THE DETECTION OF DRUGS IN BODY FLUIDS BY THIN-LAYER CHROMATOGRAPHY

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by

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Papers accepted for publication

1. The Detection of Barbiturates and related Compounds by Thin-Layer Chromatography.
   James Bogan, Edgar Rentoul and Hamilton Smith.
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2. A fatal case of Primidone poisoning.
   James Bogan, Edgar Rentoul and Hamilton Smith.
   J. For. Sci. Soc. - In press.

C O N T E N T S

INTRODUCTION

INTRODUCTION

This programme of research was undertaken to increase the rapidity and sensitivity of present methods of detection and identification of drugs in body fluids. It was felt that some rapid method of screening samples for drugs would be of great value not only in forensic toxicology but in the detection of doping in racing animals. When this study began many different techniques were available for the detection of individual drugs or groups of drugs but the only widely applicable technique was paper chromatography. Paper chromatography requires an elution time of between 4 and 12 hours. It was felt that considerable progress might be made by using one of the more recent forms of chromatography, i.e. thin layer chromatography or gas liquid chromatography. Both of these are faster and more sensitive than paper chromatography.

Gas liquid chromatography is of the order of two to three times more sensitive than either paper or thin layer chromatography. The main objections to its use were the initial cost of the equipment, and the difficulties involved in regenerating columns. This

makes it unsuitable as a routine method of testing a number of samples in a short time. Further the standard of purity of extracts requires to be much higher than that normally available by solvent extraction. Gas-Liquid Chromatography is most useful for confirming the identity of a drug after the initial separation and purification has been done by some other technique. It was decided therefore, to investigate the applications of Thin-Layer Chromatography in toxicology.

THIN-LAYER CHROMATOGRAPHY

Thin-Layer Chromatography

Tswett[1] was the first, to use adsorption chromatography when in 1903 he separated plant pigments by filtering them through calcium carbonate. His work passed relatively unnoticed until 1931 when Kuhn, Winterstein and Lederer[2,3.] separated the components of carotene by a similar method. Initially the method was mainly used for the separation of natural pigments but gradually attention turned to the separation of colourless substances as methods for their detection were introduced. This kind of chromatography, column adsorption chromatography, is now a standard procedure in modern chemistry. However it was suitable only for the separation of lipophilic substances and there was no method available for separating hydrophilic substances until Martin and Synge[4] introduced partition chromatography. In their method columns of silica gel containing known amounts of water were used. The compounds to be separated were adsorbed at the top of the column and 'developed' with suitable organic solvents. An equilibrium was set up between the 'mobile' organic phase and the 'stationary' aqueous phase. If the substances being investigated had different partition

coefficients, they travelled at different rates and were separated. In 1944, Consden, Gordon and Martin[5] replaced the silica gel support with strips of paper and so introduced paper chromatography.

Paper chromatography is used widely and has simplified greatly many complex problems such as amino acid analysis. The changeover from 'closed' (e.g. silica gel in tubes) to 'open' (e.g. paper) columns allowed the use of spray reagents directly on the columns for the detection of colourless substances. Paper chromatography is, however, of little value in separating many lipophilic substances which are easily separated by column chromatography. This limitation has been overcome to a certain extent by the use of acetylated and impregnated paper.

Various methods[6,7] of making columns between glass plates have been described but Stahl[8-12] was the first to describe a method for the coating of glass plates with an adsorbent layer containing a binding agent. He described[9] a method of preparing 'open' columns of adsorbent layer directly on plates. This form of 'open' column adsorption chromatography is known as Thin Layer Chromatography.

Thin Layer Chromatography has considerable advantages over paper chromatography in the separation of lipophilic substances. Even in the separation of hydrophilic substances it has sometimes been shown to be as good as paper chromatography.

The main advantages of thin layer chromatography over paper chromatography are:

1. Speed
2. Sharpness of separation
3. Sensitivity
4. Allowing the use of corrosive spray reagents.

A commercial apparatus is now available for spreading layers onto glass plates. In this a slurry of the absorbent layer in water is spread on glass plates, set out on a template using a device with an adjustable 'gate' which allows layers of various thicknesses to be prepared. Various other methods can be used for the preparation of chromatoplates. They involve spreading the slurry with a glass rod raised at both sides of the chromatoplate. Quite satisfactory layers can be made in this way, but generally, the preparation is time consuming.

DETECTION OF DRUGS

DRUGS FROM AQUEOUS ACID SOLUTIONS

Detection of Drugs

In toxicology, drugs are classified not by their functional groups but by their extractability from aqueous solution of different pH into immiscible organic solvents. Frequently the mode of action of drugs is related to their chemical structure and so classes of drugs are extractable under similar conditions.

