CHAPTER **11**

Column, Thin-Layer, and Paper Chromatography

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11.1 Column Chromatography 368
A. Introduction 368
B. Preparation Procedure 369
C. Methods of Detection 372
D. Laboratory Experiments 376
11.2 Thin-Layer Chromatography 381
A. Detection of Compounds 385
B. Further Procedures 389
11.3 Paper Chromatography 391
A. Ascending Chromatography 393
B. Two-Dimensional Chromatography 393
C. Descending Chromatography 394
D. Ascending-Descending Chromatography 394
E. Circular (or Radial) Chromatography 394
F. Filter Papers 396
G. Detection of Compounds 397
H. Methods of Quantitative Spot Detection 398
I. Experiments 400
Questions 403
References 404

11.1 COLUMN CHROMATOGRAPHY

A. INTRODUCTION

Chromatography may be defined as the selective adsorption and separation of a mixture of chemical substances on a column or film of adsorbent through which a suitable solvent has been passed. No chemical reaction takes place during the process, and, once separated from the mixture, any starting material is recovered chemically unchanged. This technique is used widely in many

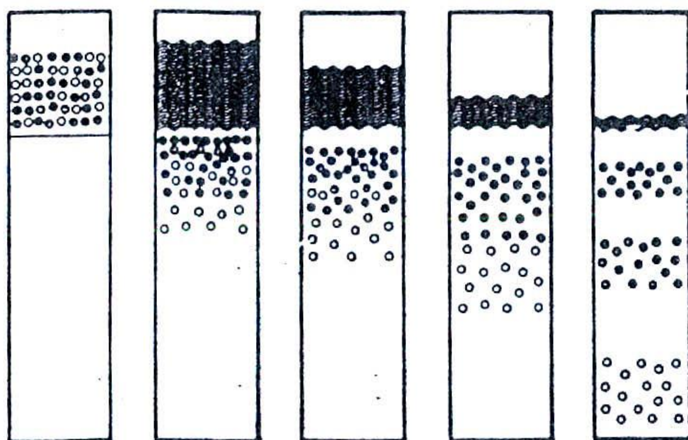

FIGURE 11.1: Schematic diagram of chromatographic separation.

fields of analysis and has resulted in numerous important advances, particularly in biochemistry.

The Russian botanist, Tswett, usually regarded as the father of chromatography, first described the process in 1906 when he filtered a petroleum ether solution of chlorophyll, extracted from leaves, through a column of calcium carbonate. As more petroleum ether was added to the column a series of colored bands separated and moved down the column at different rates. Each band represented a different pigment of the chlorophyll. Tswett referred to the column on which the separation was taking place as the chromatogram and the technique as the chromatographic method (Fig. 11.1).

As stated before, chromatography is a process of selective adsorption, which is the concentration of a gas, liquid, or solid, at the surface of a liquid or solid. A mixture of chemical substances which is only poorly separated on a column indicates that the degree of affinity of each component in the mixture for the adsorbent is approximately the same. One component is not

specifically adsorbed and retarded relative to the others, and no separation occurs. The factors influencing adsorption are not well understood, but as a general rule it can be stated that polar compounds are more readily adsorbed from nonpolar solvents, whereas nonpolar compounds are usually adsorbed more readily with increasing molecular size and degree of unsaturation. The configuration of the molecule may also affect its adsorption. The extent to which a substance is adsorbed by a particular adsorbent can be studied by means of an adsorption isotherm; this relates the amount of substance applied to an adsorbent to the amount of the substance adsorbed at constant temperature. Cassidy[1] determined the adsorption isotherms of lauric, myristic, palmitic, and stearic acids on various adsorbents but concluded that a comparison of these isotherms could not be used to predict the possibilities of separation on a column. Theoretical treatments of adsorption chromatography so far have not influenced experimental work to any great degree, and the best conditions for a chromatographic separation must be determined experimentally.

When a chemical mixture has been separated on a column by developing with a suitable solvent, further development can be stopped as necessary. To isolate the individual components, the adsorbent can be extruded from the column and split into the appropriate zones from which these components are extracted with suitable solvents. More commonly, the solvent is continually run through the column until the zones have been washed out and collected. Individual zones are often eluted with different solvents and the technique is known as *elution analysis*.

The chromatographic column can be a test tube, boiling tube, or glass column of any convenient size. A 25- or 50-ml burette with a wad of glass wool packed into the bottom makes a satisfactory column but many types are available which have a plate or disc inside to support the adsorbent. A stopcock should be fitted to regulate the flow of solvent through the column whose height should be four to ten times its diameter and, as a rough guide, should be filled to one-half to two-thirds its height (Fig. 11.2).

B. PREPARATION PROCEDURE

1. Preparation of the Column

A plug of glass wool is placed in the bottom of the column and pressed down evenly. If available, some clean washed sand can be poured on top of the wool to form a thin even layer. A slurry is made of the adsorbent with a suitable solvent, usually water, and this slurry is poured steadily into the column. After settling, the stopcock is opened and some of the solvent is allowed to drain out, but a "head" or layer of solvent should always cover the adsorbent, otherwise cracks will develop in the column. If this happens the column should be discarded. A disc of filter paper is moistened with the

solvent and placed on top of the column of adsorbent. Before developing the chromatogram, about 50 ml of solvent should be run through the column at a rate of approximately 10 ml per min.

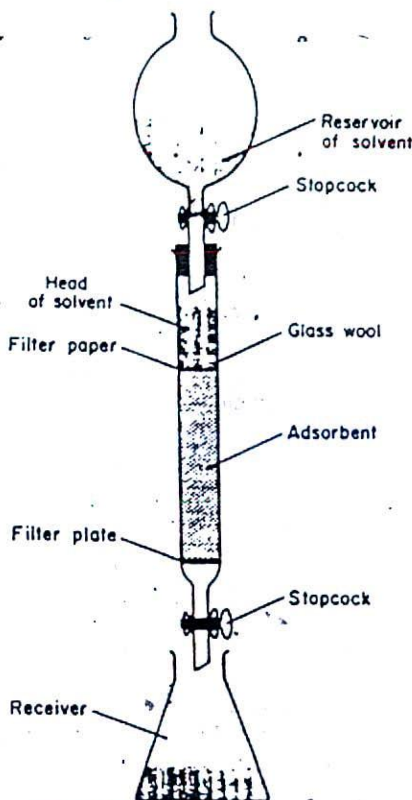

FIGURE 11.2: Chromatographic column.

2. Adding the Sample to the Column

The sample is added as a small volume of liquid to the top of the column after which the stopcock is opened and the chromatogram allowed to develop. The flow rate should be about 5 to 10 ml per min, but this may be altered with experience.

3. Developing the Chromatogram

The solvent is added continuously from a reservoir to the top of the column at a rate sufficient to ensure a "head" of liquid on the column. As the chromatogram develops, the constituents of the sample will pass down the column at varying rates.

4. Recovering the Constituents

Known volumes of eluate issuing from the column are collected in suitable receivers. These volumes may be collected in small fractions of 5, 10, or 15 ml, each of which can be assayed for chemical content, or the eluate can be collected and assayed in bulk. In a large-scale operation, these fractions are best collected by a fraction collector which monitors a certain volume of eluant from the column and electronically turns a rack containing a series of empty tubes each of which is filled in turn.

Many chromatographic adsorbents and solvents exist but the selection of the best combination is still largely a matter of trial and error. A list of the adsorbents and solvents commonly used is shown[2] in Table 11.1.

TABLE 11.1: Common Adsorbents and Solvents[2]

Adsorbents (in terms of adsorptive binding power)		Solvents (in terms of eluting power)
Sucrose	Weakest	Petroleum ether
Starch	↓	Carbon tetrachloride
Inulin		Cyclohexane
Talc		Carbon disulfide
Sodium carbonate		Diethyl ether
Calcium carbonate		Acetone
Calcium phosphate		Benzene
Magnesium carbonate		Toluene
Magnesia		Esters
Lime		Chloroform
Silicic acid		Alcohols
Alumina		Water
Charcoal	↓	Pyridine
Fuller's earth	Strongest	Organic acids

Particle size of an adsorbent is important because it influences the degree of resolution of a mixture. The smaller the particle size, the sharper is the separation achieved. Small particle size, however, can considerably reduce the rate of column flow and even stop it altogether. Some idea of the average particle size of various adsorbents can be gained[3] from Table 11.2. To

TABLE 11.2: Average Particle Size of Various Adsorbents[3]

Adsorbent	Particle size, μ
Alumina	7.0
Calcium carbonate, precipitated	1.5
Calcium sulfate, hydrated	10.5
Magnesium oxide	1.5
Fuller's earth	3.0

overcome the disadvantages of fine adsorbents and slow flow rates, common filter aids such as Celite 545, Celite 535, Celite 503, or Hyflow Super-Cel are used to make the column more permeable to the solvent, thus increasing the flow rate. Celite is a trade name for kieselguhr.

Of the inorganic adsorbents, alumina (Al_2O_3) is probably the most common. The activity of this material depends upon its water content and the acidic or basic nature of the impurities. It is prepared by washing with 0.5 *N* hydrochloric acid followed by water and then methanol and reactivated for use by heating at 200°. One gram of alumina has an adsorptive surface area of 90 $meters^2$. *Acid alumina* is prepared by mixing alumina with three or four volumes of normal hydrochloric acid, stirring well, and decanting a number of times. The alumina is washed with water until the washings are mildly acid to litmus and then dried at 100°. *Magnesia* (MgO) is too fine to allow a good flow rate and is often mixed with Celite or Hyflow Super-Cel. *Chromatographic silica* is readily obtainable and can be used after thorough washing. *Bentonite*, a hydrated aluminum silicate, can be activated by washing with acid and has been used to separate vitamin A and vitamin D. *Powdered charcoal* specifically adsorbs strongly aromatic substances such as amino acids.

The relationship between chemical constitution and chromatographic behavior has been generally classified from the most strongly adsorbed to the least strongly adsorbed as follows: acids and bases, alcohols, thiols, aldehydes, ketones, halogen-containing substances, esters, unsaturated hydrocarbons.

