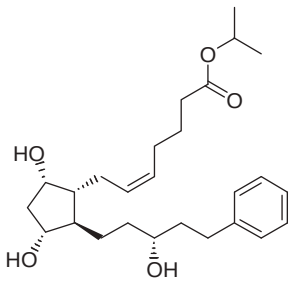
Lansoprazole



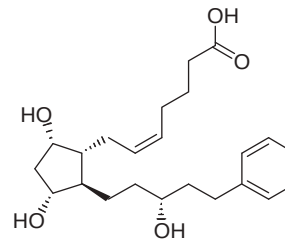
The corresponding sulfenamide



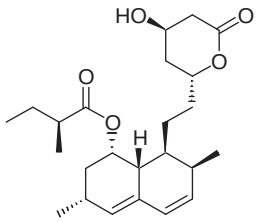
Latanoprost



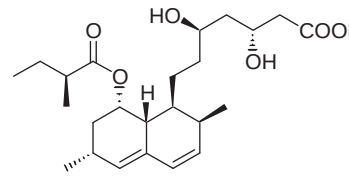
The corresponding acid



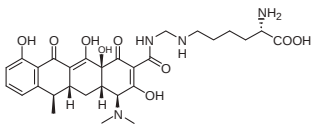
Lovastatin (lactone)



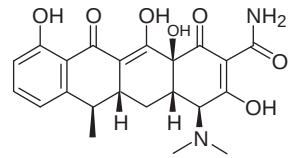
Corresponding β-hydroxycarboxylic acid



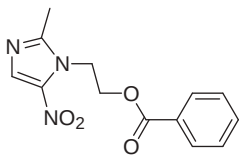
Lymecycline



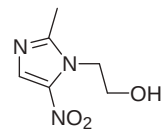
Tetracycline



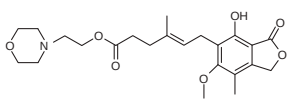
Metronidazole benzoate



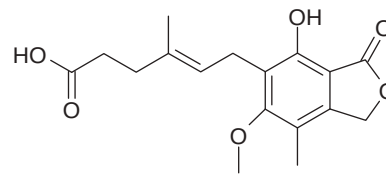
Metronidazole



Mycophenolate mofetil



Mycophenolatic acid (MPA)



(*continued*)

Table 2.4.4 (*Continued*)

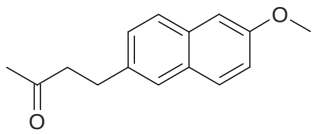
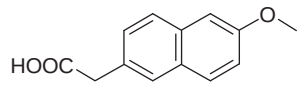
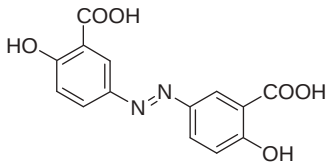
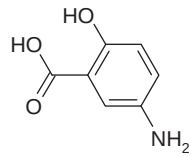
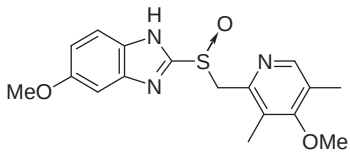
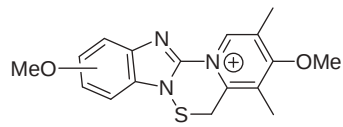
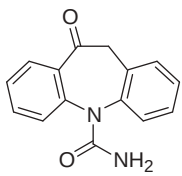
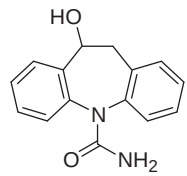
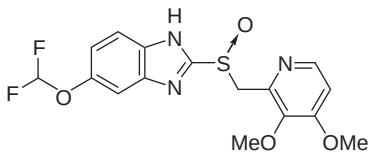
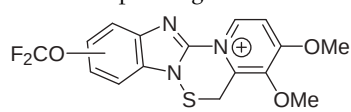
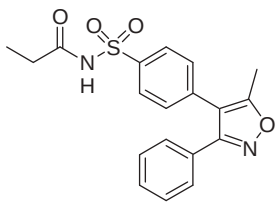
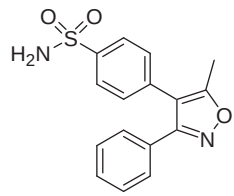
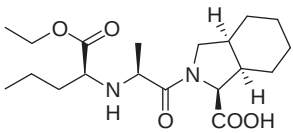
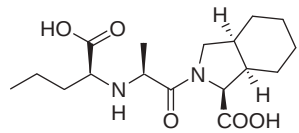
Prodrug	*Drug*
Nabumetone 	6-Methoxy-2-naphthylacetic acid 
Olsalazine 	Mesalazine 
Omeprazole 	The corresponding sulfenamide 
racemic Oxcarbazepine 	10-Hydroxy-carbazepine (MHD) 
Pantoprazole 	The corresponding sulfenamide 
Parecoxib 	Valdecoxib 

Table 2.4.4 (*Continued*)