Drugs extractable by organic solvents from aqueous acid solutions.

The main classes of drugs in this group are the barbiturates, hydantoins, monoureides and related compounds. The salicylates are also extracted in this group but are determined simply by Ultra Violet Spectrophotemetry and were not studied further.

(a) Barbiturates

The chemical names, trade names and structures of all the barbiturates and related compounds obtainable in this country and some which are only obtainable abroad are given in Table 1.

TABLE 1.

PHARMACEUTICAL NAME	TRADE NAME(S)	CHEMICAL NAME
5, 5-substituted Barbiturates		
Allobarbitone	Dial	5, 5-Diallylbarbituric acid
Allylbarbituric acid	Sandoptal	5-Allyl-5-isobutylbarbituric acid
Amylobarbitone	Amytal	5-Ethyl-5-isoamylbarbituric acid
Aprobarbital	Alurate	5-Allyl-5-isopropylbarbituric acid
Barbitone	Medinal	5, 5-Diethylbarbituric acid
	Veronal	
Butobarbitone	Sonalgin	5-Ethyl-5-n-Butylbarbituric acid
	Soneryl	
Cyclobarbitone	Phanoderm	5-Ethyl-5-(1-Cyclohexen-1-yl)
	Rapidal	Barbituric acid
Heptabarbitone	Medomin	5-Ethyl-5-(1-Cyclohepten-1-yl) barbituric
Phenylmethylbarbituric acid	Rutonal	5-Methyl-5-Phenylbarbituric acid
Probarbital	Ipral	5-Ethyl-5-Isopropylbarbituric acid
Nealbarbitone	Censedal	5-Allyl-5-Neopentylbarbituric acid
	Nevental	
Pentobarbitone	Nembutal	5-Ethyl-5-(1-Methylbutyl) barbituric acid
Penobarbitone	Barbenyl	5-Ethyl-5-Phenylbarbituric acid
	Gardenal	
	Luminal	
Quinalbarbitone	Seconal	5-Allyl-5-(1-Methylbutyl) barbituric acid
Vinbarbitone	Delvinal	5-Ethyl-5-(1-Methyl-1-Butenyl) barbituric
N-methyl Barbiturates		
Hexobarbitone	Cyclonal	5-(1-cyclohexen-1-yl),-1,5-Dimethyl barbit
Methylphenobarbitone	Phemitone	1-Methyl-5-Ethyl-5-Phenylbarbituric acid
	Prominal	
Metharbital	Gemonil	1-Methyl-5,5-Diethylbarbituric acid
Methohexitone	Brietal	1-Methyl-5-Allyl-5-(1 Methylpent-2-ynyl) barbituric
Bromobarbiturates		
Butallylonal	Pernoston	5-(2-Bromo-Allyl)-5-Sec-Butyl barbituric a
Thiobarbiturates		
Buthalitone	Transithal	5-Allyl-5-isobutyl-2-thiobarbituric acid
Thialbarbitone	Kemithal	5-Allyl-5-(1 cyclohexen-2-yl)-2-thio-barbi
Thiamylal	Surital	5-Allyl-5-(1-methylbutyl)-2-thio-barbituri
Thiopentone	Pentothal	5-Ethyl-5-(1-methylbutyl)-2-thio-barbituri
	Intraval	
Glutarimides		
Glutethimide	Doriden	a-Ethyl-a-Phenyl Glutarimide
Bemegride	Megimide	B-Methyl-B-Ethyl Glutarimide
Bromourides		
Carbromal	Adalin	1-Bromo-1-Ethylbutyrylurea
Bromvaletone	Bromural	1-Bromo-2-Methylbutyrylurea
Hydantoins		
Phenytoin	Dilantin	5, 5-Diphenylhydantoin
	Epanutin Eption	

Method of Detection of Barbiturates by Thin Layer Chromatography.

Before searching for a suitable thin layer and solvent system for the separation of these compounds a method of detecting barbiturates on chromatoplates was required. When plates spotted with different barbiturates were viewed in Ultra-Violet light, the barbiturates were observable by dark blue absorption. However the minimum quantity detectable by this method was of the order of 100 µg. and was not sensitive enough for our needs. Spraying with Ceric Sulphate in 18 N Sulphuric Acid followed by heat, a method which is frequently used for detecting unknown organic compounds on Thin Layer chromatograms failed to produce any spots. A number of known colour reactions for the identification of barbiturates were then examined to determine if they were suitable for application by sprays for the location of barbiturates on thin layer chromatoplates.