C. METHODS OF DETECTION

When chemical substances have separated on the column, they can be detected by suitable means. Obviously, if the chemical is colored it can be seen passing down the column and into the eluate. Colorless compounds should be detected by reagents specific enough to permit quantitative determination if possible. Several methods are available.

1. The adsorbent can be extruded from the column and cut into a number of sections and each section eluted with the appropriate solvent.

2. The column can be treated with a series of solvents each of which elutes a specific fraction.

3. The zones can be rendered visible by physical or chemical means.

It is possible to detect colorless substances in the eluate by measuring changes in pH, conductivity, or refractive index. Ultraviolet light is a valuable aid and is used routinely to scan the column and the eluate. If the compound fluoresces (for example, quinine) it can be readily detected. Ultraviolet light sources are available for wavelengths at 365 mμ or 276 mμ. The former wavelength is used to produce fluorescence, whereas the latter detects compounds absorbing in this region.

Specific color reactions depend upon the nature of the chemical being treated. Sugars form colored esters with *p*-phenylazobenzoyl chloride ($C_6H_5N{=}NC_6H_4COCl$), a reaction which also transforms sterols into colored esters. Phenols give colored azo-compounds with diazotized *p*-nitroaniline, whereas aldehydes and ketones form 2,4-dinitrophenylhydrazones, and alkaloids form colored reinekates. Colored indicators can be added directly to the column, and the color (or lack thereof) can be used to trace the substance as it passes down the adsorbent. Fluorescein (0.04%) can be added to the adsorbent and the column viewed under ultraviolet light. The background will fluoresce except in those areas where chemicals are present, and they will appear as dark bands. Dimethylamino azobenzene has been used on silical gel to identify alkaloids; the silica is colored red, except where the alkaloids are present. Similarly, organic compounds which react with ferric chloride to give colored compounds can be made visible by saturating the alumina with an ethereal solution of ferric chloride.[2]

The techniques of *frontal analysis* and *displacement analysis* should be mentioned. In frontal analysis the mixture is run through the column of adsorbent and the chemical least strongly adsorbed emerges first in the eluate, whereas the most strongly adsorbed emerges last. Chemicals emerging between the first and the last will occupy positions relative to their affinity for the adsorbent. By checking the eluate, frontal analysis helps determine the number of components in a mixture. In displacement analysis, the mixture is applied to the column in the normal manner, but the developing solvent contains a substance more strongly adsorbed by the adsorbent than any of the drugs in the mixture. This substance displaces all the drugs in the mixture in turn, depending upon their affinity for the adsorbent. The most weakly adsorbed is displaced first, then the next, and so on. When the displacing agent starts to emerge from the column all the drugs in the mixture have been eluted. With this technique, amino acids have been displaced one by one by ephedrine. This procedure also gives an indication of the number of components in a mixture together with their relative basic or acidic strengths. In displacement analysis, the term *specific retention colume* is used and is that volume of liquid passing through the column per gram of adsorbent before the chemical leaves the column. It gives a measure of the affinity of a variety of substances for a given adsorbent in a given solvent system.

Partition chromatography, widely used in a variety of fields, was described accurately for the first time in 1941 by Martin and Synge, in England, and for their work they were subsequently awarded the Nobel Prize in 1953. Partition chromatography is similar to liquid-liquid extraction procedures in which chemicals in a mixture can be separated from each other by shaking in two immiscible solvents. The chemicals separate because of their differences in solubility in the solvents being used. In partition chromatography one of the liquids is present as an adsorbed film on the particles of adsorbent (column partition chromatography) or supported on a sheet of paper (paper partition

chromatography). In both instances, the adsorbent or paper acts merely as an inert support for the stationary liquid. The second liquid, immiscible with the first, is used to develop the chromatogram. The moving liquid is called the mobile phase and the adsorbed liquid on the column the stationary phase. Partition chromatography is an extremely efficient method of separating a chemical mixture. Martin and Synge, investigating amino acid composition, used wet silica gel as the adsorbent and a mixture of chloroform and butyl alcohol, saturated with water, as the developing solvent. This solvent (as the mobile phase) passed over the water held in place by the silica gel, and the amino acids were carried down the column at different rates, depending upon their relative solubilities in the organic and aqueous phases.[4]

The theory developed by Martin and Synge considers partition chromatography similar to fractional distillation. By employing the concept of a "theoretical plate," that is, the length of column required for equilibrium to be established as the drug partitions itself between the stationary and mobile phases, they developed an equation to correlate the rate of movement of a chemical on a column with the partition coefficient of that chemical between the stationary and mobile phases:

$$R_f = \frac{A_L}{A_L + \alpha A_s} = \frac{\text{distance traveled by chemical}}{\text{distance traveled by solvent}}$$

A_s = cross-sectional area occupied by stationary phase

A_L = cross-sectional area occupied by mobile phase

$$\alpha = \text{partition coefficient of chemical on column} = \frac{\text{concentration of chemical in stationary phase}}{\text{concentration of chemical in moving phase}}$$

A nonmathematical approach can be developed from the simple kinetic postulate of Cremer and Muller.[5] As the chromatogram is developed the molecules of the chemical are moving backward and forward from the moving phase to the stationary phase, and the extent of this movement will depend upon the solubility of the chemical in the moving and stationary phases.

Consider a column or paper strip as in Fig. 11.3. Let x be the distance the chemical has traveled, and $x + y$ the distance the solvent has traveled. Assume also that the column is being developed with an organic solvent (lipoid phase) and on a stationary phase of water (aqueous phase). Then x is proportional to the solubility of the chemical in the moving phase, and y is proportional to its solubility in the stationary phase:

$$\frac{x}{y} \propto \frac{\text{concentration of chemical in moving phase}}{\text{concentration of chemical in stationary phase}} = k\,\frac{\text{concentration of chemical in moving phase}}{\text{concentration of chemical in stationary phase}}$$

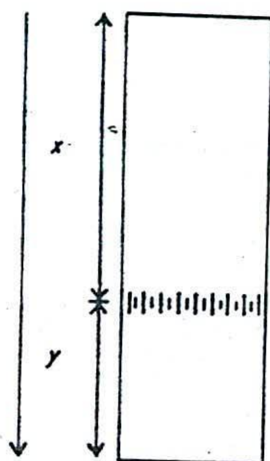

FIGURE 11.3

where k is a constant. But

$$\alpha = \text{partition coefficient of chemical}$$
$$= \frac{\text{concentration of chemical in stationary phase}}{\text{concentration of chemical in moving phase}}$$

Therefore,

$$\frac{x}{y} = \frac{k}{\alpha}$$

where k takes into account the ratio of the cross sections of the moving and stationary phases. Now,

$$R_f = \frac{x}{x+y}$$

Therefore,

$$\frac{1}{R_f} = \frac{x+y}{x} = 1 + \frac{y}{x}$$
$$= 1 + \frac{\alpha}{k}$$

k has the value of A_L/A_s in the equation of Martin and Synge. Then

$$\frac{1}{R_f} = 1 + \alpha\frac{A_s}{A_L}$$
$$= \frac{A_L + \alpha A_s}{A_L}$$

Therefore,

$$R_f = \frac{A_L}{A_L + \alpha A_s}$$

The apparatus required for column partition chromatography is similar to that already described for column chromatography. One of the most commonly used supports is Celite 545 and in packing a column approximately 1 ml of aqueous (immobile) phase per gram of Celite is the proportion which produces a column possessing suitable characteristics. Ratios such as 5 g Celite to 7 ml of water have also been found to work well. The column should be packed as uniformly and evenly as possible, particularly where the difference in partition coefficients of the drugs to be separated is small. The flow rate can be varied from about 1 ml per hr to 10 ml per min.

The order of the phases can be reversed, in which case the organic solvent is supported by the adsorbent while the aqueous phase travels down the column. This technique is known as reversed-phase partition chromatography. For example, columns of kieselguhr (Hyflow Super-Cel) have been treated with dimethylchlorosilane, a silicone fluid, and the hydrophobic alkyl groups become attached to the surface of the kieselguhr particles. Nonpolar organic solvents such as chloroform can then be used as the immobile phase, whereas the aqueous polar phase (water) travels down the column to develop it.

When drugs have been separated on a column it is sometimes possible to elute them individually with different solvents. Hyoscine and hyoscyamine can be separated on a column of kieselguhr that has been mixed with a buffer solution of pH 6.2 and acts as the stationary phase. After the alkaloids have percolated through the column, the hyoscine is eluted with ether and hyoscyamine with chloroform. Some idea of the simplicity with which partition chromatography is used can be observed from procedures involving alkaloids. Conventionally, these are isolated by "shake-out" techniques following acidification or basification of the organic phase and subsequent extraction with water. Alkaloids, however, have been isolated by partition chromatography employing a column supporting 0.3 M phosphoric acid as the stationary phase. The mixture containing the alkaloids is added to the column in ethereal solution; the alkaloids are trapped on the acidic column; the neutral and acidic substances pass through. The immobile phase is then made alkaline with ammonia-saturated chloroform and the alkaloids are eluted as the free bases.[6]

The following examples have been selected from current pharmaceutical literature to illustrate the principles of column and partition chromatography and can be used as experiments in the laboratory.

D. LABORATORY EXPERIMENTS

1. Adsorption Chromatography of Chlorophyll Extracts[7]

For chromatographic work, use a crude petroleum ether extract of grass. Shake 1 g of powdered grass with 10 ml light petroleum ether until the liquid is a rich-green color. Decant or filter for use. This filtrate contains *blue-green*

chlorophyll A, *clear-green chlorophyll B*, and yellow-orange pigments *xanthophyll* and *carotene*. By using the correct procedure, the four pigments can be separated from each other and isolated.

Prepare the column, about 15-cm high, by inserting a plug of glass wool in the tapered lower end of the column if it is not fitted with a sintered-glass disc. Place in a beaker sufficient powdered calcium carbonate to fill the column to approximately two-thirds of its height, and make a slurry by adding an equal volume of petroleum ether. Pour the suspension into the column in portions, allowing each portion to settle before adding more suspension. Always leave a "head" of petroleum ether on the calcium carbonate. Place some glass wool on top of the column to avoid disturbance when solvents are added.