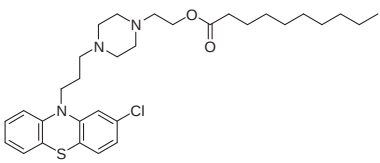
Perindopril



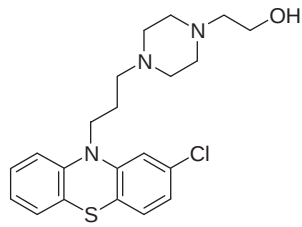
Perindoprilat



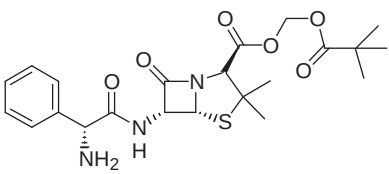
Perphenazine decanoate



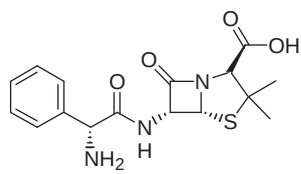
Perphenazine



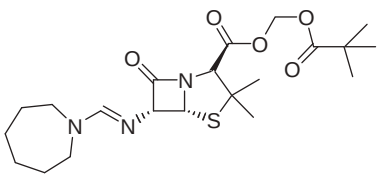
Pivampicillin



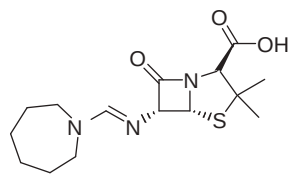
Ampicillin



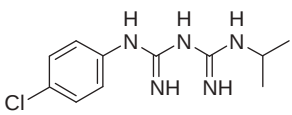
Pivmecillinam



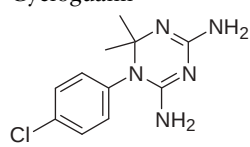
Mecillinam



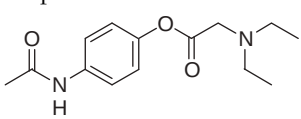
Proguanil



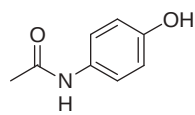
Cycloguanil



Propacetamol



Paracetamol



(*continued*)

Table 2.4.4 (*Continued*)

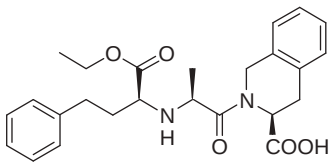
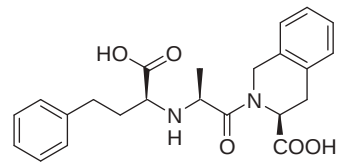
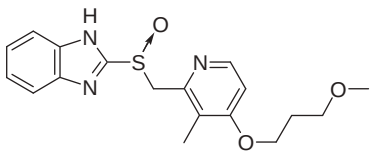
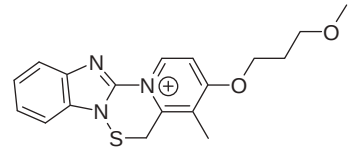
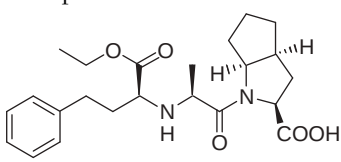
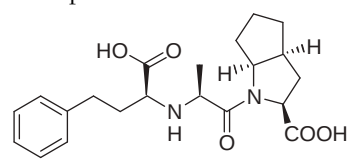
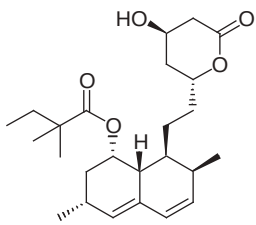
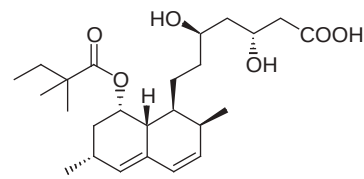
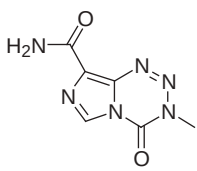
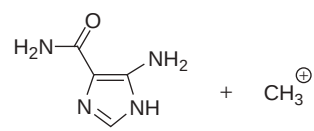
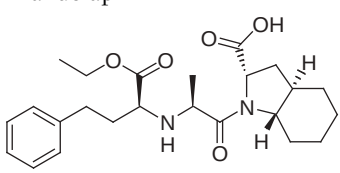
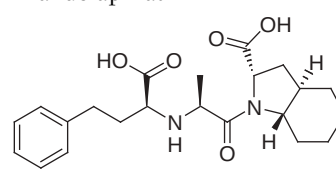
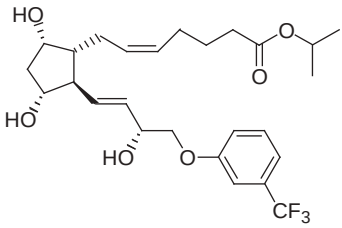
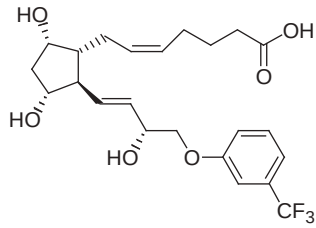
Prodrug	*Drug*
Quinapril	Quinaprilat
	
Rabeprazole	The corresponding sulfenamide
	
Ramipril	Ramiprilat
	
Simvastatin (lactone)	Corresponding β-hydroxycarboxylic acid
	
Temozolomide	The methyl cation is the active metabolite
	 major urinary metabolite
Trandolapril	Trandolaprilat
	

Table 2.4.4 (*Continued*)