The most commonly used colour reactions for the detection of barbiturates were those of Parri[13] and Zwicker[15,16].

Parri observed a pink colour when barbiturate was added to a solution of cobalt nitrate and ammonia. Koppanyi[14] modified Parri's method by using Isopropylamine instead of ammonia and made the Parri method suitable as a routine method.

Trial of Koppanyis Method as a spray technique

Reagents

Solution A. One gm. of cobaltous acetate is weighed accurately and dissolved in 100 ml. of methanol.

Solution B. Five ml. of isopropylamine are dissolved in methanol and the solution made up to 100 ml.

Method

Phenobarbitone and Amylobarbitone in varying concentrations were spotted on a chromatoplate and the plate sprayed firstly with solution A and then with solution B. No colours were observable at any concentration

Zwicker's method is based on the formation of copper-pyridine complexes with the barbiturates. The method has many adaptations and the solution which was found most suitable for spraying was that of Schmidt[17].

Reagent

Four mls of 10% aqueous Copper Sulphate, 1 ml Pyridine and 5 ml water were mixed and gave a stable

dark blue solution.

Method

Spots of barbiturates were sprayed with this solution and colours developed. These are shown in Table 3. The colours were distinctive and stable for a number of hours.

Schmidt's adaptation of Zwickers method was found most suitable as a selective spray for barbiturates and barbiturate metabolites on chromatoplates. The sensitivity of this spray was about 10 μg. for the 5,5-disubstituted barbiturates and less for the N- methyl barbiturates.

Deineger[18] and Schmidt[19] recommend immersion of paper chromatograms in saturated mercurous nitrate with resultant black spots as the most sensitive method of detection of barbiturates on paper chromatograms. When chromatoplates were sprayed with saturated mercurous nitrate, clear black spots on a white background resulted. This was the most sensitive method of detection so far tried and was used routinely while a system of separation of barbiturates was being investigated. The limit of detection using this spray was about 1 μg.

Thin Layer Chromatographic separation of Barbiturates.

Initially an attempt was made to prepare chromatoplates of Cellulose 'G' (E.Merck) as these layers should be equivalent to chromatographic paper and the solvents applicable to paper chromatography would be useful. The first few attempts at making these layers produced a very rough surface, the particle size of the cellulose powder being relatively large. Sieving through fine nylon gauze served to eliminate the larger particles but still the cellulose layers were rough, probably due to air bubbles in the slurry. By sieving and stirring with a very fast motor usable layers were prepared.

The solvent system of Algeri and Walker[20] for the separation of barbiturates on paper, n - butanol saturated with 6 N ammonia was tried on 0.25 mm. layers of cellulose 'G'. Mercurous nitrate was unsuitable as a spray when this solvent was used as it reacted with the ammonia. On viewing eluted barbiturate spots under U.V. light, all the spots were found to be long and 'tailed'. On 0.5 mm layers the same results were obtained. The thickness of the layer did not appear to affect the distance moved. The separations were not

sharp enough so other solvent systems were tried. Chloroform/Isopropylamine and Chloroform/Ethylene Diamine in ratios 5/1, 10/1 and 20/1 were tried but all produced long streaks which were of little value.

Silica Gel 'G' layers were tried next. Satisfactory layers were obtainable with this powder after very little practice. On these layers it was found that with chloroform alone and ethanol alone, all the barbiturate spots moved with the solvent front. With cyclohexane alone, none of the barbiturates moved. By varying chloroform/cyclohexane or ethanol/cyclohexane ration it was hoped to achieve a separation.. In cyclohexane/chloroform 4/1 barbiturate spots began to separate with Butobarbitone and Cyclobarbitone with Rf 0.6 (Rf value is defined as the ratio of the distance moved by the substance from the starting point to the distance moved by the solvent from the starting point) and Barbitone moving with the solvent front. However, small variations in the ratio of the constituents of the solvent mixture greatly affected the intermediate Rf values and giving spots which were long and tailed. The system was not used further.

At this time, a solvent system of chloroform/ acetone in the ratio of 9 to 1 was reported. It was suggested that this could be used for the separation of barbiturates. The system was tried and on spraying with mercurous nitrate gave clear, round black spots. Of the three barbiturates run as standards, Butobarbitone and Cyclobarbitone gave similar Rf values of about 0.33 and Barbitone 0.22.

A total of 24 barbiturates were obtained from manufacturers in this country and from abroad. This number includes all the barbiturates listed in the Extra Pharmacopoeia as being available in the United Kingdom. Their pharmaceutical names, trade names and chemical formulae are given in Table 1. Standard 1% solutions in chloroform of these barbiturates were prepared by extraction and recrystallization of the barbiturate from the standard pharmaceutical preparations. These solutions were used for Rf measurements.