Add 5 ml of the crude chlorophyll extract to the top of the column; when this has soaked in, add 5 ml of petroleum ether followed by another 5 ml as the first aliquot soaks in. *Do not let the column dry out.* If green or yellow bands separate, note the distance they have traveled from the top of the adsorbent and at the same time record the amount of petroleum ether which has entered the column.

Continue adding portions of solvent for about an hour or so; in this way colored bands may be collected as they are washed out of the column. If no colored material appears to be issuing from the column, collect the eluate as it may contain a very dilute solution of pigment. When no further separation is evident, allow the liquid to drain from the column which can then be gently extruded from the tube into a sheet of paper. Any remaining bands on the adsorbent can be extracted with petroleum ether. If a complete separation is achieved, it can be recorded by plotting a graph of the distance the bands have traveled from the top of the column against the quantity of solvent which has entered the column.

2. The Separation and Determination of Diphenylhydantoin and Phenobarbital[8]

In this assay, the diphenylhydantoin and phenobarbital are separated on a partition column buffered to an alkaline pH of 9.2. The two drugs are then eluted individually and estimated spectrophotometrically.

The columns are prepared by mixing 15 ml 0.15 *M* borate buffer (pH 9.2) with 15 g Celite. A slurry is made of this by adding 15 ml butanol/chloroform (10:90) and transferring this to the column with frequent packing. The flow rate should be about 2 to 3 ml per min.

Approximately 50 mg of diphenylhydantoin and 30 mg of phenobarbital are accurately weighed into a 50-ml beaker. To this is added 3 ml of distilled water and 3 g of Celite. This is thoroughly mixed and a slurry prepared by the addition of 5 ml of chloroform. The slurry is transferred quantitatively to the column using two 5-ml portions of chloroform and is well packed onto the surface of the column with a glass plunger. Chloroform is added to the

column, and the first 100 ml eluate are collected in a volumetric flask. The absorbance of this solution is measured for diphenylhydantoin content at 266 mμ and compared with a diphenylhydantoin standard in chloroform at the same wavelength. A second 100-ml portion of eluate is collected using a 10:90 (v/v) *n*-butyl alcohol/chloroform mixture and its absorbance measured for phenobarbital content at 266 mμ. This reading is compared with an appropriate standard.

For analytical purposes, the contents of 25 diphenylhydantoin-phenobarbital capsules are accurately weighted. Portions of this mixture containing between 50 and 90 mg of diphenylhydantoin and 8 and 16 mg of phenobarbital are accurately weighted into 50-ml beakers. Three milliliters of 0.3 *N* hydrochloric acid are added to the beaker to convert the diphenylhydantoin sodium to the free acid. The procedure is then followed as described.

Levine has used multistage columns to separate mixtures of drugs. The individual stage on each column has an acid or alkaline immobile phase, and these stages or layers can be arranged in one column or in separate columns. In the latter instance, the eluates flow from one column directly onto the next. The layers "trap" basic or acidic drugs as the mobile phase runs through the column. The trapped drugs can then be recovered by using another mobile phase of a suitable pH.[9] This technique is illustrated in a procedure applicable to atropine, codeine, dihydrocodeinone, emetine, physostigmine, quinine, and strychnine.

3. An Extraction Procedure for Alkaloids[10]

In practice, the extraction is carried out by incorporating an aqueous solution of *p*-toluenesulfonic acid together with the alkaloid sample as the stationary phase on a partition chromatographic column. Ether elutes acidic and neutral substances without removal of the alkaloids. Chloroform elutes the alkaloid-*p*-toluenesulfonic acid complex and the eluate is passed over a column, in which sodium hydroxide is the stationary phase, to remove the toluenesulfonic acid, thus yielding an eluate which contains the alkaloid as the free base.

The column can be prepared from a test tube, 25 × 250 mm, to which is attached a stopcock. Fine glass wool is packed into the column as a support.

Column 1. Dissolve 500 mg of *p*-toluenesulfonic acid in 5 ml of sample solution of the alkaloid. Add 6 g of Celite, mix thoroughly with a flexible spatula, and transfer to the column. "Dry wash" the container with approximately 1 g of Celite and add to the column content. Tamp down the Celite with a tamping rod, using gentle pressure, until the column is uniform.

Column 2. Mix 3 g of Celite with 2 ml *N* sodium hydroxide, transfer to a column, and tamp down as above. Cover with a pad of glass wool to prevent disturbance.

Use water-washed solvents throughout. Pass 100 ml of ether over column 1 and discard the eluate. Mount the columns such that the eluate from column 1 passes into column 2 and pass 100 ml of chloroform over the columns. Determine the alkaloidal content in the eluate by any standard procedure. The method can be used for tablets, suspensions, injections, and other forms.

4. Estimation of Strychnine or Quinine In Elixirs[11]

Strychnine and quinine commonly occur in elixirs, and both these alkaloids can be separated from other constituents in the elixir by being held on a "trap" layer of acidified Celite followed by elution with chloroform.

Prepare a column containing a wash layer of 1 ml of water adsorbed on 1 g of Celite. Mix 3 g of Celite with 3 ml of 2 *N* hydrochloric acid and tamp down on the wash layer. Select sufficient sample of the elixir to contain about 1.5 mg of strychnine or quinine, evaporating if necessary to a volume of 3 or 5 ml. Add 2 ml of 6 *N* hydrochloric acid, cool, and mix thoroughly with about 6 g of Celite. Transfer to the trap layer in several portions through a powder funnel, pressing it down with a tamping rod. Scrub the beaker and rod with about 1 g of Celite and add these scrubbings to the column. Repeat this with two further portions of Celite and place a wad of glass wool on top of the column. Moisten the column with about 25 ml of water-washed ether and permit 100 ml of the solvent to percolate through the column. Elute the strychnine or quinine with 150 ml of water-washed chloroform and collect the eluate in a beaker. Evaporate carefully to dryness with the aid of a current of air and dissolve the residue in 5 ml of warm 2 *N* hydrochloric acid, cool, and dilute to exactly 100 ml. Compare the absorbance of this solution at 256 mμ with that of a standard containing 2 mg of strychnine in 100 ml of 0.1 *N* hydrochloric acid or a similar standard of quinine.

5. Separation of Caffeine and Phenacetin by Partition Chromatography[12]

For a mixture of caffeine and phenacetin, a solvent combination of water as the stationary phase and a nonpolar mobile phase can be used, since caffeine has a definite affinity for water and less affinity for nonpolar solvents such as ether, whereas the opposite is true for phenacetin. A 20-mm chromatographic column, 45 cm long, is used and a glass plunger helps in packing the column.

Twenty grams of silicic acid (chromatographic grade) and 20 ml of water are thoroughly worked together in a 400-ml beaker with a spatula. Approximately 150 ml of di-isopropyl ether/chloroform mixture (75:25) is added and a slurry made by vigorous stirring. This is packed into the column in increments, care being taken to avoid air pockets. A circle of filter paper is placed on top of the column. A 5-ml sample containing 25 mg of caffeine and 25 mg of phenacetin is used, and this sample is diluted three parts by volume with di-isopropyl ether. Exactly 5 ml of this diluted solution is placed on the

prepared column. The entire phenacetin contained in the added sample is obtained in a single fraction of 100 ml by elution with the isopropyl ether/chloroform mixture. The caffeine, which remains on the column, is stripped off by elution with 100% chloroform. The flow rate should be 5 ml per min.

The phenacetin content of the first fraction and the caffeine content of the second fraction are determined spectrophotometrically. A 10-ml aliquot is taken from each and placed in a 100-ml volumetric flask. The solvent is rapidly removed by swirling the solution under vacuum, and the residue is redissolved with chloroform and diluted to the mark with the same solvent. Read the absorption at 275 mμ to assay the caffeine and at 288 mμ to determine the phenacetin. These readings are compared with the relevant standards of caffeine and phenacetin in chloroform.

6. A Chromatographic Assay of Acetylsalicylic Acid, Phenacetin, and Caffeine[9]

These three drugs, which occur very commonly in pharmaceuticals, can be separated and estimated by the use of a two-stage column prepared from Celite 545. The first stage, acidified with sulfuric acid, traps the caffeine from an ether solution, and the second stage, basified with sodium bicarbonate, traps the acetylsalicylic acid as its salt. Ether elutes the chemically neutral phenacetin, and the chloroform elutes the caffeine. The acetylsalicylic acid is recovered by acidifying the Celite with a solution of acetic acid in chloroform and the liberated acetylsalicylic acid is eluted with chloroform. The caffeine and acetylsalicylic acid eluates are received directly in volumetric flasks and their concentrations measured spectrophotometrically without further treatment.

Determine the average weight per tablet (or capsule) and grind a suitable number to a fine powder. Transfer a weighed sample containing approximately 50 mg of acetylsalicylic acid together with the appropriate amounts of caffeine and phenacetin to a 50-ml volumetric flask. Add 30 ml of chloroform and shake thoroughly. Add 0.1 ml of glacial acetic acid and make to volume with chloroform.

A suitable column can be made from a 25- × 200-mm test tube to which is attached an approximately 5-cm length of 6- to 8-mm tubing. A tamping rod is necessary to pack the column. Glass wool should be packed into the base of the column to act as a support.

To 2.0 g of Celite 545 in a glass mortar add 2.0 ml 4 *N* sulfuric acid (9 parts water plus 1 part concentrated sulfuric acid). Incorporate thoroughly by kneading with a flexible spatula and transfer to the column, compressing down with a disk. Similarly, mix 2.0 g of Celite with approximately 1 *N* sodium bicarbonate and place in the volume above the acid layer, taking care to tamp down evenly. Wash the column with 15 to 20 ml water-washed ether and discard the washings.

In the following procedure use only water-washed solvents. Dilute 5 ml of the sample preparation with 20 ml ether and pass over the column. When

the last portion of this solution has passed into the adsorbent, wash with five 5-ml portions of ether, allowing each portion to pass almost completely into the adsorbent prior to the addition of the next one. Rinse the tip of the outlet tube with chloroform and evaporate the total eluate to dryness on a steam bath in a current of air. Dissolve the residue in 5 ml of chloroform and make to volume with isooctane in a 50-ml volumetric flask. This fraction contains the phenacetin.

