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Drug separation by thin-layer chromatography

Russell Allan Knott
University of Nebraska Medical Center

DRUG SEPARATION BY THIN-LAYER CHROMATOGRAPHY

Russell A. Knott

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I. Introduction

Although thin-layer chromatography is a newer technique than either column or paper chromatography, it is related to both. Thin-layer chromatography is on a micro scale and is a relatively new technique of adsorption and a partition chromatography, which is a supplement to the known methods of column, paper, ion-exchange, and precipitation chromatography. The range of its applications has been increasing rapidly, and recent developments have closed an existing gap in the micro chromatographic method. Besides being put to use in new areas, thin-layer chromatography is replacing paper techniques in other fields. [2, 3, 4, 20, 22, 24]

In brief, thin-layer chromatography involves the coating of a glass plate with a thin, adhering layer of adsorbent. The thin layer of adsorbent represents an open column, and is the stationary phase of the system. The mixture to be analyzed is then spotted on the adsorbent. The plate is placed in a tank containing an appropriate solvent, the mobile phase, and migration of the spots occurs at a rate characteristic for each substance in the mixture. Only short travel distances of 10-14 cm. are required. The plate is then removed from the tank and dried, after which it is sprayed with reagents which make the spots visible. Identification of each spot is done by measuring the Rf, i.e., $Rf = \frac{\text{distance of migration of spot}}{\text{distance of migration of solvent}}$. [2,4]

The use of thin-layer chromatography in laboratories throughout the world is a result of the numerous advantages of this process. This technique has a much faster development time than either column or paper chromatography, ranging from 20-60 minutes for most applications, depending on the solvents used and the thickness of the layer. Economy compared to more elaborate chromatographic methods, and versatility are also important. Thin-layer chromatography has great resolving power due to the compactness of spots, being 10 to 100 times more sensitive than paper chromatography. Such high sensitivity requires only limited sample quantities, with a minimum sample of about 0.5 mcg. being sufficient for analysis. This ultra micro technique produces sharper separations than either paper or column chromatography can provide, and may be used for trace analysis. The nature of the materials used allows the application of a wide variety of harsh spray reagents which would destroy paper chromatograms. The use of pure inorganic adsorbents eliminates the impurities which cause undesirable inherent fluorescence in paper chromatograms. [2, 3, 4, 20, 24, 26]

The speed of thin-layer chromatography is one of its most important attributes, and is mainly due to the qualities of the adsorbent. Small, uniform particle size, and the thinness of the layer result in improved capillary action of the solvent flow. Short migration distances also reduce the time factor. Spots are more discrete because of limited solute diffusion, thus resulting in greater sensitivity. Variation of adsorbents, solvents, sprays,

and developing technique provides the advantage of versatility. [2, 26]

Disadvantages of thin-layer chromatography are few and are being reduced by the recent development of new techniques, which will be described later. The main difficulty lies in adapting the technique to quantitative analysis. Also, the results obtained are less reproducible in different systems, although within a given system (i.e., a single plate), the rate of movement of the spots relative to each other is constant. A minor problem lies in the fragility of the adsorbent layers, which requires careful handling. [24]

Historically, the first attempt to make chromatographic separations using a thin adsorbent layer was made in Russia in 1938 by Izmailov and Schmiber. [15] In the United States in 1949, Meinhard and Hall [18] improved the process by adding a binder to adhere the adsorbent to glass. Again in the United States in 1954, Miller and Kirchner [19] developed a practical means of applying the adsorbent to glass plates using a stainless steel applicator. Stahl [25] of Germany developed and standardized the first practical equipment for preparation of the plates. However, it was not until 1961 that standard, commercially manufactured equipment became available in this country. Since that time, numerous modifications and improvements have been developed. [2, 4]

The numerous applications of thin-layer chromatography necessarily precludes a detailed description of each. This discussion will be limited to the use of thin-layer chromatography

in the separation and identification of certain relatively large groups of drugs which are frequently encountered in cases of fatal and non-fatal poisonings, namely, the barbiturates, tranquilizers, and narcotics.

Although the value of thin-layer chromatography as a tool in forensic medicine has previously been established by experts in the field,[3, 5, 6, 20, 22, 26, 27, 28] this writer has attempted to ascertain the feasibility of applying thin-layer chromatography to use in our clinical laboratories for toxicology problems. Results of this relatively brief study will be compared with the more extensive work of others.

While the reagents used in the separation of barbiturates, tranquilizers, and narcotics are relatively specific for these drugs, the principles and procedure of thin-layer chromatography are similar regardless of the substance being analyzed.

It should be possible to separate any compound providing there is a difference in the adsorption qualities of the fractions involved. This may be accomplished by proper selection of adsorption climate, adsorbent, and solvent. Each fraction of the compound to be analyzed has a characteristic migration rate in the moving solvent. The specific tendency of the fraction to remain stationary by adsorption on the adsorbent layer is opposed by the tendency of the fraction to migrate with the moving solvent, thus determining the migration rate of each fraction. There is a state of nonequilibrium between these opposing forces. This

state results in the differential migration of each compound and its separation into fractions. [2,4]

On the basis of previously known facts, as stated in Brinkmann,[4] these fractions will separate in a distinct sequence according to the following rules:

"1. Saturated hydrocarbons are not adsorbed at all, or only slightly.

2. The adsorption of unsaturated hydrocarbons increases with the number of double bonds and with the number which are in conjugation.