Satisfactory layers of silica Gel (G) were also prepared on microscope slides instead of the standard 10 x 40 mm. plate glass plates. The only advantage of plates of this kind was economy when a large number of samples of a similar nature were being analysed.

Rf Values

Rf values are dependent on the following three factors:

1. The activity of the layers
2. The saturation of the development chamber
3. The uniform thickness of the layer

Wide variations in Rf values were found even when the following conditions were standardized:

1. The layers were always 0.25 mm. thick
2. The chromatoplates were activated for one hour at 100°C.
3. The solvent mixtures were allowed to equilibra for 24 hours
4. The development chambers were lined with filter paper to promote saturation

However the order of development was always the same and so standards were run for comparison. The standard used routinely is a mixture of barbiturates place on a spot beside the unknown. The position of unknown substances with relation to the known spots allows a near estimate of their identity, which may then be proved by the reactions with the various reagents described below. Table 2 shows the range of Rf values expected and compare

them with the following proposed system. Two barbiturates (Barbitone and Thiopentone.) are selected as standards and both are spotted onto the positions nearest the edges of the chromatoplate. The unknown substances are spotted between the standards and then the spots are developed. The lower spot (Barbitone) is given the arbitrary value 0.00. The distance the unknown has gone beyond the lower spot is measured and divided by the distance between the two standards giving a comparative position value (R_{t-b}) which generally may be reproduced with twice the accuracy of the R_f values, even though the accuracy of measurement of the R_{t-b} values is decreased due to the shorter distances being measured. In Table 2 the limits of error determined on 12 different elutions for each barbiturate, are shown.

TABLE 2.

COMPOUND	R_f	R_{t-b}
Phenylmethyl barbituric acid	0.12 ± 0.03	= 0.20 ± 0.04
Phenytoin	0.20 ± 0.06	= 0.06 ± 0.02
Barbitone	0.22 ± 0.07	0.00
Probarbital	0.24 ± 0.06	0.06 ± 0.03
Phenobarbitone	0.24 ± 0.07	0.06 ± 0.02
Bromural	0.26 ± 0.07	0.09 ± 0.03
Allobarbitone	0.27 ± 0.11	0.15 ± 0.05
Vinbarvitone	0.28 ± 0.07	0.17 ± 0.02
Butallylonal	0.28 ± 0.08	0.17 ± 0.04
Aprobarbital	0.30 ± 0.14	0.18 ± 0.07
Heptabarbitone	0.31 ± 0.06	0.20 ± 0.03
Cyclobarbitone	0.32 ± 0.10	0.22 ± 0.03
Butobarbitone	0.33 ± 0.12	0.25 ± 0.06
Amylobarbitone	0.33 ± 0.09	0.25 ± 0.04
Pentobarbitone	0.33 ± 0.10	0.25 ± 0.04
Allybarbituric acid	0.36 ± 0.15	0.32 ± 0.03
Nealbarbitone	0.38 ± 0.13	0.34 ± 0.07
Bemegride	0.39 ± 0.14	0.37 ± 0.06
Quinalbarbitone	0.44 ± 0.10	0.40 ± 0.06
Carbromal	0.50 ± 0.06	0.57 ± 0.04
Hexobarbitone	0.50 ± 0.10	0.59 ± 0.07
Doriden	0.52 ± 0.06	0.70 ± 0.06
Metharbital	0.55 ± 0.10	0.72 ± 0.06
Methylphenobarbitone	0.59 ± 0.08	0.75 ± 0.04
Methohexitone	0.67 ± 0.10	0.96 ± 0.06
Thiopentone	0.68 ± 0.09	1.00
Buthalitone	0.68 ± 0.12	1.00 ± 0.02
Thialbarbitone	0.69 ± 0.11	1.03 ± 0.05
Thiamylal	0.70 ± 0.13	1.07 ± 0.03

In practice, in Scotland, the barbiturates encountered in routine forensic analysis are few in number (Table 8) and it is simpler to run standards of Phenobarbitone, Amylobarbitone and Quinalbarbitone beside an unknown. As much information about the identity of the unknown can be found by direct comparison as from R_{t-b} measurements. R_{t-b} values are of most value when an unusual barbiturate is encountered or when an authentic sample of the unknown is not available. A number of other, non-barbiturate, sedatives which are commonly encountered in toxicology and which are extracted in the weak acid fraction was obtained. Their formulae, R_f and R_{t-b} values are also given in Tables 1 & 2. Also included is the glutarimide, Bemegride, which is frequently used as an antidote in barbiturate overdosage.