Immediately after the passage of the last portion of ether through the column, replace the receiver with a 100-ml volumetric flask. Pass 95 ml of chloroform through the column, rinse the tip with chloroform, and dilute the eluate to volume. This fraction contains the caffeine.

Replace the receiver with a 100-ml volumetric flask. Pass a solution of 0.5 ml glacial acetic acid in 5 ml of chloroform through the column followed by 90 to 92 ml of a 1% solution of acetic acid in chloroform and dilute to volume. This fraction contains both the acetylsalicylic acid and the salicylic acid.

In a suitable spectrophotometer read the absorbances of the solutions and compare the values with those determined simultaneously on solutions of the pure materials. Chloroform containing 1% acetic acid is used for the solution of salicylic acid and acetylsalicylic acid. This latter standard must be prepared daily.

The absorbance of phenacetin at a concentration of 50 mcg/ml is about 0.505 at 285 mμ; of caffeine at 10 mcg/ml is about 0.485 at 276 mμ; of acetylsalicylic acid at 100 mcg/ml is about 0.790 at 280 mμ and 0.010 at 310 mμ; and of salicylic acid at a concentration of 250 mcg/ml calculated as acetylsalicylic acid, 0.113 at 280 mμ and 0.573 at 310 mμ.

11.2 THIN-LAYER CHROMATOGRAPHY

Thin-layer chromatography (TLC) is a technique whereby thin layers or films of silica gel are spread on glass plates, and chemical mixtures applied to these films are separated into their respective components by means of suitable solvents. The thin-layer films can be developed rapidly and the mixtures resolved with a high degree of sharpness. In addition, a much greater variety of reagents are available to develop specific colors on the plate than is the case with either column or paper chromatography. There are several methods by which thin layers of silica or alumina can be prepared. Glass plates, 8-in. square, are commonly employed but smaller plates, 4 × 5 or 3 × 4 in., and even microscope slides can be used satisfactorily.

1. Spreaders

There are several spreading devices available commercially to prepare plates, and they all function on the same principle.[13] Essentially, a spreader

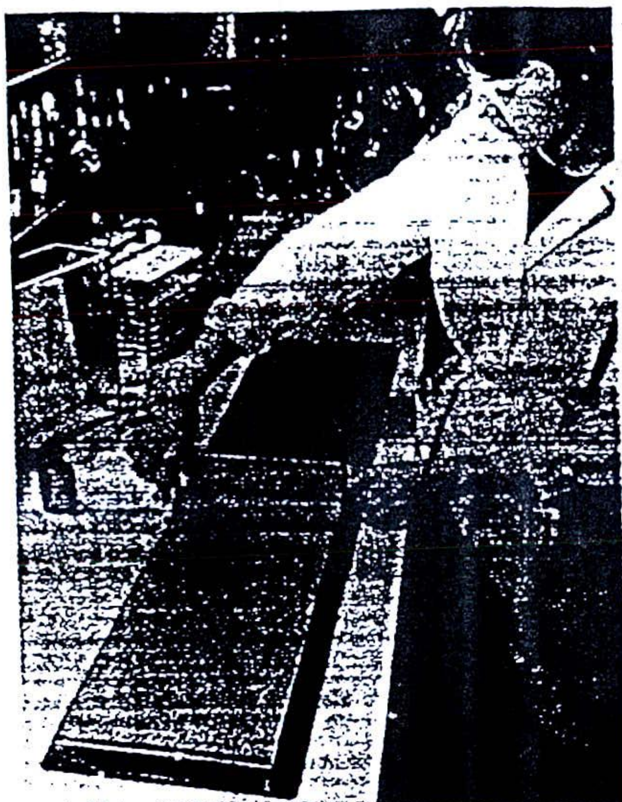

FIGURE 11.4: Thin-layer chromatography spreading apparatus. (Reproduced by permission of Brinkmann Instruments, Ltd.)

FIGURE 11.5: Thin-layer chromatography applicator. (Reproduced by permission of Brinkmann Instruments, Ltd.)

consists of a stainless steel rectangular box which acts as a form of trough. The glass plates are placed on a carrying tray and the spreader, which usually has a guide arm fitting flush with the tray, is placed on top of the plates. The silica gel in the form of slurry is placed in the spreader, which is then pushed smoothly and steadily along the tray: As it does so, the slurry flows out and coats each plate with adsorbent. The thickness of the film is controlled by adjusting the distance between the spreader and the plates by means of adjustable screws or small metal gauges. When the layer of silica gel has hardened the plate is activated for use in an oven at about 105° (Figs. 11.4 and 11.5).

2. Adhesive Strips

Small plates or microscope slides can be fixed to the bench top with strips of adhesive tape. The silica gel is made into a slurry as before and poured onto the plates. The slurry is then spread lightly and evenly with a glass rod held loosely between the fingers. The tape acts as a guide line to prevent the gel overflowing. When the layer is set it is activated for use in an oven after the tape has been removed.

3. Hand Pouring

The slurry is prepared in a mortar and pestle, transferred to a beaker, and poured onto small plates or microscope slides which are tilted backward and forward by hand until a smooth even film results. A glass rod can be used to assist spreading. The silica is then activated for use in an oven. This technique is suitable for 3- by 4-in. plates or microscope slides but not for 8- by 8-in. plates.

In the above methods a satisfactory slurry can be made by mixing 25 g of silica gel G in a mortar with 65 ml of water. The gel can be prepared at a neutral pH (6.7) if the water is replaced by 0.1 *N* sodium hydroxide. The amount of water can be varied as required—the less water used the thicker the slurry and the resulting film, and vice versa. A higher proportion of water is recommended for the hand-pouring technique: 25 g of silica gel to 75 ml water is satisfactory.

When the plates have been spread, they are activated for use in an oven at 105° to 110° for 1 hr. Meanwhile, a suitable chromatographic tank is lined with filter paper and enough solvent is poured in to give a layer about one-quarter inch thick on the bottom. The lid is placed on the tank and the atmosphere allowed to become saturated.

The plate is removed from the oven and allowed to cool. A straight line is ruled along one side of the plate about 1 in. from the edge and on the side opposite to this about 5 μliters or 10 μliters of the solution to be analyzed are applied to the plate by small micropipettes or capillaries. These applied spots should be about ¾ in. apart and about 1 in. from the bottom of the plate.

When the filter paper in the tank has become saturated, place the spotted plate in the solvent with the edge nearest the spots facing downward. The layer of solvent should not be deep enough to cover the spots. The solvent will gradually climb up past the spots and will eventually reach the ruled line. The plate is then removed from the tank, allowed to dry, and sprayed with the required color reagent.

Various adsorbents can be used in TLC and most usually have a particle size of about 5 mμ. Adsorbents in column chromatography have particle sizes in the range 70 to 200 mμ. Silica gel G, alumina G, and kieselguhr G are available commercially. Silica is slightly acidic and alumina slightly basic, but films of varying pH can be prepared by using buffer solution in place of water in preparing the gel. Alumina films are dried at temperatures of 250° to 500°C. Other adsorbents used are finely powdered ion exchange resins, calcium sulfate, and powdered cellulose. The cellulose slurry is made with acetone instead of water and when the acetone has evaporated the film is ready for use.

Particle size influences the rate at which the solvent climbs. Particles less than 1 mμ form films which develop slowly; with larger particles the film develops more rapidly, but the sharpness of resolution deteriorates with particles larger than 5 mμ. Adsorbents used in column chromatography have particles too large to be used in thin-layer work and the adsorbents used to prepare thin films are much too fine to be used in column chromatography, since they block the column and stop the solvent flow.

For average thin layer plates, about 250-mμ thick, samples containing up to 250 μg can be applied for normal development. The sample should contain about 100 to 300 μg for radial development. In radial development a small hole is bored in the plate and, after spreading, a wick is fitted into the hole. The plate is placed horizontally in a shallow trough and the solvent is fed to the silica gel by means of the wick. Development takes place in a circular or radial manner. The volume of solution applied to the plate should be about 2 to 20 μliters, and the spot of application should be kept as small as possible. The chemical can be prepared as a 1 to 2% solution in an organic solvent such as chloroform or alcohol. To assist rapid drying, a stream of warm air from a hot-air drier can be blown gently over the plates while the solutions are applied.

For larger quantities the solution to be chromatographed can be pipetted onto the plate in the form of a line drawn along one edge. It is then developed in the normal way and the chemical is isolated as a band of material instead of a spot. Much more material can be applied in this way (up to about 25 mg) since the plate is used to maximum efficiency. This method is used to purify extracts from plant sources.

There is no firm rule for selecting any particular solvent to separate a mixture. The simpler the solvent mixture the better, but the solvent or mixture has to be found by preliminary trial and error. It is useful to

remember that ordinary microscope slides coated with silica gel can be developed in a 250-ml beaker covered with a watch glass. A slide is developed in about 10 to 15 min and 30 to 40 solvents can be examined in an afternoon. The list of solvents in Table 11.3[14] is presented as a rough guide.

TABLE 11.3: Eluting Power of Chemical Solvents[14]

Solvent	
Light petroleum	Increasing eluting power ↓
Cyclohexane	
Carbon tetrachloride	
Benzene	
Chloroform	
Ether	
Ethyl acetate	
1,2-Dichloroethane	
Acetone	
Ethanol	
Methanol	
Water	
Pyridine	
Acetic acid	

The R_f value in thin-layer chromatography is affected by many factors, and any published values for a given drug and solvent system should always be confirmed before undertaking practical work. The term R_b value is sometimes used and is as follows:

$$R_b = \frac{\text{distance traveled by the compound}}{\text{distance traveled by a test mixture}}$$

It is felt that the results are more reliable when the chemical is compared to a test mixture rather than the distance traveled by the solvent front. Two test mixtures suggested are indophenol blue, *p*-dimethylaminoazobenzene, and Sudan red G (50 mg of each in 50 ml of benzene: load 2 μliters per spot) or *p*-dimethylazobenzene (butter yellow). It is suggested that R_b values are less variable than R_f values.