3. If functional groups are introduced into a hydrocarbon, the adsorption affinity increases in the following sequence:

— CH_3
— O-Alkyl
— C=O
— NH_2
— OH
— COOH "[4]

These influences may be schematically illustrated (Figure I).[4]

II. Equipment and Materials

The equipment used in thin-layer chromatography is inexpensive and uncomplicated in operation. It is desirable to use glass plates of a standard size, 20 x 20cm, 10 x 20cm, or 5 x 20cm,

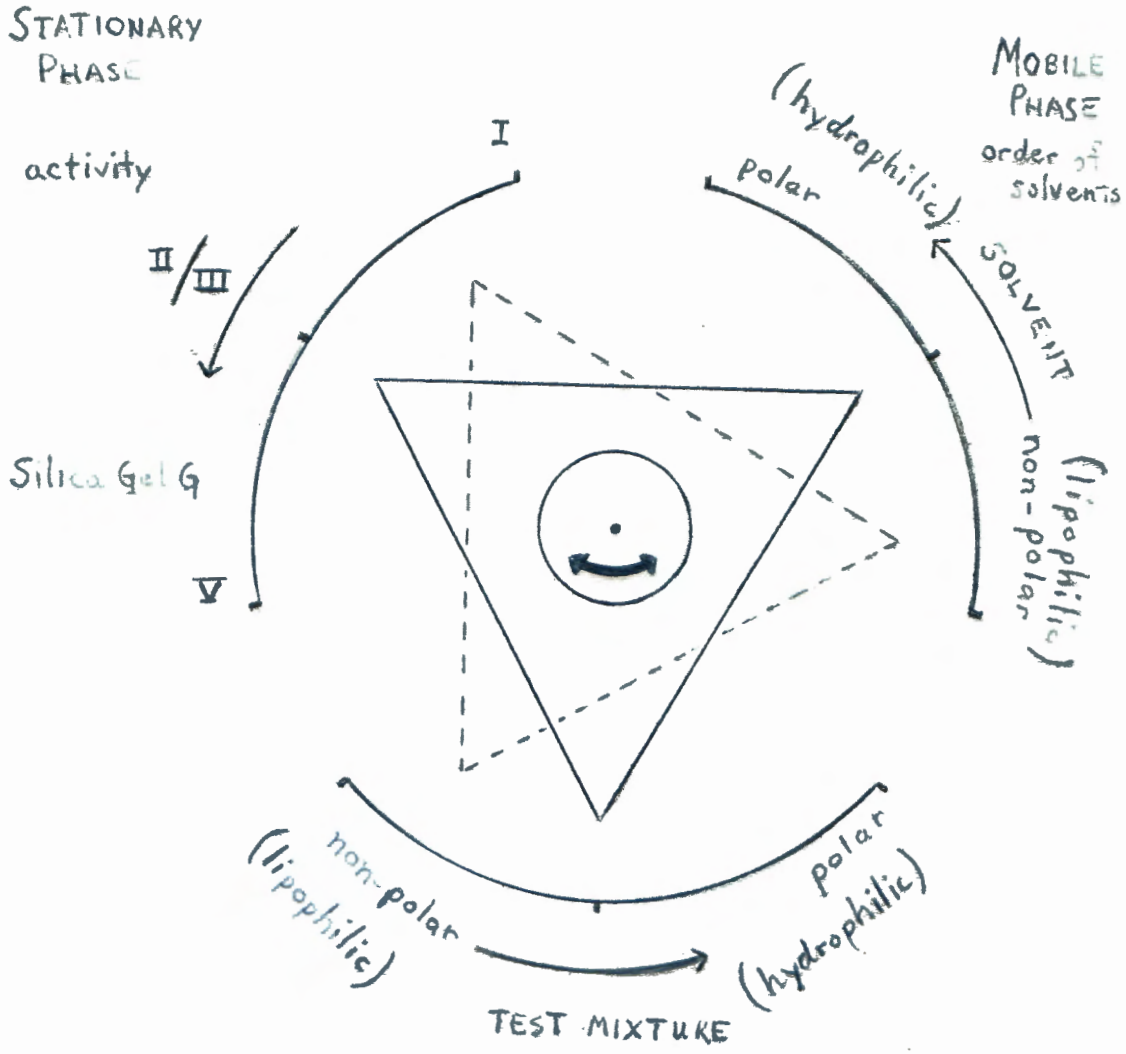

FIGURE I

The Three Major Variables in Adsorption Chromatography

The Roman numerals refer to the Brockmann activity scale. The triangle is rotated about its center. If a quantity is given, one point of the triangle is rotated toward it, and the two remaining points indicate possible applications. If two quantities are given and two points are rotated accordingly, then the third point indicates the remaining choice. [4]

and about 3/16" in thickness. A plastic template is needed to secure the plates. A stainless steel, adjustable applicator is used for coating the plates with adsorbent. A standard rack for storing the prepared plates, a drying oven, a dessicator, and a labeling template for marking the plates are required. Spots are applied with a microsyringe or micropipet. Additional requirements are a suitable developing tank and a sprayer for applying chemical reagents to the plates. [2, 4, 20, 26, 28, 30] All reagents and solvents used are commercially available analytical grade chemicals.

Many adsorbents are available for thin-layer chromatography, but the one most suitable for drug separation is Silica Gel G. This adsorbent is composed of silicon dioxide and contains a calcium sulphate binder. The binder allows for a faster operation time. Silica Gel G is excellent for the rapid separation of neutral, acid, and basic substances. This adsorbent provides the uniformity and smallness of particle size (5 to 25 microns), which is essential for adherence to the plates, speed of development, and concentration of the spots. [2, 4, 20, 26]

III. Procedure

Prior to preparing the adsorbent slurry, five 20 x 20cm clean glass plates are mounted on the plastic template. The plates should be good quality flat glass, finished for safe handling and cut with 90° angles for proper fit on the template. [2, 4, 26]

The standard procedure for coating five 20 x 20 cm plates with adsorbent is to mix 30 gm. of Silica Gel G with 60 to 70 ml. of distilled water. This mixture is shaken in an Erlenmeyer flask and the resulting suspension is called a slurry. This slurry must be used immediately, as it sets rapidly. Adding water will somewhat extend the drying time. [4, 20, 26]

The quick setting time of the slurry necessitates preparation of individual batches and immediate use, which is often inconvenient. Fike and others [11] have devised a procedure whereby a large amount of slurry may be prepared for use in coating many plates over a long period of time. They use a mixture of 2.5 parts water or other aqueous solution (e.g., 0.1M sodium hydroxide or 0.1M potassium bisulphate) to one part Silica Gel G. Such slurries have been used to coat plates over a period of three months, and use over a longer period seems feasible. No aggregates of Silica Gel G formed with any of the slurries prepared in the above manner. Coatings of adsorbent from these slurries showed as good adhesion to the plates and resistance to abrasion as did conventional coatings. Finally, results of chromatography obtained on these coatings fell within the range of values found when using conventional coatings.