In conjunction with the R_{t-b} values the chemical reactions of the barbiturates and related compounds may be used to give the absolute identity. For this purpose the plates are sprayed with the reagents, or some of the substance is treated with Sulphuric Acid after the preliminary separation.

The test reagents and their reactions are as follows:

1. Mercurous Nitrate. A saturated solution is sprayed onto the plates, when black spots develop. These spots fade after a few minutes, so they should be marked immediately. This reagent reacts with all the compounds being examined, except Carbromal and Bromural, which are included because of the similarity of their action to that of the long acting barbiturates and because they are extracted from acid solution by organic solvents.

2. Potassium Permanganate. A 2% (w/v) solution is sprayed onto the plates, when yellow-brown spots on a purple background appear. These spots indicate the presence of barbiturates with unsaturated side chains. This reagent may be applied to the plate after the mercurous nitrate.

3. Zwicker's Reagent. 4 ml of copper sulphate solution (10% w/v) are mixed with 1 ml Pyridine and 5 ml water to give a clear dark blue solution. This reagent is sprayed onto the plates causing pink or green spots to appear.

4. Fluorescein-Eosin. This method[21] for the detection of Bromobarbiturates on paper chromatograms was found suitable for use with Thin-Layer Chromatography. It was also found suitable for the detection of carbromal and Bromural. For these latter prior hydrolysis of the

extract with Sodium Hydroxide was found necessary. 10 ml of a saturated solution of fluorescein in acetic acid is mixed with 15 ml glacial acetic acid and 25 ml of 100 volume hydrogen peroxide. This reagent must be prepared fresh as required. The plates are sprayed initially with a sodium hydroxide solution (10% w/v)(unless bromo-barbiturates are being sought) and heated to 100°C for five minutes. They are sprayed with the fluorescein reagent and heated at 100°C for five minutes, when pink spots form.

5. Sulphuric Acid. Maynert and Washburn[22] first observed that barbiturates are frequently attacked when heated with sulphuric acid at 100°C. Curry has used this fact for identification by noting the change in U.V spectra after treatment with sulphuric acid. This was used as follows:

A little of the substance obtained from the preliminary extraction is heated with concentrated sulphuric acid for twenty minutes at 100°C. After cooling the solution is diluted with water, re-extracted with chloroform and evaporated to about 0.1 ml. This is placed on a chromatoplate and analysed as an ordinary sample. Whether it has been destroyed or not by the acid gives a further indication of identity.

Method of Identification

The initial extract is prepared and spotted onto the chromatoplate in the following order - standard; unknown; standard; unknown; the plate is then developed and the unknown spot nearest the edge covered. The remainder of the plate is sprayed with mercurous nitrate solution to reveal the standards and the unknown, if it is a barbiturate, Doriden, Nemegride or Phenytoin. If the unknown spot develops, its position is marked and the R_{t-b} calculated and compared with those listed in Table 2. The same area is then sprayed with potassium permanganate to detect unsaturated compounds. When this is complete, the other part of the plate is sprayed with Zwicker's reagent, which reacts with two groups of barbiturates. The results obtained are compared with the reactions listed in Table 3. If further information is required, the treatment with sulphuric acid described above may be applied. When this investigation is completed, the identity of most of the substances will be known. A few barbiturates will be impossible to distinguish, due to their similar structures and hence their similar chemistry and R_{t-b} values. Fortunately these have very similar biological action.

When the mercurous nitrate spray fails to reveal a spot, the other part of the plate should be treated with the fluorescein spray, when Carbromal and Bromural may appear.

It is often possible to remove the spot from the plate, extract the drug from aqueous acid solution, and rechromatograph it if further tests are required and no further material is available.