A. DETECTION OF COMPOUNDS

When a plate has been developed, the compounds, if colorless, can be detected by various methods which are discussed below.

1. Ultraviolet Light

The plate can be examined in the usual way to detect fluorescent substances. Alternatively, the silica gel can be made to fluoresce by incorporating phosphors such as zinc silicate and zinc cadmium sulfide (0.5% of each) in the adsorbent and preparing the slurry in the usual way. Any chemical in the

adsorbent will shield the phosphor from the ultraviolet light, and the fluorescence will be quenched in that particular area. The spots appear as dark spots on a light background. Instead of adding phosphors the slurry can be made with a 0.04% aqueous sodium fluorescein instead of water. Again in ultraviolet light the spots appear as dark areas on a yellowish-green background. It is also suggested that better results are obtained by spraying the fluorescein on the plate rather than incorporating it as above.

2. Spray Reagents

Most reagents which are used to produce colors with specific chemicals can be used in thin-layer work. The range of reagents which can be used is wider than that available for paper chromatography, since strong reagents such as concentrated sulfuric acid destroy the paper of the chromatogram but leave the silica gel unaffected. The atomizers used for spraying the reagents on the plates are available from any scientific supply house. The plates should be gently and evenly sprayed in a fume hood and should be placed against a wooden board or glass sheet to protect the wall of the hood.

The following spray reagents can be used for a variety of drugs.[14]

Fluoresceine/Bromine. Spray the plate with 0.05% aqueous fluorescein and expose it while still damp to bromine vapor. Examine the plate in normal and in ultraviolet light.

Sulfuric Acid. Spray the plate with concentrated sulfuric acid and heat at 200°C for 10 to 30 min. This treatment carbonizes organic compounds and these appear as black, brown, or grey spots on a white background.

Antimony Trichloride. Spray the plate with a saturated solution of antimony trichloride in water-free chloroform and heat at 150° for 10 min. A variety of colors are often obtained. The plate can be heated and then sprayed while hot. On cooling, expose the plate to chlorine vapor for a few seconds and additional compounds may be detected. The spots should be marked immediately.

Ferric Chloride/Sodium Molybdate. A saturated aqueous solution of ferric chloride is sprayed on the plate which is then sprayed again with 0.1 *M* sodium molybdate. Heat the plate to about 150° for 10 min.

Iodine. Place the plate in an enclosed tank containing a few crystals of iodine. The compounds, particularly those containing nitrogen, absorb the iodine and the spots appear as brown areas on a faint-yellow background. When the spot areas have been marked, allow the iodine to evaporate (overnight if necessary) and the compound can then be eluted from the silica gel unchanged.

Water. Spray the plate with water. The spots may appear as opaque areas on a translucent background when the plate is held up to the light. After marking, the compounds can be recovered unchanged.

3. Specific Reagents

Acids. 1% aqueous bromocresol blue, adjust to pH 6. The acids appear as yellow spots on a greenish-blue background.

Amino Acids. 2% ninhydrin in ethanol and heat the plate at 120° for 20 min.

Ketones and Aldehydes. Acidified solution of 2,4-dinitrophenylhydrazine in ethanol and heat to 120° for 10 min.

Sugars. Spray with a solution of aniline phthalate prepared by dissolving 0.03 g of aniline and 1.6 g of phthalic acid in 100 ml of butanol saturated with water. Heat the plate to 120° for 10 min.

Alkaloids. Dragendorff's reagent. (Soln. A: 0.85 g bismuth subnitrate + 40 ml water + 10 ml acetic acid; Soln. B 8 g potassium iodide + 20 ml water. To use, take 5 ml A + 5 ml B + 20 ml glacial acetic acid + water to 100 ml). The alkaloids appear as red spots on a pale orange background.

Most spray reagents will produce colors with about 1 or 2 μg of material.

The technique of *two-dimensional chromatography* is easily adapted to thin-layer films. The solution to be developed is spotted onto one corner of the plate, which is then developed in the usual way. When complete, the plate is removed from the tank and allowed to dry. The plate is then rotated through 90° and developed with a second solvent at right angles to the first. Since the entire surface of the plate is used, two-dimensional chromatography can resolve complex mixtures.

4. Recovery of the Sample

When a mixture has been resolved or a compound separated from its impurities the sample can be recovered in various ways. The relevant area of silica gel can be scraped off the plate with a spatula or razor blade and collected in a sintered-glass crucible, from which it is subsequently eluted. It can also be removed by a readily constructed vacuum apparatus, which enables the silica gel to be collected directly in an extraction thimble (Fig. 11.6)

After elution from the silica gel, the extract can be evaporated to dryness or a specific derivative made directly from the extract.

5. Quantitative Determinations

Probably the most common method is the elution of the chemical from the relevant area of silica gel. There are, however, two disadvantages to this

technique. In the first place, some substances are eluted from the silica gel and contribute quite significantly to the ultraviolet absorption spectra of the eluted solution particularly in the region of 245 to 260 mμ. Extraction treatment of the silica prior to its use as an adsorbent can decrease the amount of interference but not completely eliminate it. Consequently, overestimation frequently results by this method. Second, the compound may not be completely eluted from the silica. The most satisfactory technique is the elution of the compound as described, followed by treatment with a specific

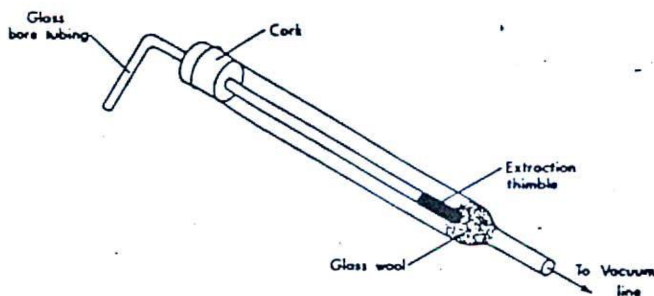

FIGURE 11.6: Apparatus for collecting silica gel from developed plates.

color reagent which shifts the absorption peak from the critical 245 to 260-mμ region. This nullifies the effects of interfering substances. The color reagent should react only with the compound under investigation, and where such a reagent is available 100% recoveries have been achieved by this procedure.

Several methods have been devised which relate the concentration of the applied chemical to spot size. Purdy and Truter,[15] found that there is a linear relationship between the square root of the area of the spot (in mm^2) and the logarithm of the weight of compound applied (in mg). The plate is developed in the usual way and the areas of the spots are determined by laying a sheet of tracing paper on the chromatogram, outlining the spot, transferring the tracing to millimetre graph paper, and counting the squares. Rather than prepare a calibration graph each time an unknown solution is developed, the following method is suggested. Three solutions are prepared: a solution of the unknown sample, a solution of known dilution prepared from the unknown, and a standard solution of the compound being estimated. Duplicate samples of equal volumes of each of the three solutions are spotted on the same plate, which is then developed. When the areas of the spots have been measured the weight of material is calculated from the following formula:

$$\log W = \log W_s + \frac{\sqrt{A} - \sqrt{A_s}}{\sqrt{A_d} - \sqrt{A}} \log d$$

where W = weight of the unknown in milligrams; W_s = weight of the standard in milligrams; A = area of the unknown solution in square millimeters; A_d = area of the diluted unknown solution in square millimeters; A_s = area of the standard solution in square millimeters; and d = the dilution factor involved in diluting the unknown solution; for example, if 1 ml of the unknown was diluted to 2 ml with solvent, then d would be 0.5.

B. FURTHER PROCEDURES

The following procedures are used for purposes of illustration and can be employed as experiments.

1. Separation of Leaf Pigments by TLC[16]

Crush some green leaves in a mortar with a few milliliters of alcohol or acetone and twice this volume of petroleum ether. Transfer the solution to a separatory funnel and swirl with an equal volume of water. Allow the phases to separate and discard the lower water phase. Repeat the water washing twice. Decant the petroleum ether phase and add 2 g of anhydrous sodium sulfate. If the solution of pigment is too weak, concentrate it by evaporating in a stream of nitrogen.

To 5 g of silica gel G in a beaker, add 12 ml of water and stir until dispersed. Pour 1 ml on a clean microscope slide and spread the slurry with a glass rod and tap the slide gently till an even film results. Dry in an oven at 110° for 15 to 30 min.

When dry, place 1 drop of the pigment extract near the bottom of the slide and place it vertically, spot end down, in a 250-ml beaker containing some benzene/acetone (7:3 v/v). Cover with a watch glass. The solvent rises rapidly and the pigment separates immediately. Carotenes move most rapidly, followed by chlorophyll a, chlorophyll b, and xanthophylls.

An alternate procedure is the use of benzene/acetone (95:5 v/v) and continued development for ½ hr with the cover glass removed from the beaker.

2. Separation of Aspirin, Phenacetin, and Caffeine[17]

Plates about 3- by 4-in. should be prepared from an aqueous slurry of silica gel G and poured by hand. Sample solutions of aspirin, phenacetin, and caffeine (separately and in a mixture) should be prepared in methanol in concentrations of 5 to 10 mg/ml. Samples of the drugs and of the mixture are spotted on the plate by means of a micropipette or small capillary. Place the plate in a suitable jar containing a solvent system of absolute methanol-glacial acetic acid/diethyl ether/benzene (1:18:60:120 v/v). After development, the plate is sprayed with 0.1 N potassium permanganate in 0.5 N sulfuric acid and heated for a few minutes in an oven. The zones appear as yellowish-green spots on a violet background. R_f aspirin, 0.97; phenacetin, 0.5; caffeine, 0.2.

3. Vitamin C Content of Orange Juice[7]

Place 20 μliters (or other suitable volume) of orange juice on plates of silica gel G and develop with an ether/formic acid/water system (5:2:1 v/v). Spray the dried plate *either* with 0.1 *N* silver nitrate followed by 0.1 *N* ammonia *or* with 0.1% 2,6-dichlorophenolindophenol in ethanol. Vitamin C should have an R_f of about 0.5. A quantitative estimation can be made by preparing a known dilution of orange juice with water and comparing with a suitable standard using the formula of Purdy and Truter. The areas of the spots can be measured with millimeter graph paper.