It is desirable to standardize the activity of the adsorbent layer in order that satisfactory separations may be obtained. Water content of the adsorbent layer is a decisive factor in determining the grade of activity. Water content can be

controlled by drying time and temperature, as well as the period of activation. Testing the activity of the layer is done by means of the separation behavior of a three-dye mixture, which is commercially available. [26]

Coating of the plates with a uniform film is very important, and is best accomplished with the Desaga/Brinkmann adjustable applicator. The applicator can be adjusted to control layer thickness. A layer 250 microns thick is satisfactory for most routine separations. The slurry is poured into the applicator chamber, the chamber is opened by rotating a large lever, and the applicator is rapidly pulled across the series of plates and the chamber closed at the end of the run. The layer must be even and uniform, but this is easily achieved with practice. [2, 4, 26]

If large or variable sized plates are desired, the commercial applicators are inadequate, being limited to use with plates of a single width. Large plates would have the advantage of enabling many samples to be run at once or allow the recovery of fractions of a large amount of any one sample. Lees and DeMuria [17] have devised an inexpensive and rapid technique for applying a uniform adsorbent layer on almost any size rectangular plate. Using their method, narrow strips of adhesive tape are applied to opposite edges of a plate. The thickness of the adsorbent layer depends on the thickness of the tape. A sufficient amount of slurry is poured along an untaped edge of the plate. Using a thick, uniform glass rod, the slurry is drawn between the taped edges, gliding

the rod along the tape surface. After air drying for ten minutes, the tape is removed and the plate is ready for activation. Simplicity, versatility, and economy are attractive advantages of this technique.

After the coated plates have air dried for 10 to 30 minutes, they are placed in a special rack and activated. Activation for toxicological work is done by heating the plates for 30 minutes at 110°C. To protect the activated plates from moisture, they are stored in a dessicator until needed. Plates stored for long periods may be reactivated by heating again. [2, 4, 26]

Prior to the application of samples to a plate, a series of parallel columns 1.2 cm apart are ruled on the plate using a pencil and the labeling template. Again using the labeling template, ethanol or chloroform solutions of the samples are applied with a micropipet in the center of each column 1.5 to 2.5 cm from the bottom of the plate. Samples should always be applied as a small spot to limit the sample to a minimum area and improve separation quality. Sample quantities should be 5-10 mcl. per spot. Known standards may be spotted alternately with unknowns to facilitate identification of the latter.[2, 4, 26, 28]

After the plates have been prepared in the above manner, they are ready for development. One or two plates are placed vertically in a glass tank of proper size and which provides a good seal. The tank should contain the proper solvent in sufficient quantity to cover the lower edge of the plate to a

height of 1.0 cm. The choice of solvent is critical and varies with the compound being analyzed. In the separation of barbiturates, tranquilizers, and narcotics, one-dimensional development is used. In the separation of various other compounds, either multiple or two-dimensional development may be used. [4, 26, 28]

In the use of certain solvents, the "edge-phenomenon" may be encountered, resulting in zones of similar samples not being located at the same height. This is due to evaporation of solvent from the plate edges, causing the Rf values to increase from the center of the plate toward the edges. This is easily counteracted by lining the tank walls with filter paper which has been immersed in solvent. This creates a saturated atmosphere, which both offsets the edge phenomenon and reduces developing time. [4, 26]

As the solvent migrates up the adsorbent layer, a "front" is formed by the leading edge of solvent. When the front reaches a predetermined mark, the plate is removed from the tank and air dried. The interrupting line is usually drawn at 10 cm, and it takes the solvent 20-60 minutes to reach this point. Vizualization of the characteristic fractions of a separated compound is accomplished by spraying the dry plate with an appropriate reagent. Distinct spots are developed by the spray, and are identified by staining characteristics and Rf values. [2, 4, 26, 28]

A particularly frustrating problem, which occurred during the early stage of plate development, was occasionally encountered by this writer. As the solvent front began to migrate past the

spotted samples, the lower edge of the adsorbent layer would crack and flake off the plate, often destroying the spot before adequate migration occurred. Valuable time was lost in preparing new plates. This event would occur unpredictably with random plates regardless of how thoroughly the plates were cleaned prior to application of the adsorbent, and regardless of the fact that Silica Gel G contains a calcium sulphate binder. No solution to this problem was found during the time available for the project on drug separation.

However, a relatively new technique has been developed which appears to circumvent the problem of peeling of the fragile adsorbent layer, which seems to be due in part to the poor adhesive surface offerred by the smooth glass plates. Kabara and others [16] found that by etching the clear glass plates, a more adhesive surface could be provided.