TABLE 3

CLASS	COMPOUND	MERCUROUS NITRATE	ZWICKER'S REAGENT	POTASSIUM PERMANGANATE	FLUORESCEIN	SULPHURIC ACID
5, 5-substituted barbiturates	Allobarbitone	+	Pink	+	-	Destroyed
	Allybarbituric acid	+	"	+	-	"
	Amylobarbitone	+	"	-	-	Unchanged
	Aprobarbital	+	"	+	-	Destroyed
	Barbitone	+	"	-	-	Unchanged
	Butobarbitone	+	"	-	-	"
	Cyclobarbitone	+	"	+	-	Destroyed
	Heptabarbitone	+	"	+	-	"
	Phenylmethyl barbituric acid	+	"	-	-	"
	Probarbital	+	"	-	-	"
	Nealbarbitone	+	"	+	-	Partially destroyed
	Pentobarbitone	+	"	-	-	Destroyed
	Phenobarbitone	+	"	-	-	"
	Quinalbarbitone	+	"	+	-	"
	Vinbarbitone	+	"	+	-	"
1, 5, 5-substituted barbiturates	Hexobarbitone	+	-	+	-	Destroyed
	Metharbital	+	Faint Pink	-	-	Unchanged
	Methylphenobarbitone	+	"	-	-	Destroyed
	Methohexitone	+	"	+	-	Partially destroyed
Bromobarbiturates	Butallylonal	+	Faint Pink	+	+	Destroyed
Thiobarbiturates	Thiopentone	+	Green	+	-	Destroyed
	Buthalitone	+	"	+	-	"
	Thiamylal	+	"	+	-	"
	Thialbarbituric acid	+	"	+	-	"
Bromoureides	Bromural	-	-	+	+	
	Carbromal	-	-	+	+	
Glutarimides	Bemegride	+	-	-	-	
	Doriden	+	-	-	-	
Hydantoins	Phenytoin	+	-	-	-	

Sensitivity

Mercurous nitrate is the most sensitive spray and reveals spots when about 1 μg of barbiturate is present. The other reagents are less sensitive and require the following weights of material before a definite spot is seen:-

Permanganate spray	...	...	8 μg
Zwicker's reagent	...	...	10 μg
Fluorescein spray	...	...	10 μg

These figures compare well with the other commonly used techniques for the detection of barbiturates

1. Paper Chromatography	...		10 μg
2. Ultra-Violet Spectrophotometry	...		10 μg
3. Colorimetric	...	...	100 μg

Paper chromatography and Thin Layer Chromatography are relatively unaffected by the purity of extract since systems are chosen which separate the barbiturate from interfering substances as much as possible whereas Ultra-Violet Spectroscopy and Colorimetric methods require a fairly high purity.

Separation From Biological Material

Blood.

The direct extraction of barbiturate is possible from small quantities of fresh blood. With putrefying blood and with increasing quantities, emulsions are liable to form with organic solvents. Using small quantities of fresh blood, chloroform is a suitable extraction medium. 5 ml of blood is shaken directly with two 30 ml portions of chloroform. The chloroform layer is filtered and evaporated to dryness under an infra-red lamp. The residue is dissolved in 0.2 ml chloroform and used to spot the chromatoplates. If a quantitative estimation is required the chloroform is extracted with 5 ml of 0.45 N Sodium Hydroxide and determined on an Ultra-Violet Spectrophotometer using the method of Broughton[23,24]. After measurement, the extracts are recombined, the aqueous solution adjusted to PH 2-3 using 10% hydrochloric acid and re-extracted with 5 volumes of chloroform. This chloroform layer is filtered and evaporated and used to spot the chromatoplate.

Quantitative Estimation of barbiturates in blood[23,24]

5 ml. of blood were extracted with two 30 mls volumes of chloroform. The chloroform layers were filter and the blood discarded. The combined chloroform layers were extracted with 5 ml. 0.45 N sodium hydroxide. The lower chloroform layer was discarded and the aqueous layer centrifuged. To 1.5 ml of the sodium hydroxide extract was added 1.5 ml. 0.45 N sodium hydroxide. The ultra-violet absorbance of this solution was measured between 325 m μ and 200 m μ with 0.45 N sodium hydroxide the reference solution. To a further 1.5 ml of the sodium hydroxide extract was added 1.5 ml. of boric acid buffer. (37.3 g. Boric Acid and 45 g. Potassium Chloride per litre). The ultra-violet absorbance of this solution was recorded with a reference solution of 1.5 ml 0.45 N sodium hydroxide and 1.5 ml boric acid buffer.

The criteria for the presence of barbiturates were:-

1. In borate buffer a maximum at 230-240 m μ.
2. In sodium hydroxide solution a maximum at 252-255 m μ.
3. Isobestic points at 227-230 m μ and 247-250 m μ.

For calculation of the concentration present, the following equation was used:-

$$C = F(D_n - D_b)$$

where C = concentration of barbiturate in solution measured in μg/ml.

D_n = Absorbance of sodium hydroxide solution at 260 mμ

D_b = " " borate " " " "

F, a calibration factor for different barbiturates, has been calculated as

Phenobarbitone	43.7
Barbitone	37.5
Butobarbitone	38.8
Amylobarbitone	41.7
Cyclobarbitone	44.7
Quinalbarbitone	47.5
Pentobarbitone	48.1

Using these methods a quantitative and a qualitative estimation of barbiturates can be accomplished in 1-1½ hours. Using direct extraction the recoveries of 0.2 mgms of barbiturate added to 5 ml of blood were determined. These were:

Butabarbital	86%
Quinalbarbitone	90%
Heptabarbitone	89%

If the blood sample was putrefying or if an analysis was being made for other groups of drugs the prior

precipitation of protein is necessary. Deproteinization can be accomplished by trichloroacetic acid[25] or sodium tungstate[26,27]. The adaptation made by Curry[28] of the tungstate precipitation method was found most suitable and gave very pure extracts.