4. Determination of Dichlorophene and Hexachlorophene[18]

Spread 0.25 ml of a solution containing 40 mg of each substance per 10 ml (that is, 1 mg of each substance) along the origin of two silica gel plates over a length of 4.5 cm and develop the plates with *n*-heptane saturated with acetic acid. Both plates are developed together but only one is sprayed with the reagent, which is a freshly prepared solution (equal parts) of 1% aqueous ferric chloride and 1% aqueous potassium ferricyanide solutions. The sprayed plate is used to obtain the position of the drugs on the untreated plate, although this can be determined by examining the two bands under ultraviolet light. The zones of silica gel are scraped off and transferred to a small column or flask. The compounds are eluted with *n*-heptane saturated with acetic acid to a total volume of 50 ml. The ultraviolet absorption is determined at 290 mμ and the concentration calculated from a standard curve prepared from dichlorophene and hexachlorophene in *n*-heptane saturated with acetic acid. R_f: dichlorophene, 0.32; hexachlorophene, 0.63.

Note: Both dichlorophene and hexachlorophene are purified by dissolving each in hot 95% ethanol, adding water until a slight turbidity appears, boiling until the solution clears, and filtering. Repeat twice until the melting point of dichlorophene is 164° and hexachlorophene is 167°.

5. Identification of Meprobamate[19]

Meprobamate can be extracted from commercial preparations (tablets, capsules, etc.) by ethanol. Samples of the extract are micropipetted on thin-layer plates, which are then developed with cyclohexane and absolute ethanol (85:15 v/v). After development, spray the dried plates with concentrated sulfuric acid and heat at 115° for 2 to 3 min. Spray lightly with distilled water and reheat as before for a few minutes until yellow spots of meprobamate appear (R_f: meprobamate, 0.3).

For clinical investigation, meprobamate can be isolated from urine by adding 0.2 ml *N* sodium hydroxide and 10 ml ether to 5 ml urine. Centrifuge and decant the ether and add 60 mg of charcoal. Filter the ethereal solution and evaporate to dryness in a stream of air. Dissolve the residue in 0.1 ml

ethanol and spot the plate. A quantitative determination can easily be made by cutting out the spot and transferring the silica gel to a test tube. One milliliter distilled water is added and, after careful shaking, 1 ml of 0.2% hydroquinone in concentrated sulfuric acid. The test tube is heated in a boiling-water bath for 20 min, until a yellow color is obtained. Filter the solution, make up to volume, determine the absorbance at 420 mμ, and compare with an appropriate standard.

6. Separation of Theophylline, Caffeine, and Theobromine[20]

Prepare solutions in the strength of 1 mg/ml of each of the above and place 2 μliters of the solutions on a silica gel plate. On the same plate spot a mixture of the three alkaloids (same strength) and develop with ethyl acetate-methanol/12-N hydrochloric acid (18:2.0:0.05 v/v). To detect the bases, spray with two solutions in the following order:

Solution 1: 1 g iodine and 1 g potassium iodide are dissolved in 100 ml ethanol;

Solution 2: 25% hydrochloric acid 95% ethanol (1:1). Caffeine and theophylline are colored reddish and theobromine grey.

R_f: theophylline, 0.41; caffeine, 0.36; theobromine, 0.25.

7. Separation of Sulfonamides[21]

Prepare plates of silica gel G as outlined. Dissolve 50 mg each of sulfamerazine, sulfadiazine, and sulfacetamide in separate 50-ml solutions of dilute hydrochloric acid. Spot 2 μliters of each solution on the plate and prepare a fourth spot consisting of 2 μliters of each solution superimposed on each other. Develop the plates in a solvent system of chloroform (160 ml), anhydrous methanol (40 ml), and distilled water (2.5 ml). After development, spray the plate with the following reagents:

Solution 1: 1.0 N hydrochloric acid.

Solution 2: 5% aqueous sodium nitrite.

Solution 3: 0.1% alcoholic solution of N-(1-naphthyl)ethylenediamine dihydrochloride (Bratton–Marshall reagent).

The sulfonamides appear as reddish purple spots on a white background. R_f: sulfamerazine, 0.72; sulfadiazine, 0.65; sulfacetamide, 0.47.

11.3 PAPER CHROMATOGRAPHY

Paper chromatography is similar in principle to column chromatography except that the mixture to be resolved is chromatographed on a sheet of filter paper (essentially cellulose) which acts as the adsorbent. The chemical mixture is applied in a small drop of solution to a strip of filter paper about 1 in. from the end. When the spot has dried, the paper is allowed to stand in

the developing solvent which flows up past the applied spot by capillary action. When the solvent has run its prescribed length, the paper is removed, dried, and the drug or chemical is rendered visible or eluted by a suitable method. The mechanism involved in the transport of the chemical on the paper may not be a single one. Adsorption of the chemical to the cellulose fibers of the paper may have an effect. The three recommended rules to be observed are[22]:

1. The paper chromatogram should remain in an enclosed chamber whose atmosphere is saturated with the developing solvent. The solvent should be added 24 hr before the chromatogram is developed. Filter paper in a water-saturated atmosphere will absorb 22% of its weight of water.

2. The developing solvent should move at a fairly slow rate, about 2 to 3 cm/hr.

3. The various components of the mixture should be partially soluble in the developing solvent. A drug which is insoluble in the solvent will not migrate and will remain at its point of application. A drug which is very soluble will be carried along at the solvent front.

As before, the term R_f is used to denote the distance traveled by a drug in relation to the distance traveled by the solvent. This value is important, since it is characteristic of a drug in a particular solvent. It is defined as

$$R_f = \frac{\text{distance traveled by drug}}{\text{distance traveled by solvent}}$$

Although the R_f of a compound depends upon its solubility in the solvent, it is not enough to identify an unknown substance as such. A term related to the R_f value is R_m:

$$R_m = \log\left(\frac{1}{R_f} - 1\right)$$

The R_m value is an additive value to which every functional group in the molecule contributes and is a constant for a given solvent system and the type of paper used; that is, a drug may have a given R_m value in a particular solvent system such as ethanol, but if the drug molecule is altered by the addition or subtraction of a hydroxyl or nitro group, the R_m value will alter in that solvent.

Various techniques have been developed in paper chromatography and these are best described individually. In all instances, a well-sealed chromatographic jar is used during development. Glass jars with ground-glass edges to give a close fit are commonly used but an ordinary graduated glass cylinder fitted with a tight stopper serves adequately. A 1-liter measuring cylinder, sealed with a rubber stopper or aluminum foil makes a good developing chamber.

The four types of paper chromatography employed are *ascending*, *descending*, *ascending-descending*, and *circular* (*radial*) (Fig. 11.7).

A. ASCENDING CHROMATOGRAPHY

A filter paper sheet of any suitable size is selected and the spots to be analyzed are placed about 1 in. from the end of the paper. The spots themselves should be about 1 in. apart. The paper is rolled into a cylinder and the ends are clipped or stapled together in such a manner that they do not touch. The paper cylinder, spot end downward, is inserted upright in the solvent mixture which has been placed in the jar or tank long enough to ensure a saturated atmosphere. The tank is sealed by placing the lid firmly in position

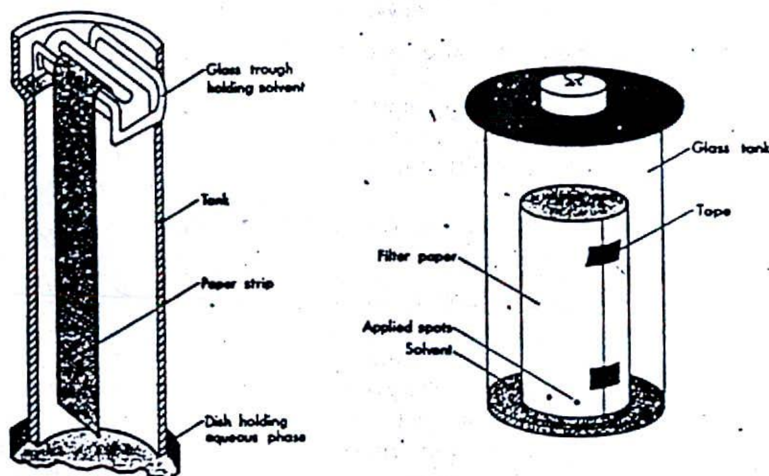

FIGURE 11.7: (*a*) Apparatus for descending chromatography; (*b*) apparatus for ascending chromatography.

and the solvent allowed to creep up the paper until it almost reaches the top. The paper can then be removed, dried, and the spots identified by a suitable spray if necessary.

B. TWO-DIMENSIONAL CHROMATOGRAPHY

In this technique, a mixture is developed in one direction as described in ascending chromatography. After drying, the paper is again developed with another solvent in a direction at right angles to the first. This uses the paper to its maximum efficiency and allows greater separation of a drug mixture. In practice, a spot is placed near one corner of the paper which is rolled into a cylinder as before. After its first development, the cylinder is removed, dried, and again rolled into a cylinder such that the spots now will be developed at right angles to their previous direction of flow. After this development, the

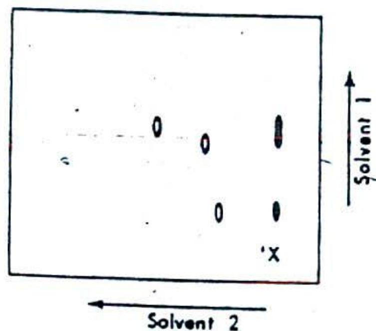

FIGURE 11.8: Two-dimensional chromatography.

substances can be identified by appropriate means. The solvent climbs relatively slowly in ascending chromatography since, the rate of solvent advance is determined by capillary attraction (Fig. 11.8).

C. DESCENDING CHROMATOGRAPHY

In this method the upper end of a paper strip is immersed in a trough containing the development solvent. The chamber must be airtight and the atmosphere saturated with solvent. The trough includes a glass bar over which the paper passes to prevent siphoning of the solvent by capillary action. Usually, a sharp fold is made in the paper about 1 in. from the bottom of a strip of filter paper about 10 in. long. The spot to be analyzed is placed on this line and the area of application kept as small as possible. The folded end of the paper is immersed in the trough and held in place by the glass bar. The chamber is sealed and the paper allowed to hang down from the trough while the solvent gradually descends. When the solvent has advanced the necessary distance, the strip is removed and the position of the solvent front marked. After drying, the strip is sprayed with a suitable solvent or chemical to develop the spot. Descending chromatography is faster than the ascending technique since the solvent is aided in its descent by gravity.