To etch the plates, the surfaces were sand-blasted with a variety of substances, which produces surfaces of varying degrees of roughness. Besides providing better adhesion qualities, the coarseness of the plate surface effected Rf values. Rf values lower than those obtained on plain glass were the result of a very coarse surface. Conversely, Rf values were increased by a finely etched surface. By experimentation it was found that the ideal surface was prepared by sand-blasting the plates with a light medium grain of sand, giving the glass a frosted appearance. [16]

In using these frosted plates for routine thin-layer chromatography, the silica adsorbent layer did not crack and peel from the plate. Rf values were slightly higher in comparison to clear glass plates, when the same solvent was used. The increased mobility of compounds chromatographed on frosted plates was apparently due to the capillary action of the etched surface, and was most noticeable for compounds with a small Rf value. These frosted plates are now commercially available. [16]

Once the finished chromatograms are sprayed, the spots which are developed by the spray tend to rapidly vanish as the reagent dries. This is especially true in the case of the barbiturates. This writer used two methods which are commonly employed to mark the spots before they fade. Usually, the spots were marked with a sharp stylus and the various migration distances measured directly from the chromatogram using a calibrated plastic labeling template. On occasion, photographic equipment was available, and the plates were photographed in color before the spots had time to fade. A centimeter rule was included alongside each plate before it was photographed to facilitate calculation of Rf values directly from the finished prints. As it is impractical to store finished plates for future reference, photography is a useful means of providing permanent documentation of results.

Less expensive techniques than photography have been divised for permanent documentation of results. It is possible to place

a piece of transparent drawing paper over the chromatogram and manually copy the spots. Colored spots may be duplicated on the paper by using crayons. [26] It is possible to preserve the chromatogram itself by spraying the finished plate with Neatan, a commercially available watery dispersion of polyvinylpropionate. This forms a film which can be peeled from the plate, allowing reuse of the plate. The removed films may be pasted on paper and stored in a file for future reference. [4, 26]

Other means of preparing accurate and permanent records are readily available to those with access to some of the modern copying machines. Getz and others [13] describe the use of the photorapid photocopier. Two prints of the plate are obtained, a negative and a positive. Rf values may be calculated directly from the print. Hilton and Hall [14] describe the successful recording of chromatograms using a Xerox 914 office copier. Spots that are too faint to be copied may be marked with a stylus or by scraping away the adsorbent layer at the spot. Connors and others [7] describe a method for direct reading of fluorescent spots using the Turner fluorometer. Rapid, permanent records of chromatograms may also be made without expensive equipment. Zietman [31] describes the use of high speed blue print paper for documentation of thin-layer chromatograms. Development of the paper is simple and inexpensive. Notes may be written directly on the finished blueprint, and photographs of the paper may be obtained for

publishing purposes.

Drug separation by thin-layer chromatography is essentially a qualitative procedure. The analysis of isolated fractions is based on the color and intensity of spots, as well as Rf values calculated on the basis of spot position. The Rf values of identical fractions vary with each plate due to several factors. These factors include such variables as minor variations in different batches of the same adsorbent, adsorbent layer thickness, moisture content, the nature of the sample, and minor impurities in the system. This lack of reproducibility of Rf values may be offset by calculating the Rf ratio of each fraction, using a known or reference compound. Thus,

$$\text{Rf Ratio} = \frac{\text{Rf of unknown spot}}{\text{Rf of reference spot}}$$. 2, 26, 28

Although thin-layer chromatography does not readily lend itself to quantitation, such analysis is feasible. The simplest method of quantitation is to directly measure the area of the spots using the labeling template. It has been determined that there is a linear relationship between the square root of the spot area and the logarithm of the weight of the substance. [2] If Neatan is used to preserve the adsorbent layer, the spot may be removed directly from the film, eluted, and analyzed. [4] It is also possible to scrape the spot from the plate, elute the substance from the adsorbent, and analyze the extract by colorimetry or spectrophotometry. This latter technique has the disadvantage of low recoveries, since some of the substance adheres to the glass plate after the

adsorbent is removed. [2, 21, 26] Direct quantitation may also be performed by using a densitometer to measure spot density, or by tagging the substances with radioisotopes and using a scanner on the finished plates. [2]

A promising new and simple quantitation technique, which obviates removal of the adsorbent from the plate is described by Rabenort. [21] Aluminum foil is fixed to the glass plate and the adsorbent is applied directly on the foil. The plate is processed in the usual manner, and after chromatography the spots are cut out. Elution and analysis of the spot is then carried out. This technique profits from the flatness of the glass plates, and adds the advantage of total substance recovery for quantitative analysis. [21]

IV. Extraction of Samples

When applying drug separation by thin-layer chromatography to forensic toxicology in the investigation of suspected poisoning cases, drug samples may be extracted from several sources. In non-fatal cases, either blood, urine, or stomach contents may be used for analysis. In fatal cases, tissue samples may be used for drug extraction, in addition to the above sources. In the project on drug separation, the procedures described by Sunshine [28] were used for all extractions.

A. Barbiturates and Weak Acids

Whole blood samples are extracted directly with chloroform. Urine, stomach contents, and drugs for standards are first either made alkaline or dissolved in NaOH. These alkaline solutions are filtered and acidified with H_2SO_4, the filtrate is extracted with chloroform, and the extract washed with a pH 7.0 buffer. Tissue samples are homogenized and the homogenate is mixed with chloroform and extracted.[28]

Aliquots of the chloroform extracts obtained from the above procedures are made alkaline and separated from the chloroform. The alkaline extract is made acid and re-extracted with fresh chloroform. This fresh extract is separated from the acid, filtered and evaporated to dryness. The residue is dissolved in a small quantity of ethanol and is ready for direct application to the chromatoplate.[28]

In preparing barbiturate standards using the above procedure, this writer extracted the drugs from commercially available barbiturate capsules. The result of using these concentrated residues dissolved in ethanol was tailing of the spots. This tailing effect made it very difficult to measure the spot centers for calculation of Rf values. After several trials, it was determined that the tailing of spots was due to overloading the capacity of the adsorbent layer. When the ethanol solutions containing the standards were further diluted with ethanol (1:20),

well-defined spots were obtained with good correlation of Rf values.