Tungstate Precipitation

5 ml of blood, 30 ml of water and 1 ml of sodium hydroxide solution (10% w/v) were shaken together and allowed to stand for ten minutes. Then 10 ml of sodium tungstate solution (10% w/v) were added with shaking, followed by 3.5 ml of sulphuric acid (10% w/v) a drop at a time. The resulting mixture was heated on a boiling water bath for ten minutes and allowed to cool, when the precipitated proteins were removed by filtration. When the filtrate was quite cold it was shaken with 2 vols. of chloroform. The aqueous layer was discarded, and the resulting chloroform solution evaporated to dryness by an infrared lamp. The residue was dissolved in 0.2 ml of chloroform and used to spot the chromatoplates. Using this method, the recoveries of 0.2 mgms of barbiturate added to 5 ml blood were determined. These were

Butabarbital	64%
Quinalbarbitone	60%
Heptobarbitone	54%

The two methods have been compared in determinations of barbiturate blood levels in post-mortem samples.

	Concentrations. (mgms %)	
Barbiturate	Direct Extraction (1)	Tungstate Ppt (2)
Amytal	1.85	1.25
Amytal	0.64	0.34
Tuinal	4.10	3.60
Phenobarb.	9.20	7.66
Cyclobarb.	3.22	2.28

There is obviously a considerable variation in the barbiturate blood levels found using the two mekthods and more cognisance should be taken of this.

Urine

Methods of extraction of barbiturates were decided by the identification technique being used. Techniques requiring very pure extracts (e.g. micro-crystalline techniques.) involve laborious and often wasteful extractions. The purity of extract required for thin layer chromatography was not high and extraction was done principally to extract completely both barbiturate and metabolites. Chloroform or ether could be used for extraction provided the urine to solvent ratios were kept high to prevent the formation of troublesome emulsions. Schmidt[29] recommended

Ethyl Acetate as an extraction medium for urine.. This solvent was found to extract more chromogenous material than ether or chloroform but was much less liable to form emulsions. Metabolites of barbiturates are also more soluble in ethyl acetate. For routine testing of samples in the detection of doping in racing animals where the quantity of barbiturate present was low and where metabolites could be important, ethyl acetate was the best solvent.

Method.

10 ml. of urine were made acid to pH 2-3 with hydrochloric acid (10% v/v) and then shaken with 2 vols. of ethyl acetate. The aqueous layer was rejected and the ethyl acetate shaken with 5 ml. Sodium Bicarbonate (5% w/v). The aqueous layer was discarded and the ethyl acetate layer was shaken with 4 ml Sodium Hydroxide (10% w/v). The ethyl acetate layer was discarded, the aqueous layer was adjusted to pH 2-3 with hydrochloric acid (10% v/v) and re-extracted with 2 vols ethyl acetate. The ethyl acetate layer was dried with anhydrous sodium sulphate, filtered and evaporated under an infrared lamp. The resultant residue was treated as for 'Blood'.

Stomach Contents

The sample, usually only a few ml was diluted to 20 ml and made acid with hydrochloric acid (10% v/v). The resulting solution was extracted by shaking with 40 ml of chloroform. The chloroform layer was then cleared by filtering through a paper previously wetted with chloroform, and re-extracted into 4 ml of sodium hydroxide solution (2% w/v). The chloroform layer was rejected and the aqueous layer was acidified with hydrochloric acid (10% v/v). This solution was then extracted with 10 ml chloroform and the aqueous layer rejected. The chloroform solution was evaporated to dryness and the residue redissolved as described above.

Viscera

Viscera were macerated in a blender with an equal volume of saline. The mixture was adjusted to pH 2-3 with hydrochloric acid (10% v/v) and extracted as described for stomach contents.