D. ASCENDING-DESCENDING CHROMATOGRAPHY

This is merely a combination of both techniques. The filter paper containing the spot is draped or hung over a glass rod. One end of the paper is immersed in the solvent which climbs up the paper as in ascending chromatography and down the other side.

E. CIRCULAR (OR RADIAL) CHROMATOGRAPHY

In this method, the various drugs are resolved into circular zones instead of spots or bands. Special glass tanks (resembling pie plates) are available but

two upper or two lower sections of Petri dishes are quite adequate. A circular sheet of filter paper, slightly larger in diameter than the Petri dish section, has a slice about 2 mm wide cut out along one radius, that is, from the center to the circumference of the paper. This slice is then cut at right angles to the plane of the paper and cut to a suitable length. The slice acts as a wick for feeding solvent to the paper. A drop of the solution to be analyzed is applied

FIGURE 11.9: Simple apparatus for circular paper chromatography.

to the center of the paper, which rests on the rim of the dish, and the second dish is placed on top of the first to act as a cover or seal. The solvent rises through the wick and spreads out radially from the center of the paper, and the various solutes separate to produce a series of concentric circles each representing a different solute. The solvent, spreading by capillary action, has its rate of development controlled by the width of the wick and the distance between the solvent and the paper. Separations can be accomplished in as little as 20 min (Fig. 11.9).

It should be remembered that a small strip of filter paper can be developed by the ascending technique in a test tube. A small amount of solvent is placed in the bottom of the tube, and one end of the strip of filter paper is immersed in this. The strip is held in place by the rubber or cork stopper which is used to seal the tube and to ensure a saturated atmosphere. Numerous samples

can be analyzed at one time by this simple method since only a few test tubes and a rack are required.

F. FILTER PAPERS

Any of the common filter papers can be used in paper chromatography. Whatman No. 1 is probably the most widely employed, but other grades can be used for specific purposes, as illustrated in Table 11.4. Schliecher and Schull

TABLE 11.4: Whatman Filter Paper Guide

Grade no.	Flow rate	Paper characteristics	Applications
1	Medium	Smooth surface, medium weight	Widely used for all types of chromatography
2	Slow	heavier and denser than 1	Popular with some workers for amino acids, peptides, proteins, and electrophoresis
3	Medium	rough-surfaced thick paper	Inorganic work and electrophoresis
3MM	Faster than 3	Softer and smoother than 3; thick	Inorganic work and electrophoresis; will handle larger amounts of test solution than No. 1 or 4
4	Fast	Open texture, medium weight	Widely used for amino acids, sugars, etc.
7	Medium fast	Rough surface	Intermediate in speeds to Nos. 1 and 4
17	Medium	Soft paper, very thick	For small-scale preparative chromatography and electrophoresis
20	Very slow	Very uniform, close texture	Produces very even and compact spots for most types of compounds which have been chromatographed
31 extra thick	Fast	Very thick, acid washed	Very widely used for electrophoretic separations
54	Fast	Single acid-washed, hardened, great wet strength	Very popular for sugar separations and impregnation with absorbents such as alumina, starch, magnesia, etc., for large sheets in 2-dimensional work, avoiding spreading in manipulation
540	Medium	Double-acid washed, hardened	These three grades also used for many inorganic and organic separations where minimum metallic impurity in the paper is required
541	Fast		
542	Slow		

offer a variety of paper grades which can be adopted for various uses. Normally, preliminary tests are run to determine which of the several types available is best for a specific purpose.

The sample is usually applied by small micropipettes which may be calibrated or not. Self-filling micropipettes (1 to 50 μliters) work well. These fill to a fixed height and are emptied by touching them onto the paper. The spot of application should always be kept as small as possible, but where a large volume of solution is to be applied it should be added a little at a time drying each portion in a current of hot air after it has been added. Very small spots can also be obtained with the fine capillary tubes used for taking melting points. These are easily drawn from glass tubing. The tubes will always fill to the same height whenever they are dipped in solution and they can be calibrated by weighing them both empty and full in a sealed weighing bottle. The usual volumes applied are 2 to 200 μliters, and a series of spots are placed about 1 in. apart so that they do not merge during development. Alternatively, the sample can be drawn along the filter paper about 1 in. from the edge. On development, the components of the mixture resolve themselves into lines or bands parallel to the solvent front. This method does not give particularly fine resolution but allows larger quantities of sample to be applied. Distinct separation will be achieved with components which differ markedly in R_f value.

G. DETECTION OF COMPOUNDS

The spot or zone can be identified after development by normal chemical methods. Naturally, no treatment is necessary if the spot is colored. The paper can be sprayed with an atomizer, dipped in the specific reagent, or painted on with a brush. Iodine may be used as a general reagent, the paper strips being hung in a sealed tank containing a few crystals of iodine. Brown spots gradually appear with many substances but these often disappear after the paper is removed from contact with the iodine vapor. Thus the spot can be eluted unchanged. Dilute potassium permanganate or dilute sulfuric acid are useful reagents which reveal the chemical as a white or yellow spot on a brown background. Substances such as acids and bases can be sprayed with indicators. Another technique is to hold the paper over a volatile acid (acetic) or base (ammonia) to detect any difference in pH between the background and the spot. Again, the paper should be routinely examined under ultraviolet light to detect any fluorescent substances. Amino acids are detected by ninhydrin used as a 0.2% solution in water-saturated butanol.

For aromatic amines, Bratton-Marshall's reagent is a sensitive reagent. The paper is sprayed with 2.5% sodium nitrite in 0.5 N sulfuric acid and then with 0.1% N-1-naphthyl ethylenediamine in water. A reddish-purple color results. Amines also give an intense yellow color with Ehrlich's reagent, 0.5% dimethylaminobenzaldehyde dissolved in butanol with some glacial acetic acid added. Primary and secondary amines can be detected with an alkaline solution of sodium naphthoquinone sulfonate. Alkaloids can be detected by Dragendorff's reagent. Fatty acids of low molecular weight have

been detected by spraying with a 0.5% solution of bromocresol green. The acids appear as yellow zones. Phenols can be detected by spraying with an alcoholic solution of diazotized sulfanilic acid (Pauly's reagent) followed by exposure to ammonia. Ferric chloride or ammoniacal silver nitrate can also be used. Some polyphenols fluoresce under ultraviolet light.

Carbohydrates can be detected by spraying with ammoniacal silver nitrate (equal volumes of 0.3 N silver nitrate and 5 N ammonium hydroxide are mixed immediately before use). On standing or heating, brown or black spots appear in the presence of the reducing agent. This reagent is effective for the detection of reducing sugars but not for nonreducing sugars.[2]

H. METHODS OF QUANTITATIVE SPOT DETECTION

There are several methods by which a spot can be estimated quantitatively on paper.[22]

1. Measurement of Spot Length

It has been reported that the logarithm of the drug concentration in the spot is directly proportional to the logarithm of the spot length. This was found to be valid for sucrose over the range of 4 to 450 μg., that is,

$$\log \text{concentration} = K \cdot \log (\text{spot length})$$

2. Planimetric Measurement

The area of the spot size is determined by measurement with a planimeter. Accuracy has been claimed to 2%.

3. Method of Counting Squares

A sheet of millimeter-ruled graph paper is placed over the spot (or a tracing of the spot) and the number of squares equal to the spot area counted. The method can be tedious but results accurate to 5% have been achieved.

4. Weighing the Excised Spot

The spot is dried after development, weighed, and compared with a blank piece of the paper chromatogram of equal area. Results have been claimed accurate to 5%.

5. Method of Visual Comparison

Since the intensity of the color and the size of the spot are proportional to the concentration of drug, a rough estimation of the quantity of substance in an unknown concentration can be obtained by developing on the same chromatogram a series of dilutions of the standard and unknown solutions.

Where a spot of unknown concentrations matches a spot of known concentration relative to area and density of color, it is assumed that the unknown is equal to the standard. The same volume of standard and unknown must be used throughout, since the volumes of solution applied alter the area of the spot. Volumes of 1 to 100 μliters are used.

A variation of this technique can also be employed. A series of drops containing known concentrations are placed on the paper. Then a series of dilutions of the unknown are superimposed on the same spots and on blank spaces of the paper. The volume of solution for standards and unknown must remain the same. After development, the spots of the standard solutions are compared with those containing the unknown plus an added quantity of standard. From this the concentration of unknown is calculated. The method can give errors up to 30%.

6. Elution

This is probably the most widely used method. The section of the developed filter paper is cut out and eluted with an appropriate solvent such as ethanol or chloroform. The eluate is then made up to volume and measured in a spectrophotometer or colorimeter and compared with a standard of known concentration. Recoveries of 95% or more are frequently achieved. The excised spot is usually placed in a small volume of solvent, shaken, removed, and rinsed with some fresh solvent, after which it is made up to volume. Alternatively, the piece of paper containing the required substance is placed between two glass slides resting in a Petri dish of water. The water rises between the slides by capillary action and eventually drops off the end of the paper into a little beaker. The beaker containing the slides must be placed in a large outer vessel to prevent evaporation.

It should also be remembered that in descending paper chromatography the paper can be cut in zig-zag fashion at the bottom of the strip. The solvent gradually drips off the tip of the strip and can be collected in a beaker. In the process, the solute is eluted.

Partition chromatography can also be carried out on paper. The paper must be kept in a closed chamber where the atmosphere is saturated with the vapor of the solvent system. For example, if the solvent is water-saturated chloroform, equal volumes of water and chloroform are shaken together until mutually saturated. The chloroform is then separated and placed in a container. It will be used as the mobile or developing phase on the paper strips. The aqueous layer is transferred to a second container. Both the aqueous and organic phases are placed in a sealed chamber and allowed to stand until the atmosphere is saturated with the vapor of the solvents. The water absorbed by the filter paper is regarded as the stationary or immobile phase. A drop of the solution to be analyzed is applied to the strip and, after drying, the paper is placed in the chromatography chamber and equilibrated

with the vapor for about an hour. The strip is then developed by either the ascending or descending method.