B. Neutrals (meprobamate group of tranquilizers)

Following the extraction procedures described above for drug, blood, urine, and tissue samples, the original chloroform solution remaining after alkali extraction is washed with water, filtered, and air dried. The residue is dissolved in ethanol and resulting solutions are analyzed.[28]

C. Organic Bases (phenothiazine group of tranquilizers and narcotics)

Biological materials are made alkaline and extracted with chloroform. An aliquot of the chloroform extract is filtered and the filtrate made acid. The acid solution is separated from the chloroform, made alkaline, and re-extracted with fresh chloroform. The fresh chloroform extract is filtered and air dried, and the residue dissolved in ethanol. The ethanol solutions are then analyzed.[28]

D. Separation of Metabolites

Acid hydrolysis is used to separate drugs which are conjugated in the process of metabolism. Concentrated HCl is added to the biological sample and heated over a steam bath for 30 to 45 minutes. These hydrolyzed samples are then extracted using the same procedures as for unhydrolyzed samples.[28]

For the preparation of spray solutions, consult Table I.

TABLE I [28]

Spray Solutions	Preparation of Spray Solutions
(A) For barbiturates	
(1) $HgSO_4$	Suspend, with mixing, 5 Gm. of HgO in 100 ml. H_2O. Add, while mixing, 20 ml. of concentrated H_2SO_4. Cool, dilute to 250 ml. with H_2O.
Diphenyl-carbazone	Dissolve 5 mg. in 50 ml. $CHCl_3$. Store in dark bottle.
(2) Fluorescein	To 500 ml. H_2O add 20 Gm. NaOH and 20 mg. sodium fluorescein.
$KMnO_4$	0.1 per cent aqueous solution.
(B) For phenothiazines	
FPN	Mix 5 ml. of 5 per cent $FeCl_3$ (w/v), 45 ml. of 20 per cent $HClO_4$ (v/v), and 50 ml. of 50% HNO_3 (v/v).
(C) For carbamates	
Furfural	(1) Furfural freshly redistilled and stored in the cold in a dark bottle. Shelf life approximately one month.
Hydrochloric acid	(2) Concentrated HCl.
(D) For organic bases	
Potassium iodo-platinate	1 Gm. of $PtCl_4$ in 100 ml. H_2O is added to 10 Gm. of Kl in 300 ml. H_2O. This mixture is diluted to 500 ml.
(E) For doriden	
Chlorine-starch iodide	Spray each in the following sequence, (1) 20 ml. household bleach diluted to 100 ml.; (2) 5 Gm. phenol in 100 ml. H_2O; (3) 2 Gm. soluble starch, 1 Gm. Kl dissolved in 100 ml. H_2O

Preparation of Spray Solutions

Information concerning solvents and types of sprays will be incorporated into tables listing the results of various drug separations.

V. Experimental Results

A. Barbiturates

In a paper on barbiturate detection using thin-layer chromatography, Sunshine and others [27] used pure drugs to present data concerning twenty-three barbiturates (Table II). The table represents the average of 15 to 20 determinations, with 90% of these giving Rf ratios within ±0.10 of those listed in the table. Absolute Rf values varied slightly with each plate, but the ratio of the Rf of any sample to phenobarbital were reproducible and significant. The relative activity of the barbiturates is indicated by the Rf ratio, from 1.0 for long acting barbiturates to over 2.0 for the very short acting. [27, 29]

Reaction to the various spray reagents provides more data for a more specific identification, but this is not absolutely essential for clinical purposes. The $KMnO_4$ spray identifies thio-barbiturates and barbiturates with unsaturated substituents by producing yellow spots. Cyclobarbital and barbiturates with an allyl group give dark blue to violet spots after spraying with diphenylcarbazone. Comparison of the spots with a standard

TABLE II [28]

	Rf	Rf Ratio	Reactions to Spray Reagents		
			$HgSO_4$	DPC	$KMnO_4$
Phenobarbital	0.33	1.00	W	V	
Barbital	0.33	1.00	W	V	
Vinbarbital (U)	0.36	1.19	W	V	Y
Butabarbital	0.42	1.28	W	V	
Pentobarbital	0.47	1.42	W	V	
Amobarbital	0.48	1.44	W	V	
Secobarbital (A)	0.55	1.67	W	B	Y
Thiopental	*		W		Y
Cyclobarbital (A)	*		W	B	Y

Adsorbent = Silica Gel G

Solvent = chloroform and acetone (9:1)

Spray Reagents = (1) $HgSO_4$ (2) Diphenylcarbazone (3) $KMnO_4$

A = allyl substituent present in 5 position

U = unsaturated side chain present in 5' position

B = blue; V = violet; Y = yellow; W = white

* = run with solvent front

Ratios of Rf Values of Barbituric Acids to the Rf of Phenobarbital

provides a semiquantitative analysis as to their concentration.[27]

The thin-layer technique was also successfully applied to biologic samples from over one hundred victims of barbiturate intoxication. Within 90-120 minutes after starting the extraction, it was possible to report which drug or drugs were present. It was also possible to roughly estimate how much drug was present by comparison of spot concentrations. Thus, a rapid and reasonable answer as to whether or not the patient ingested barbiturate was readily available.[27]

Cochin and Daly[5] demonstrated the practical applicability of thin-layer chromatography in the identification of barbiturates from urine specimens of patients receiving clinical dosages of commonly used barbiturates. Varying amounts of metabolites of the administered drugs were also detected. It was found that the number of metabolites detected varied directly with how soon urine specimens were collected after drug ingestion. Urines collected within four hours after drug ingestion demonstrated the largest amounts of the original compound.

Cochin and Daly[5] also found thin-layer chromatography useful in the determination of barbiturates in tissues and blood. High blood and tissue levels of pentobarbital were found in guinea pigs receiving injections of the drug. In a patient receiving 30 mgm. of phenobarbital three times daily,

phenobarbital was detected in blood samples taken one hour after the second dose. No interfering spots were detected in samples taken from patients receiving barbiturates who were heavy coffee drinkers and/or were taking aspirin in addition to barbiturates, even though the mercurous sulphate spray reagent reacts with caffeine and aspirin.