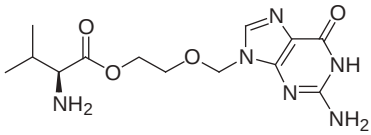
Travoprost



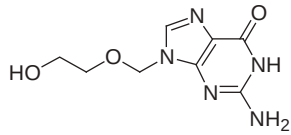
The corresponding acid



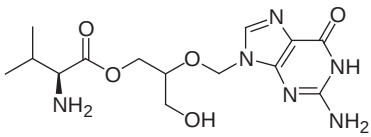
Valaciclovir



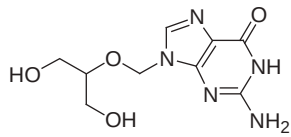
Aciclovir



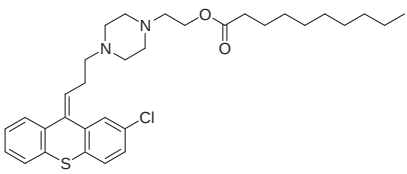
Valganciclovir



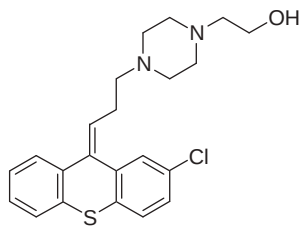
Ganciclovir



Zuclopentixol decanoate



Zuclopentixol



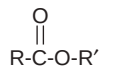
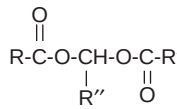
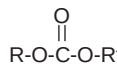
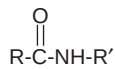
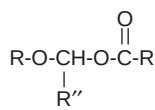
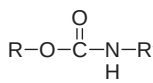
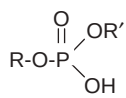
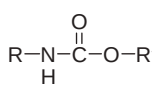
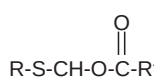
Strategies to improve the permeability by prodrug formulation may be to develop prodrugs that have increased passive diffusional permeability properties across the enterocytic membrane compared to the parent compound, or to develop prodrugs as substrates for absorptive intestinal membrane transporters such as the peptide transporter PEPT1. However, regardless of the strategy, a prerequisite is that the drug candidates contain functional group(s) such as carboxylic acid, alcohol, thiol or amine functionalities which can be applied in the formation of bioreversible linkages between the pro-moiety and the drug candidate. The most frequently applied prodrug linkage is the ester linkage; however, many other

linkages may also be used. Some of the most conventional examples are shown in Table 2.4.5, together with chemical structures of derivable functional groups.

Thus, drug candidates that are alcohols, amines, thiols, or carboxylic acids may be formulated as the corresponding ester, amide, thioester and ester prodrugs, respectively. Conventional ester linkages are in general rapidly cleaved in the gastrointestinal fluids and thus characterised by very short half lives *in vivo*, due to esterase-catalysed hydrolysis. In cases where the prodrug linkage is hydrolysed in gastrointestinal fluids, the prodrug formulation does not necessarily increase the oral bioavailability of the parent drug candidate. However, in cases where the ester linkage is sterically hindered by either the drug substance or a bulky promoiety, its hydrolysis may be only slowly catalysed either by esterases or by acids and/or bases. This sterically hindered ester linkage will normally lead to a decreased release rate of the parent drug candidate from the prodrug compared to drug release from prodrugs, which are not designed to have sterically hindered ester linkages. Consequently, in cases where the prodrug permeability rate is faster than its gastrointestinal metabolism rate, the prodrug candidate may show an increased bioavailability compared to the parent drug candidate.

Such a prodrug strategy was applied in the formation of a bioreversible ester linkage between the β-carboxylic acid in the Glu-Sar promoiety and the alcohol functionalities in hydroxyethyl-thymine candidate and aciclovir (Thomsen *et al.*, 2003a,b). In this prodrug candidate approach, the promoiety but not the linker is stabilised by N-methylation (see Figure 2.4.2). The resulting Glu(aciclovir)-Sar and Glu(hydroxyethylthymine)-Sar prodrug candidates, which show affinity to the intestinal absorptive peptide carrier hPEPT1, release aciclovir/hydroxyethylthymine in a non-enzyme-catalysed but specific base-catalysed manner, whereas the promoiety Glu-Sar itself is fully stabilised. Thus, aciclovir is released by a half life of 6.1 days in upper intestinal fluid (pH 6.0) and 344 minutes in blood (pH 7.4), respectively, and consequently the Glu(aciclovir)-Sar prodrug candidates should hypothetically survive during the intestinal transit time of approximately 3 hours while releasing aciclovir in a controlled manner in blood (Thomsen *et al.*, 2003a,b). The Glu(aciclovir)-Sar prodrug candidate and its release of aciclovir is shown in Figure 2.2.8, the pH–rate profile describing the pH-dependent release rate of aciclovir in Figure 2.3.2 and the pH of various biological fluids in Table 2.1.2. Glu(aciclovir)-Sar and Glu(hydroxyethylthymine)-Sar have both been shown to have strong affinities for PEPT1; however none of them were translocated by the transporter, and consequently their bioavailabilities in rats was

Table 2.4.5 Examples of functional groups that may be applied for prodrug formation

Functional group	*Prodrug form*	*Linkage*
		Ester
		α-Acyloxyalkyl esters
		Carbonate esters
		Amides
R-OH		Esters
R-OH		α-Acyloxyalkyl ethers
R-OH		Carbamate
R-OH		Phosphate esters
$R\text{-}NH_2$		Carbamate
R-SH		Thioesters
R-SH		α-Acyloxyalkyl thioethers
R-SH	R–S–S–R′	Disulphides

shown to be almost zero (Eriksson *et al.*, 2005; Thomsen *et al.*, 2004). The use of an N-methyl amide bioisoster as peptide bond replacement to stabilise against cleavage by peptidases and yet keep affinity for absorptive PEPT1 carrier seems not to be a generally applicable approach to increasing PEPT1-mediated bioavailability, whereas the ketomethylene promoiety approach seems more promising (Våbenø *et al.*, 2004a,b; Andersen *et al.*, 2006).