The paper may be soaked or treated with a specific substance to aid the separation. For instance, paper strips have been impregnated with alumina by soaking them in a solution of ammonia alum, draining, and leaving to hang overnight in a chamber containing ammonia gas. The paper is then washed in running water for 6 hr and dried. Progesterone, estrone, estradiol, and the acetates of various corticosteroids can be separated on these strips using benzene as the developing solvent. Estrone, testosterone, and androsterone are chromatographed with tetralin as the solvent. In reversed-phase partition chromatography, the stationary phase is organic and is usually absorbed by the paper which is then developed with the aqueous mobile phase.

I. EXPERIMENTS

Paper chromatography is used extensively in all branches of pharmaceutical chemistry both for the quantitative estimation of drugs and for their identification. It is also used in pharmacognosy as a rapid and simple method for identifying plant constituents. The following examples, which can be attempted as experiments, have been chosen to illustrate the use of this technique in pharmaceutical analysis.

1. Paper Chromatography of the Tetracycline Antibiotics[23]

Tetracycline, chlortetracycline, and oxytetracycline, in tetracycline preparations can be separated and detected individually. Whatman No. 1 filter paper is soaked with pH 2.5 phosphate buffer, dried, and 40 μg of the antibiotic applied. Reference standards of the three antibiotics are run at the same time. The paper is developed by descending chromatography using butanol-acetic acid/water (2:1:1), air dried, treated with ammonia vapors for a few minutes, and the spots located by irradiation with ultraviolet light. The sensitivity of this reaction is about 1 μg.

2. Determination of the Vitamin C Content of Orange Juice[7]

Place 20 μliters (or other suitable volume) of orange juice on a strip of Whatman No. 1 paper and develop with ether/formic acid/water (5:2:1). Spray the dried paper either with equal volumes of 0.1 N silver nitrate and 0.1 N ammonia, or with 0.1% 2,6-dichlorophenolindophenol in ethanol. Vitamin C should have an R_f of about 0.6. The concentration of vitamin C can be determined by comparison with a suitable standard developed simultaneously on the paper.

3. Separation and Identification of a Mixture of Free Amino Acids

Prepare a mixture of leucine and aspartic acids in ethanol and spot a drop of this mixture onto an 8- by 8-in. piece of Whatman No. 1 paper. Place

individual samples of leucine and aspartic acids at suitable distances on the paper. Develop the chromatogram with 70% ethanol and spray the dried paper with 0.2% ninhydrin in water-saturated butanol, followed by heating in an oven for a few minutes. The amino acids appear as bluish-purple spots. Aspartic acid has an R_f of 0.5 and leucine an R_f of 0.8.

4. Clarification of Identity of Powdered Cascara and Powdered Rhubarb[7]

Verify that these powders contain anthraquinone glycosides by boiling 1 g of each powder with about 5 ml dilute sulfuric acid for 2 min. This will hydrolyze the glycosides to yield aglycones which are soluble in hot but not in cold water. Filter the hot solution, cool the filtrate, and extract with a little benzene, shaking gently. Apply spots of this solution to Whatman No. 70 paper and develop with pure toluene by the ascending method. About 90 min should be sufficient. Examine the dried paper in daylight and ultraviolet light and note the colors of spots in both. Spray with 0.5% magnesium acetate in methanol and heat gently. This changes the colors and increases the permanency of the spots. Re-examine the chromatogram in daylight and ultraviolet light. For identification purposes, use the following approximate reference values.

Constituent	R_f
Chrysophenol	0.9
Aloe-emodin	0.8
Emodin	0.5
Rhein	0.0

The cascara solution gives red spots at approximately 0.5 and 0.8, denoting the presence of emodin and aloe-emodin. The rhubarb solution gives yellow spots at 0.0 and 0.9 denoting the presence of rhein and chrysophenol.

5. Identification of Barbiturates[24]

Solutions of phenobarbital, butobarbital, amobarbital, pentobarbital, and secobarbital, in a strength of 20 mg/ml in ethanol are prepared. An 8- by 8-in. Whatman No. 1 filter paper sheet is spotted with 1 μliter of each of the solutions 1 in. from the base of the sheet. The paper is mist sprayed with 0.5 *M* sodium carbonate so that uniform dampness is achieved without soaking. The paper is immediately placed in a tank containing ethylene chloride as the mobile solvent and development allowed to proceed for 4 to 6 in. The paper is removed, dried, and sprayed with 0.05 *N* silver nitrate in ethanol, and placed in an oven about 100° until tan spots appear in a brown

background. Standards must be developed on the same sheet as the unknowns for purposes of comparison.

Drug	R_f
Phenobarbital	0.02
Butobarbital	0.18
Amobarbital	0.35
Pentobarbital	0.47
Secobarbital	0.56

6. Chromatographic Separation of Dichlorophene and Hexachlorophene[18]

Strips of Whatman No. 1 paper (6 × 30 cm) are soaked in glacial acetic acid and then developed in a tank with *n*-heptane saturated with glacial acetic acid. Parallel chromatograms are run with the pure compounds and with a mixture of the two compounds at room temperature. After development and drying, spray the strips with a freshly prepared mixture of equal parts of 1% aqueous ferric chloride and 1% potassium ferricyanide solutions. The compounds can be eluted from the paper with *n*-heptane saturated with acetic acid. Collect a total volume of 50 ml. Determine the ultraviolet absorption at 290 mμ and compare with an appropriate standard. R_f dichlorophene, 0.32; hexachlorophene, 0.63.

7. Separation and Estimation of Phenylephrine, Chlorpheniramine, and Dextromethorphan[25]

A two-dimensional ascending paper-chromatographic method is applied to the separation of the above drugs. Standard spots containing 20 μg chlorpheniramine maleate, 75 μg phenylephrine hydrochloride, and 100 μg dextromethorphan hydrobromide, are applied to an 8- by 8-in. sheet of Whatman No. 1 paper. The paper is rolled into a cylinder and placed in a solvent tank containing a chloroform/ether (1:1) solvent system which separates the chlorpheniramine maleate from the other two drugs in about 2 hr. The paper is removed and dried. The chlorpheniramine will be very close to the solvent front, and this area is lightly sprayed with Dragendorff's reagent. The chlorpheniramine will appear as a red spot on an orange background and this area of the chromatogram can be cut off. The paper is turned at right angles to previous direction of solvent flow and again rolled into a cylinder. It is developed with butanol/acetic acid/water (5:1:3), and about 3 or 4 hr are required to separate phenylephrine and dextromethorphan. Phenylephrine is located by scanning with long wave ultraviolet light (365 mμ) and the dextromethorphan is located with Dragendorff's reagent (Fig. 11.10).

The area of the three spots can be measured in square millimeters, using

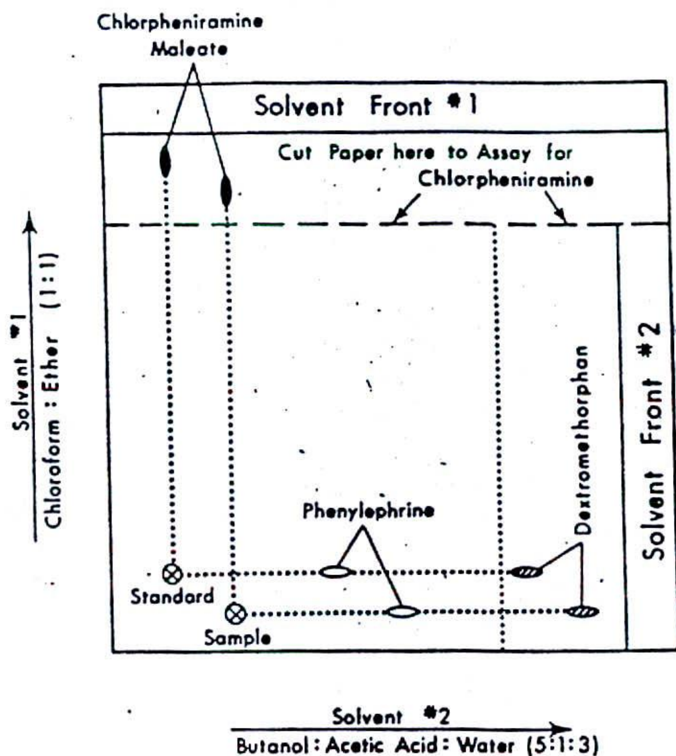

FIGURE 11.10: Paper-chromatographic separation of phenylephrine, chlorpheniramine, and dextromethorphan.

millimeter-ruled graph paper. The concentration of the sample can be determined by comparison with a standard using the relationship

$$\frac{\log \text{standard (mg)}}{\log \text{sample (mg)}} = \frac{\sqrt{\text{area of standard (mm}^2)}}{\sqrt{\text{area of sample (mm}^2)}}$$

QUESTIONS

Q11.1. Outline the procedure to be followed in preparing a column for chromatographic analysis.

Q11.2. How may chemical substances which have been separated on a column be detected?

Q11.3. Describe briefly what is meant by partition chromatography. Derive the equation which correlates the rate of movement of a chemical on a column

to the partition coefficient of that chemical between the stationary and mobile phases, that is,

$$R_f = \frac{A_1}{A_1 + \alpha A_s}$$

where

$$R_f = \frac{\text{distance traveled by chemical}}{\text{distance traveled by solvent}}$$

A_s = cross-sectional area occupied by stationary phase

A_1 = cross-sectional area occupied by the mobile phase

$$\alpha = \frac{\text{concentration of chemical stationary phase}}{\text{concentration of chemical in moving phase}}$$

Q11.4. By what methods may thin-layer chromatograms be prepared?

Q11.5. Name *twelve* reagents which may be used to detect compounds on thin-layer plates.

Q11.6. Discuss the procedures used in quantitative thin-layer chromatography?

Q11.7. Describe the experimental procedure to be followed in preparing a descending paper chromatogram.

Q11.8. Discuss the methods available for quantitative paper chromatography.

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