In this writer's project on drug separation by thin-layer chromatography, sufficient samples of barbiturates were not available to obtain such extensive data as in Table II. The data in Table III represents the average of numerous determinations. Results were not absolutely reproducible from plate to plate, but remained consistent within individual chromatograms. The drugs in Table III were extracted from barbiturate capsules, and used as standards in subsequent procedures.

A small series of chromatograms were run using blood and urine specimens from patients receiving clinical doses of known barbiturates. These samples were collected one and one half hours after the drugs were given, and all extracted in the manner described above; but none was hydrolyzed. Positive results corresponding to the drugs received were obtained in all cases, although the spots which developed were faint compared to those of the standards. In view of this, it was felt that more distinct spots would have been obtained if the samples had first been hydrolyzed. However, it was found that by concentrating the

TABLE III

Drugs	Rf	Rf Ratio	Reactions to Spray Reagents $HgSO_4$	DPC	$KMnO_4$
Phenobarbital	0.31	1.00	W	V	Y
Secobarbital	0.47	1.47	W	V	Y
Amobarbital	0.43	1.34	W	V	Y
Tuinal *	0.43	1.34	W	V	Y
	0.47	1.47	W	V	Y
Cyclobarbital	0.37	1.16	W	V	
Vinbarbital	0.36	1.12	W	V	Y
Pentobarbital	0.41	1.28	None	V	Y
Barbital	0.30	0.94	W	V	Y

* Tuinal contains amobarbital and secobarbital, and formed two distinct spots within that column.

Adsorbent = Silica Gel G

Solvent = chloroform and acetone (9:1)

Spray Reagents = (1) $HgSO_4$; (2) DPC; (3) $KMnO_4$
N.B. The $KMnO_4$ failed to give satisfactory results, in that colors were faint and spots were indistinct. Therefore, its use was discontinued.

V = violet; W = white; Y = yellow

Ratios of Rf Values of Barbituric Acids to the Rf of Phenobarbital

extracts, it was possible to develop clear spots without hydrolysis. An interesting development occurred in patient "F", who received a known quantity of sodium amytal. Besides the spot corresponding to the Rf of amobarbital, another spot which corresponded to the Rf of phenobarbital developed in the column used for patient "F". Examination of the chart showed that this patient was also receiving Tedral three times daily for asthma. Tedral contains 8 mgm. of phenobarbital, thus illustrating the sensitivity of thin-layer chromatography in detecting small amounts of drugs. Consult Tables IV and V for data concerning this series.

B. Tranquilizers

Although the barbiturates are most frequently involved in cases of fatal and non-fatal poisonings in adults, the increasing use of the tranquilizers and their generally ready availability among the public have increased the importance of these drugs as possible instruments of suicide or homocide. In addition, the widespread use of tranquilizers has led to their implication in numerous cases of accidental poisoning. Simple and rapid methods for the identification and analysis of these drugs would be extremely useful in both forensic medicine and toxicology. Thin-layer chromatography has proven to be admirably suited to this purpose. [6, 27]

Numerous studies of the mode of action and metabolism of

TABLE IV

Drugs & Samples	Rf	Rf Ratio	Reactions to Spray Reagents	
			$HgSO_4$	DPC
Phenobarbital	0.36	1.00	W	V
Patient "Su"	0.36	1.00	None	V
Secobarbital	0.56	1.55	W	V
Patient "Hs"	0.55	1.53	None	V
Amobarbital	0.50	1.39	W	V
Patient "Fs"	0.36	1.00	None	V
	0.50	1.39	None	V
Tuinal	0.50	1.39	W	V
	0.56	1.55	W	V

Adsorbent = Silica Gel G

Solvent = chloroform and acetone (9:1)

Spray reagents = (1) $HgSO_4$; (2) Diphenylcarbazone

"Su" = from urine of Patient "S", receiving 30 mgm of phenobarbital, twice a day, for treatment of epilepsy

"Hs" = from serum of Patient "H", who received 3 grs. of Seconal (secobarbital) for sedation

"Fs" = from serum of Patient "F", who received 3 grs. of Sodium Amytal (amobarbital) for sedation. Also receiving Tedral three times daily for asthma (Tedral contains 8 mgm of phenobarbital).

W = White

V = Violet

Ratios of Rf Values of Barbituric Acid Standards and Patient Samples to the Rf of Phenobarbital

TABLE V

Drugs & Samples	Rf	Rf Ratio	Reations to Spray Reagents	
			$HgSO_4$	DPC
Patient "Ss"	0.34	1.00	W	V
Phenobarbital	0.34	1.00	W	V
Patient "Su"	0.34	1.00	W	V
Patient "Hs"	0.52	1.53	W	V
Secobarbital	0.52	1.53	W	V
Patient "Hu"	0.51	1.50	W	V
Patient "Fs"	0.34	1.00	W	V
	0.48	1.41	W	V
Amobarbital	0.48	1.41	W	V
Patient "Fu"	0.35	1.03	W	V
	0.49	1.44	W	V

Adsorbent = Silica Gel G

Solvent = chloroform and acetone (9:1)

Spray reagents = (1) $HgSO_4$; (2) Diphenylcarbazone

"Ss" = from serum of Patient "S" receiving 30 mgm of phenobarbital, twice a day for treatment of epilepsy

"Su" = from urine of Patient "S"

"Hs" = from serum of Patient "H", who received 3 grs. of Seconal (secobarbital) for sedation

"Hu" = from urine of Patient "H"

"Fs" = from serum of Patient "F", who received 3 grs. of Sodium Amytal (amobarbital) for sedation. Also receiving Tedral three times daily for asthma (Tedral contains 8 mgm of phenobarbital.