Santos and co-workers designed and synthesised several 5′-*O*-dipeptide ester prodrug candidates of azidothymidine (AZT) in which the peptide bond is not stabilised. Some of these prodrug candidates show affinity for PEPT1 and are suggested to release the parent AZT by plasma amino- and diaminopeptidase-catalysed hydrolysis. Whether these prodrugs are stable within gastrointestinal environments has not yet been investigated (Santos *et al.*, 2008).

Other ester linkages are stable towards esterase activity in gastrointestinal fluids, but the hydrolysis is catalysed by enterocytic esterases. In such cases the ester prodrug is cleaved within the enterocyte and consequently the parent drug candidate is released in the intracellular compartment of the enterocytes. This is suggested to be the case for the valine ester prodrugs of aciclovir and ganciclovir (valaciclovir and valganciclovir, Table 2.4.4), which are both shown to be substrates for hPEPT1 (Han *et al.*, 1998a,b; Thomsen *et al.*, 2003a,b). It is suggested that the ester linkage between the carboxylic acid in the valine promoiety and the alcohol functionality in aciclovir is cleaved by the intracellular esterase valase which is present in both enterocytes and hepatocytes (Kim *et al.*, 2003). Consequently no parent valaciclovir prodrug but only aciclovir reaches the blood circulation *in vivo* (Beauchamp *et al.*, 1992; Burnette and Miranda, 1994). The bioavailability of aciclovir is approximately 20%, which increases to approximately 60% when administered as valaciclovir. The chemical structure of valaciclovir is shown in Figure 2.2.4 and Table 2.4.4.

Other more chemically advanced prodrug linkages are the acyloxyalkoxy- (AOA), coumarinic-acid- (CA) and oxymethyl modified coumarinic acid (OMCA)-based linkers made by Borchardt and co-workers in the cyclic prodrugs of the [Leu5]-enkephalin analogue DADLE (Bak *et al.*, 1999; Wang *et al.*, 1999; Ouyang *et al.*, 2002). The chemical structures of these prodrug candidates are shown in Figure 2.4.5. The parent drug candidate is suggested to be released by enzyme-catalysed hydrolysis (Wang *et al.*, 1999). The strategy behind the design of these prodrug candidates was to increase the lipophilicity and thus oral bioavailability of DADLE. However, other factors than lipophilicity

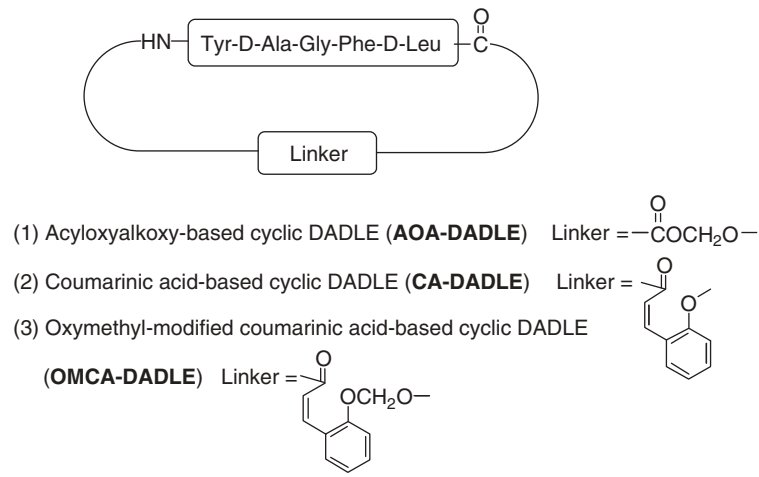


Figure 2.4.5 Chemical structures of DADLE and its cyclic prodrugs (AOA-DADLE, CA-DADLE, and OMCA-DADLE). Reproduced from Ouyang H, Chen W, Andersen TE, Steffansen B, Borchardt RT (2009a). Factors that restrict the intestinal cell permeation of cyclic prodrugs of an opioid peptide (DADLE): (I) role of efflux transporters in the intestinal mucosa. *J Pharm Sci* 98: 337–348.

restricted the intestinal cell permeation of these cyclic prodrugs. The restricting factors have recently been partially elucidated, the prodrugs being substrates for exsorptive intestinal transporters such as P-gp, breast cancer-resistant protein (BCRP) and multidrug-resistance-associated protein (MRP2) (Ouyang *et al.*, 2009a).

All together the prodrug approach may improve drug bioavailability when designing prodrug candidates with improved permeability or solubility properties. Strategies for improving permeability across biological membranes such as the membrane of the small intestine may be to design prodrug candidates that are more lipophilic than the parent drug candidate or to design prodrug candidates as substrates for absorptive membrane transporters such as hPEPT1. Intestinal absorptive transporters have recently been reviewed (Steffansen *et al.*, 2004).

2.4.5 Conclusions

This chapter has presented some examples of how chemical approaches, i.e. salt formation, bioisosteric replacement, and prodrug formation, may be applied as strategies to improve the bioavailability of drug candidates. Optimising bioavailability is classically based on Lipinski's rule of five; however, many other factors have also been proven to limit the

bioavailability of drug candidates. These factors may involve metabolic enzymes and/or membrane carriers that may increase or limit the bioavailability and cause interactions between drug candidates (US Department of Health and Human Services, FDA, CDER and CBER, 2006). Modern drug development should be based on such challenges, and consequently subsequent chapters describe how membrane transporters that influence drug bioavailability may be investigated.

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2.5

Preformulation in the industry step by step

Heidi Lopez de Diego

In the pharmaceutical industry, preformulation studies are initiated in the early stage of drug discovery and extend all the way through the development of the drug until registration. The results from the preformulation studies form an integral part of the registration package. In this chapter, the preformulation studies performed at the different stages of development of a new drug will be described.

The development is divided into the following stages: early discovery, where the lead series are identified; lead optimisation; late discovery, approaching the selection of the development candidate; early development, that is prior to the clinical studies; and full development, which means during the clinical studies. Of course it would be an advantage to know everything about the physicochemical properties of the compound at an early stage, so that these properties could be taken into account in all aspects of development. It is, however, time consuming to undertake salt selection and polymorphism screening, and it is waste of resources to do this on the vast amount of compounds that never even enter clinical trials. On the other hand, a salt shift, or appearance of a new stable polymorphic modification with significant different properties at a late stage of development may involve a lot of extra work and cause delay in registration of the new drug. Therefore it has to be decided at which stage of development each investigation should be performed, in a way that minimises the risk of delay, and at the same time uses as few resources as possible working on compounds that never turn into a marketed product.

Every company has its own preformulation programme and differences in the strategy are observed. These variations reflect differences in company politics, the size of the company, resources, specialist knowledge and available in-house techniques. As an example, high-throughput salt screening and polymorph screening are performed at an early stage of development in large companies with their own high-throughput facilities, while smaller companies without this equipment normally do salt

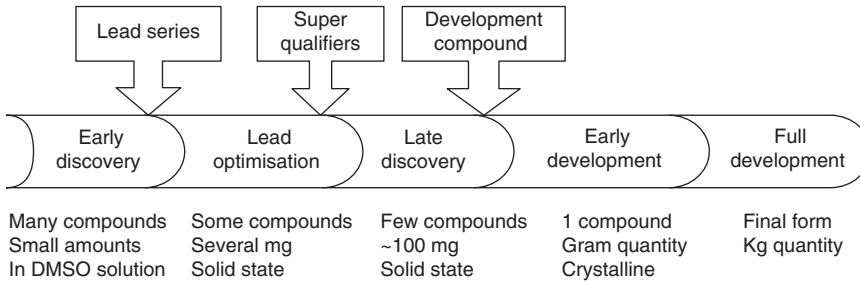

Figure 2.5.1 Schematic representation of the development stages.

selection and polymorphism investigation at a later stage. Furthermore, every compound and project has its own peculiarity, so even though a preformulation programme and policy exist, deviations from the programme will occur to solve specific challenges for a given compound.

The number of compounds, the amount and purity of each compound, and the demands in relation to precision of the analytical results change throughout the development. In the following, the preformulation studies performed at each stage of development are described. A schematic representation of the development stages is given in Figure 2.5.1.

2.5.1 Early discovery

In the early discovery phase the purpose of preformulation studies is to guide the chemist to compounds with properties that are acceptable for a drug. It is not enough that the compound is biologically active; it should also be able to reach the target in the human body. If, for example, compounds in a series are insoluble, very lipophilic, or very unstable, it will be impossible, or at least very difficult and time consuming to transform one of these compounds into a bioavailable drug. It would be preferable if another series is selected or the compounds are modified in a way that gives better physicochemical properties.

At the early discovery stage, the keyword is ranking. A large number of compounds are being investigated and they have to be ranked from a physicochemical point of view. We are not seeking precise values; the compounds at this stage often only exist in very small amounts and with low purity, so the aim is to divide the compounds into groups as: good, acceptable, bad or unacceptable, using a small amount of compound and a short time on each compound. The techniques at this stage are often denoted 'high-throughput screening'. Even placing the compounds into

the groups itemised above may be difficult. At the early stage of development, there is usually no information available about the biological potency. It is clear that a very potent compound will be administered in low dose and therefore the requirement for solubility is much less than for a compound that ultimately has to be given in a dose of 500 mg. Furthermore, the expected route of administration may affect the grouping. The demands on solubility, lipophilicity and stability differ for a compound in a cream as compared to a compound for oral administration.

The typical parameters of interest at this point are: acid/base properties, stability, solubility and lipophilicity. The available substance for the investigations is often only few microlitres of a dimethylsulfoxide (DMSO) solution.