"Fu" = from urine of Patient "F"

W = White; V = Violet

Ratios of Rf Values of Barbituric Acid Standards and Patient Samples to the Rf of Phenobarbital

tranquilizers have resulted from the extensive use of these medications in the treatment of mental illness. The extreme sensitivity of the thin-layer chromatography technique, often being able to detect less than 5 mcg. of a drug, should allow investigators to follow the metabolism of tranquilizers during both their long term administration and their withdrawal. [6]

Tables VI and VII contain data relevant to the standardization of the phenothiazine tranquilizers and the meprobamate or neutral group of tranquilizers. In addition to the spray reagents used by Sunshine [28] to develop chromatograms of the tranquilizers, Cochin and Daly [6] described another spray reagent useful in differentiating the phenothiazine from their sulfoxide metabolites. This reagent is a 2% aqueous solution of ferric chloride, which forms red to violet colors with the phenothiazines; but not with their sulfoxides.

Being unable to obtain the wide variety of tranquilizers used in Sunshine's [28] experiments, this writer was limited to standardizing but some of the more commonly used tranquilizers. Obtaining reproducible Rf values for the tranquilizers was generally successful, in that consistent results were produced in almost every trial. Excellent drug separation on the plates was achieved, even when as many as four different drugs were spotted in the same column. On spraying the finished plates, colors proved to be sharp and consistent for each drug. Due to the

TABLE VI [28]

Drug	Rf	Rf Ratio	Color with FPN
Promazine	0.65	1.31	Flesh
Promethazine	0.62	1.25	Pink
Chlorpromazine	0.77	1.55	Pink
Prochlorperazine	1.51	1.02	Pink
Perphenazine	0.08	0.15	Pink
Triflupromazine	0.76	1.52	Flesh
Trifluoperazine	1.52	1.03	Flesh
Fluphenazine	0.05	0.10	Flesh
Thioridazine	0.68	1.35	Turquoise
Propiomazine	0.50	1.00	Pink

Adsorbent = Silica Gel G

Solvent = cyclohexane and diethylamine (9:1)

Spray reagent = FPN

Ratios of Rf Values of Phenothiazines to the Rf of Propriomazine

TABLE VII [28]

Drugs	Rf	Rf Ratio	Color with Spray Reagents	
			E	C
Gluthetimide	0.53	2.7	Blue	White
Meprobamate	0.18	0.18	Blue	Black
Carisoprodol	0.33	0.33	Blue	Black
Librium	0.28	0.28	Blue	Black
Mebutamate	0.20	0.20	Blue	Black

Adsorbent = Silica Gel G

Solvent = cyclohexane and ethyl alcohol (8:2)

Spray reagents = chlorine-starch (E)

Furfural and concentrated HCl (C)

Ratios of Rf Values of Tranquilizers to the Rf of Meprobamate

unavailability of furfural and potassium iodoplatmate, it was impossible to prepare spray reagents for the carbamates and organic bases. See Table VIII for data concerning the phenothiazine standards. T

Tests conducted by this writer using samples of blood and urine from patients receiving known amounts of tranquilizing drugs were entirely unsuccessful. Although the drug dosages these patients were receiving were known to be high, no trace of drug could be found in serum or urine. Concentration of extracts was not successful as it was with the barbiturates. Several futile attempts to obtain satisfactory chromatograms exhausted available supplies of patient samples. Insufficient sample quantities remained to carry out hydrolysis. However, it would seem likely that elimination of metabolites by hydrolysis would have produced extracts containing sufficient drug to produce satisfactory chromatograms.

C. Narcotics and Related Compounds (Organic Bases)

In the routine separation of the organic bases, Silica Gel G is used as the adsorbent. To obtain movement of the spots, acidic or alkaline solvent systems are required, since neutral organic solvents are not effective. Data on the Rf values of the organic bases separated in this manner may be found in Table IX. It has been suggested that an organic alkali be incorporated into the adsorption layer, thus creating an alkaline environment. This

TABLE VIII

Drugs	Rf	Rf Ratio	Color with FPN
Prochlorperazine	0.48	1.00	Pink
Thioridazine	0.62	1.29	Turquoise
Chlorpromazine	0.71	1.48	Pink
Imipramine	0.78	1.62	Turquoise
Promazine	0.60	1.25	Flesh
Fluphenazine	0.09	0.19	Flesh
Trifluoperazine	0.47	0.98	Flesh
Perphenazine	0.09	0.19	Pink
"X" ("A"	0.48	1.00	Pink
"X" ("B"	0.60	1.25	Turquoise
"X" ("C"	0.71	1.48	Pink
"X" ("D"	0.77	1.60	Turquoise

Adsorbent = Silica Gel G

Solvent = cyclohexane and diethylamine (9:1)

Spray reagent = FPN

"X" = four drugs spotted in same column

A = prochlorperazine

B = thioridazine

C = chlorpromazine

D = imipramine

Ratio of Rf Values of Phenothiazines to the Rf of Prochlorperazine

TABLE IX [28]

Drugs	A		B	C	
	Rf	Rf Ratio	Rf Ratio	Rf	Rf Ratio
Cocaine	0.73	1.05	Top		
Codeine	0.41	0.58	0.68	0.11	0.19
Demerol	0.66	0.94	Top	0.66	1.20
Dilaudid	0.26	0.38	0.38	0.03	0.07
Dolophine	0.51	0.72	Top		
Heroin	0.59	0.85	0.16	0.01	0.03
Morphine	0.41	0.59	0.66	0.09	0.17
Pontopan	0.39	0.56	0.67	0.10	0.18
Propiomazine	0.70	1.00	1.00*	1.00	0.55

Adsorbent = Silica Gel G

Solvents:

A = methonal and ammonia (100:1.5)

B = chloroform and diethylamine (9:1)

C = cyclohexhane and diethylamine (9:1)

* The Rf of propiomazine in this system is 1.00

Ratios of Rf Values of Organic Bases to the Rf of Propiomazine

facilitates chromatography by increasing the mobility of basic drugs in two ways. First, the adsorption activity of the silica layer is decreased, and second, the solubility of basic drugs in organic solvents is increased. Thus, it is possible to obtain spot mobility without adding aqueous alkaline components to the solvent system. [10, 28]