2.5.1.1 Acid/base properties

The acid/base properties give information about how other properties changes with pH (e.g. lipophilicity and solubility) and about whether the solubility may be changed by selecting a salt for development. pK_a values are not measured at this stage, but from the structure of the molecule it can be judged whether the compound has any acid- or base character within the pH range of interest (pH ~1–10). Some companies use computer programs for calculation of approximate pK_a values.

2.5.1.2 Stability

The stability is seldom measured at this stage, but the molecular structure gives information about whether obvious stability problems may be expected.

2.5.1.3 Solubility

Low solubility is one of the major challenges in the modern pharmaceutical industry. Nephelometry may be used for solubility ranking. Microtitre plates are loaded with different concentrations of the compounds in buffer solution and placed in the nephelometer, where laser light is used to measure the turbidity of the solutions. If a solution has a concentration that is higher than the solubility of that particular compound, precipitation will occur, leading to a turbid solution. One of the major drawbacks of this method is the danger of formation of supersaturated solutions due to lack of precipitation of crystalline material. Another drawback, if the solutions are prepared from DMSO solutions, is that effect of the

presence of DMSO may differ from compound to compound and thereby upset the ranking.

Some companies use computer programs for calculation of approximate solubility.

2.5.1.4 Lipophilicity

The lipophilicity of a homologous series of compounds may be ranked by high-performance liquid chromatography (HPLC). In this method, the retention times of a series of compounds with known log*D* values are measured on reverse-phase HPLC (RP-HPLC) through a lipid column using a buffer solution, and a standard plot of retention time versus log*D* is constructed. Then, from the plot, the log*D* values of the unknown compounds may be estimated from their retention times.

The major drawback of this method is that the compounds should be of similar structure (homologous series) to get the correct order of magnitude.

2.5.2 Lead optimisation

The purpose of preformulation studies in the lead optimisation phase is to help the medicinal chemist in optimising the series as regards physicochemical properties. If, for example, low solubility is the problem, it may be improved by introducing a polar group into the molecule. At this stage the compounds are solids and the amount available for preformulation studies is typically ~10 mg.

The properties of principal interest are pK_a, log*P*/log*D*, aqueous solubility and stability. Not all parameters are measured on all compounds. Depending of the general properties of the series, focus is placed on different parameters. Furthermore the solubility in different vehicles (e.g. cyclodextrins) is important information for animal experiments.

2.5.2.1 pK_a

The pK_a values are determined by titration. Often the solubility of the compound is too low to allow titration in pure water. In these cases the titration is performed in water–methanol mixtures with variation in methanol content, and the pK_a value in pure water is determined by extrapolation to zero methanol content.

2.5.2.2 Log*D*

The log*D* profile may be determined by titration if the compound has a pK_a value in the relevant range and it is known. Titration is performed with known amounts of octanol present. The shift in the apparent pK_a value is related to the water–octanol equilibrium and makes it possible to calculate the entire log*D* versus pH profile.

Another possibility is to use the shake-flask method.

2.5.2.3 Solubility

The solubility is determined at a fixed pH value. Phosphate buffer pH 7.4 is often chosen as a solvent. The solubility is typically determined using the shake-flask method (e.g. by adding excess of solid to the buffer, leaving it shaking at constant temperature (often 22 °C or 25 °C) for 1–2 days and then measuring the concentration in the supernatant using HPLC).

If sufficient compound is available (~20 mg), the solubility is determined in relevant vehicles (e.g. cyclodextrin solutions) to support the choice of formulation for animal experiments.

2.5.2.4 Stability

The stability is measured in solution, often under very stressed conditions (e.g. exposed to light in a light cabinet, placed at high temperature in solution of different pH). The concentration is measured by HPLC before and after exposure to the stressed condition.

2.5.3 Late discovery

At a later stage in the discovery process, a few promising compounds are selected. These are sometimes called super qualifiers. At this stage the compounds exist as fairly pure solid compounds and in larger amounts. This means that normally 100–200 mg can be used for preformulation studies. The preformulation task is to characterise them and judge whether they have suitable properties for development, highlight problems, and give input to possible solution.

At this point, pK_a values and the log*D* profile are normally already known. The crystallinity is verified by X-ray powder diffraction (XRPD). If the compound is not crystalline, efforts are made to crystallise it. The aqueous solubility is re-determined at e.g. pH 7.4, and after the solubility measurement, the precipitate is analysed for crystallinity and identity

(XRPD). It is important for solubility determination that the solid is crystalline, as amorphous material has higher solubility. If it subsequently crystallises the solubility will drop significantly (easily by up to 10 times). From the solubility at pH 7.4 and the pK_a value, the solubility profile (solubility versus pH) can be calculated. This profile is used for judging whether, for example, solubility problems may be overcome by selecting another salt. If so, a mini salt screening is considered.

Properties such as pK_a, lipophilicity, stability in solution at fixed pH and solubility at fixed pH (in the pH range where the solubility of pure base (acid) determines the overall solubility) are inherent compound properties that are independent of the salt form selected. Therefore they cannot be improved by selecting another salt.

Other properties such as crystallinity, melting point, thermal stability and hygroscopicity are salt dependent. Also, the solubility at low pH for bases and at high pH for acids depends on the salt.