Emmerson and Anderson [10] have described a method for the thin-layer chromatography of the organic bases which is more versatile and convenient than the technique using alkaline silica gel layers. In the separation of narcotics and related compounds, it is frequently necessary to incorporate a preliminary step to convert all samples to the common chemical form of either a salt or a free base. Although the unknowns are usually isolated as free bases, the known compounds are generally available as hydrochloride or sulfate salts. If the plates are developed in an ammonia saturated atmosphere, single organic solvents may be used to separate the organic bases and their salts. If the sample being analyzed is the salt of a basic drug, exposure to the ammonia atmosphere converts the compound to the free base. Thus, the organic bases and related synthetic drugs will travel on the chromatogram as bases, regardless of whether the compound spotted on the plate was in the form of the base or a salt. Saturating the atmosphere with ammonia meets the requirement that the spots be continually exposed to an alkaline environment during migration of the solvent front.

Use of the above technique eliminates the need for specially prepared plates. Drugs separated in the ammonia development tank exhibited greater overall mobility, and the results obtained were comparable to those found using alkaline silica gel layers in the absence of ammonia.[10]

An evaluation of the use of thin-layer chromatography in the analysis of narcotics was not done by this writer. The potassium iodoplatinate spray reagent was not available, and narcotics samples for standardization could not be obtained without access to a federal narcotics license. Therefore, the time allotted this project was utilized in the analysis of the barbiturates and tranquilizers.

VI. Clinical Applications

In patients with coma of unknown cause, thin-layer chromatography is well-suited for crucial qualitative identifications of barbiturates and other drugs. Until recently, statistics indicated that if coma were the result of a drug, it was probable that this agent was one or more of the barbiturates. However, this is no longer a valid assumption in view of the now widespread use of tranquilizers and sedatives such as meprobamate and gluthetimide (Doriden). Thus, a negative analysis for barbiturates would not exclude drugs as the causative agents of coma. Thin-layer chromatography is useful in forensic medicine as well as

toxicology, providing rapid and specific analysis of drugs used in suicides and poisonings. [2, 28] Another application might be in the identification of quantities of unknown drugs found in the possession of addicts, etc.

Also in the field of toxicology and pharmacology, thin-layer chromatography can be used in the analysis of antibiotics, pesticides, sulfonamides, and antihistamines. In pharmacy, essential oils, resins, balsams, waxes, fatty dyes, tars, terpenoids, carbohydrates, glycosides, etc., may be analyzed by this technique. [1, 3, 9, 22, 25]

Thin-layer chromatography is finding general use in almost all fields of organic chemistry. In biochemistry and clinical chemistry, the majority of papers have dealt with steroids, vitamins, enzymes, lipids, phospholipids, nucleotides, amino acids, piptides, proteins, radioactive tracing studies, etc. Antioxidants, dyes, explosives, antifreeze, photochemicals and stabilizers have all been studied using thin-layer chromatography in the field of synthetic organic chemistry. [1, 3, 8, 9, 12, 22, 23, 25]

VII. Summary

A brief description of the technique of thin-layer chromatography, a short history of the subject, the theory involved, and advantages and disadvantages of this technique are included in the introductory material. Qualitative and quantitative analysis using thin-layer chromatography is discussed. The

employment of thin-layer chromatography in the separation of barbiturates, tranquilizers, and narcotics is described; especially as applied to toxicology and forensic medicine.

Details concerning the essential equipment and materials for the application of this technique are presented. For those who might be interested in conducting work on drug separation, yet who are unfamiliar with thin-layer chromatography, the procedure is described in detail. Included in the procedure are descriptions of several relatively new techniques which expedite the process, allow the use of less expensive equipment, and which provide simple means of adapting thin-layer chromatography to quantitative analysis. The method of extraction of barbiturates, tranquilizers, and narcotics from biological samples is summarized; as is the method of separating these drugs from their metabolites.

The experimental results of experts in this field are discussed, and data tables are presented for these three large groups of drugs. Data acquired by this writer on similar drugs separated by thin-layer chromatography is presented to illustrate both the reproducibility of results and the feasibility of inexperienced personnel applying this technique. Finally, the clinical applications of thin-layer chromatography in toxicology and forensic medicine are described, as well as listing applications in other fields.

VIII. Conclusions

1. Thin-layer chromatography has proven to be an excellent supplement to the other methods of chromatography currently in use. It has been adopted by laboratories on a world-wide scale.

2. Equipment and materials are relatively inexpensive, and well within the budget of most laboratories.

3. While appearing somewhat complex in theory, the technique is comparatively simple in practice. Inexperienced technicians can acquire the necessary skill to produce useful results within a relatively short period of time.

4. Sufficient variations in technique are available to allow adaptation to individual needs. These variations do not significantly affect results.

5. Results are sufficiently reproducible to allow comparison and evaluation with those obtained by other workers.

6. Although best suited for rapid and specific qualitative analysis, adequate means are available to adapt the technique to quantitative analysis.

7. Thin-layer chromatography is an ultra micro technique suitable for trace analysis. Conversely, large amounts of samples can also be analyzed.

8. Thin-layer chromatography is ideally suited for drug separation, particularly the analysis of barbiturates. It is possible to ascertain whether or not barbiturates are present in

a sample, which ones are present, their relative concentration, and the activity of the drugs. Tranquilizers and narcotics may be identified with equal ability.

9. Drug samples may be extracted from blood, urine, stomach contents, and tissue samples.

10. Thus, thin-layer chromatography is a valuable tool in both forensic medicine and toxicology. The technique is especially valuable to physicians treating the comatose victim of drug poisoning, and to pathologists involved in medico-legal work.

11. Finally, the practical clinical applications of thin-layer chromatography are so numerous and varied that the technique may be applied to virtually any medical and/or scientific field.

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