The obtained form of the compound is characterised with respect to crystallinity (XRPD), melting point (DSC), thermal stability (thermogravimetric analysis (TGA)), hygroscopicity (dynamic vapour sorption (DVS)) and solubility in water without pH adjustment. Furthermore, the solid-state stability may be determined under highly stressed conditions (light and high temperature and humidity).

From the results of these investigations, it is judged whether the present form may be used for the early development, or another salt is needed.

2.5.4 Early development

In this stage, the compound has to be turned into a new drug; nevertheless, its properties may be problematic. Hopefully the preformulation work during discovery has resulted in selection of a compound with adequate properties, and in this case it is 'just' a matter of optimising the compound in relation to selecting the best salt, finding the more stable polymorphic modification and characterising its properties. However, if it happens that a particular compound with a unique pharmacological profile is insoluble, instable and very lipophilic, and if all attempts to modify the molecule towards better physicochemical properties lead to lack in biological activity, the problems with bioavailability have to be overcome in the development.

The purpose of the preformulation studies is to find the best form of the compound for development into a drug, and to characterise it with respect to all physicochemical properties. The first step is to evaluate

whether the compound may be developed into a stable bioavailable drug or whether it should be considered for developing a prodrug. Then the best salt for development has to be identified (if the compound has acid or base properties). When the salt is selected it has to be characterised. Part of the characterisation is performed during the salt screening, as physicochemical properties form the basis for the selection (e.g. aqueous solubility, intrinsic dissolution rate, stability, thermal behaviour, hygroscopicity) but solubility versus temperature, solubility in organic solvents, possible solvate formation and polymorphism screening also remain to be carried out.

The characterisation is described here as a case study (compound XX).

2.5.4.1 Background

XX is a new development candidate. The following parameters have been determined in the discovery phase: it is a base with one pK_a value = 8.5. It has a solubility of 180 μg ml^{-1} at pH 7.4. Log$P$ = 2.6 and log$D_{7.4}$ = 2.2. The log$D$ profile has been determined by titration. It is unstable in acidic solution. It will be developed for oral administration. So far it has been precipitated as an oxalate salt.

It is not expected to give problems where physicochemical properties are concerned, although acidic salts should be avoided as they could give rise to an acidic microenvironment in the formulation, leading to possible degradation.

So far the free base of the compound has not been isolated as a crystalline solid. The solubility at pH 7.4 may therefore be overestimated. Efforts are made to crystallise the base.

2.5.4.2 Preformulation work

The free base is crystallised and used for re-determination of the aqueous solubility at pH 7.4. The result gives 30 μg ml^{-1}. The new solubility versus pH curve is shown in Figure 2.5.2, together with the previous one. It appears that the compound is much less soluble than expected from the initial investigation.

At the same time it is observed in the biology department that they cannot make the cyclodextrin solutions at 15 mg ml^{-1} for animal dosing as they could previously. The reason is that the free base precipitates. New solubility determinations in cyclodextrins are performed and the dosing programme has to be changed.

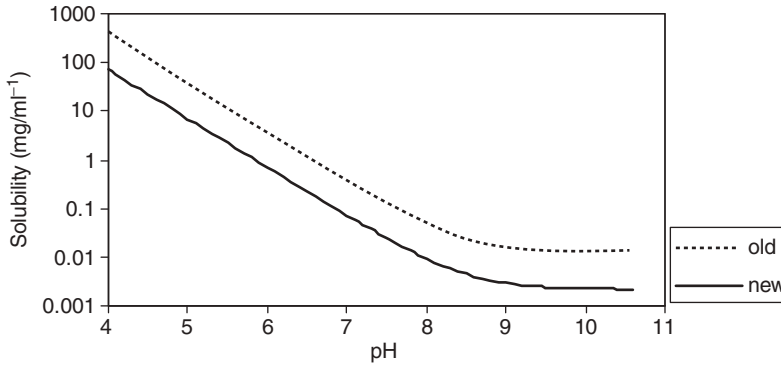

Figure 2.5.2 Solubility versus pH calculated from the old value at pH 7.4 (180 μg ml^{-1}) and from the new value (30 μg ml^{-1}).

The free base is characterised. It is found to be non-hygroscopic and crystalline, with low solubility and a low intrinsic dissolution rate in hydrochloric acid. It is therefore clear that a salt screening should be performed. The more important parameters for this salt screening are the solubility and the dissolution rate, although the salt must not give rise to acidic solution.

2.5.4.3 Salt screening

The properties of the oxalate salt are already known. A series of other acids are selected, and a small amount of salt of each acid is made. The first criterion for an acceptable salt is that it is crystalline and has a well-defined stoichiometry. For most compounds these properties are closely connected, a crystalline compound is well defined; but in some cases the solubility of a 1:1 salt and a 2:1 salt is so close that the mixtures precipitate, which gives the overall precipitation odd stoichiometry.

The prepared salts are analysed by XRPD. The stoichiometry of the crystalline ones is then checked by microanalysis (carbon hydrogen nitrogen (CHN)). The solubility of those that are well defined and crystalline is then determined. pH is measured in the saturated solution, and the precipitate from the solubility determination is analysed by XRPD to check that the solubility measure is really the solubility of the crystalline salt.

The three more-soluble salts are selected for further characterisation, and larger amounts of these salts are made (200–500 mg of each). XRPD is performed to ensure that the same polymorphic modification as last time is obtained. It happens very often that the first small amount made is a metastable modification.

The larger amount is characterised by:

- intrinsic dissolution rate
- thermal properties (TGA and DSC)
- solid-state stability (light, heat and humidity, e.g. 80°C, 80% relative humidity); the stability is tested under very stressed conditions in order to be able to rank the compounds in a short time
- hygroscopicity (DVS).

From these results the most promising salt is selected.

Now the basic properties of this salt are known. A large batch is made for general early development activities, and part of the batch is used for further characterisation. The first task is to check that the same crystal modification is obtained (XRPD). Upscaling from small batches only prepared for salt-screening purposes to larger scale may lead to other crystal forms (polymorphism).

Then the solubility in different organic solvents is measured. This has two purposes. One is to actually determine the solubility of the salt in organic solvent and the other is to get information on possible solvate formation and polymorphism.

The solubility in organic solvents is determined by addition of excess of compound to the solvent and then shaking the suspensions for several days (shake-flask method).

The solubility is determined as the concentration of compound in the supernatant.

The precipitate is isolated and analysed by XRPD. In methanol and ethanol, the X-ray powder diffractogram of the precipitate is similar to the pattern of the salt batch used.

In toluene, the diffractogram of the precipitate differs from the original diffractogram. This implies that either a new polymorphic modification is obtained or a solvate is formed. The precipitate is then analysed using TGA. The TGA thermogram is shown in Figure 2.5.3.

The curve shows a weight loss of about 10% corresponding to a solvate (hemi-toluenate). This means that a solvate has been formed from toluene. The sample is heated to 120°C, and subsequently analysed by XRPD. From the TGA curve it is evident that at this temperature the solvent is liberated. The XRPD differs from both the solvate diffractogram and the original batch diffractogram. This means that a new polymorph has been formed. The modification obtained was less stable than the original form (proved afterwards by cross-seeding).

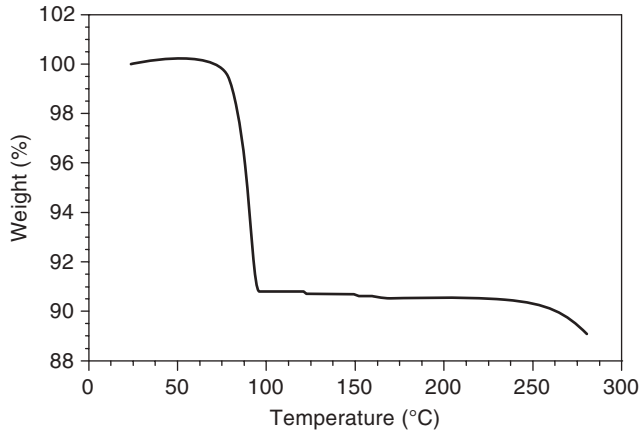

Figure 2.5.3 TGA thermogram of the precipitate from toluene.

Other aromatic solvents also led to formation of solvates. All these solvates formed metastable polymorphic modifications after desolvation. Therefore, it is concluded that aromatic solvents should be avoided in the final step of the process.

The saturated solutions from the solubility determinations are left for slow evaporation of the solvents. The solids obtained from these experiments are analysed by XRPD to see if new polymorphs are obtained. No new form was obtained. Some of the precipitates seem to be larger crystals (slow evaporation is the optimal condition for crystal growth). These are investigated under the microscope, and the crystals obtained from methanol seem to be large single crystals. Therefore this precipitate is used for crystal structure determination by single crystal X-ray analysis.

2.5.4.4 Polymorphism investigation

Polymorphism investigation is one of the most demanding tasks in the preformulation package. Some information has already been obtained from the experiments for determination of solubility in organic solvents. Furthermore, all the batches produced are followed up in the chemical development process.

Polymorphism screening is performed by making crystallisations from a large number of solvents and solvent mixtures under different conditions (high and low temperature, fast and slow cooling, stirring, no stirring etc), always avoiding the presence of crystal seeds.

All precipitations are analysed by XRPD. If new diffractograms are obtained, TGA is used to check whether it is a solvate or a new polymorphic modification.

If new polymorphs are obtained, the next step is to determine the relative thermodynamic stability of the forms. It has to be clarified whether it is monotropy (the same form is the more stable at all temperatures) or enantiotropy (a transition temperature exists where one form is the more stable form at temperatures below this temperature and the other form is stable above this temperature). The relative thermodynamic stability at room temperature is determined by cross-seeding experiments, where saturated solutions of each polymorph are seeded with another one and the mixtures are left for days to weeks. Subsequently the precipitate is analysed. The metastable form is then transformed into the stable form. The DSC-thermograms (melting point and enthalpy) are used for more information of the relative stability issues (the higher-melting form is the more stable in the case of monotropy; the lower-melting form has the higher enthalpy of fusion in the case of enantiotropy). The solubility of each form is measured at different temperatures to find the eventual transition temperature (in the case of enantiotropy).

If a more stable form appears, that one is normally chosen for development.

In that case the entire characterisation has to be repeated for that form.

2.5.4.5 Compatibility

Examination of compatibility with excipients is performed when formulation development is initiated.

2.5.5 Full development

Now all should be done. It may, however, happen that new polymorphic forms appear during scaling-up of the process. Therefore the new batches should be followed, not only by the analytical team, but also by the preformulation group.

Further characterisation may also be needed for particular formulations.