Extraction of Doxiden

If 'Doxiden' was being looked for, another technique was used, since this substance and its principal metabolites are rapidly destroyed in alkaline solution. The technique recommended was an adsorption chromatographic method and may also be used to prepare a purer extract of barbiturates. The method was as follows:-

The sample as described before was made acid with hydrochloric acid (10% v/v) and extracted with twice the volume of ethyl acetate. Sufficient anhydrous sodium sulphate was added to free the ethyl acetate of water. It was then filtered and passed through a column (1 cm x 5 cm long) of aluminium oxide (100 - 200 mesh, Brockmann Grade 1). This adsorbed 'Doxiden', its metabolites, and barbiturates. The 'Doxiden' etc. may be removed by passing 25 ml of a mixture of chloroform and methanol (9:1) through the column. This eluant was evaporated to dryness and the residue dissolved in 0.2 ml of chloroform as described before.

Determination of Barbiturates in Biological Material.

1. Effect of washing barbiturate extracts with Sodium Bicarbonate solution.

Curry[30] recommended the use of a Sodium Bicarbonate wash (5% w/v) to separate barbiturates from more strongly acidic material. To find the value of this, two dogs were given doses of Phenobarbitone and Amylobarbitone. Phenobarbitone was chosen as it was one of the most acidic (p Ka 7.45) of the barbiturates and, therefore, most likely to be extracted by Sodium Bicarbonate solution. It had been observed that the size of the spot obtained was roughly proportional to the quantity of barbiturate present and extracts were designated strongly positive, positive, slightly positive, and negative. On this basis a comparison of both methods is shown in Table 4. All doses were given orally.

TABLE 4.

Barbiturate	Dose	Time of Sample	Method of Extraction	Volume Used	Result		
Phenobarbitone	6mg/kg	1 hour	(a) Direct	10 ml	Strongly Positive.	Phenobarbitone.	No Metabolites
			(b) Washed	10 ml	Strongly Positive.	Phenobarbitone.	No Metabolites
		5 hours	(a) Direct	10 ml	Strongly Positive.	Phenobarbitone	No Metabolites
			(b) Washed	10 ml	Strongly Positive.	Phenobarbitone.	No Metabolites
Amylobarbitone	6mg/kg	1 hour	(a) Direct	10 ml	Positive	Amylobarbitone.	Metabolite Rf 0-0.02
			(b) Washed	10 ml	Positive	Amylobarbitone	Metabolite Rf 0-0.02
			(a) Direct	10 ml	Strongly Positive	Amylobarbitone	Metabolite Rf 0-0.02
			(b) Washed	10 ml	Strongly Positive	Amylobarbitone	Metabolite Rf 0-0.02

In all cases some difficulty was experienced in transferring the unwashed extract on to the chromatoplates on account of the interfering substances extracted. This was not found with the washed extract. In no case was the size of the spot on the chromatoplate from the washed extract smaller than in the unwashed. Thus it would appear that although there might be slight losses involved in washing with sodium bicarbonate solution, these are offset by the ease of transference of the extract on to the chromatoplate and by the clarity of the barbiturate and metabolite spots.

Washing with sodium bicarbonate (5% w/v.) was incorporated in the general extraction from urine.

2. Test of general method and determination of Excretion Patterns.

A set of experiments was devised to investigate the usefulness of the general method for the extraction and detection of barbiturates. These experiments were also used for the determination of the onset of excretion and for the determination of the Rf values of any metabolites found. The results are shown in Table 5.

Conclusion.

The method was successful in the detection of barbiturate in every sample. From the experiments where Butobarbitone was given, it was obvious that little could be said about the administered dose from urine concentrations. The three dogs given equal doses of Butobarbitone under similar conditions all gave different excretion rates and metabolite production.

TABLE 5.

BARBITURATE	DOSE	TIME OF URINE SAMPLE	VOLUME USED	RESULT
Amylobarbitone	3mg/Kg	1 hour	10 ml.	Faintly Positive. Amylobarbitone. No metabolites.
		5 hours	10 ml.	Strongly Positive. Amylobarbitone. 1 Metabolite Rf 0.0.02
Phenobarbitone	10mg/Kg.	1 hour	10 ml.	Strongly Positive. Phenobarbitone. No metabolites.
		5 hours	10 ml.	Strongly Positive. Phenobarbitone. No metabolites.
Barbitone	10mg/Kg.	1 hour	10 ml.	Positive. Barbitone. No metabolites.
		5 hours	10 ml.	Strongly Positive. Barbitone. No metabolites.
Butobarbitone	2mg/Kg.	1 hour	10 ml.	Positive. Butobarbitone. 1 Metabolite Rf 0.05
		5 hours	10 ml.	Positive. Butobarbitone. 1 Metabolite Rf 0.05
Butobarbitone	2mg/Kg.	1 hour	10 ml.	Strongly Positive. Butobarbitone 1 Metabolite Rf 0.05
		5 hours	10 ml.	Positive. Butobarbitone 1 Metabolite Rf 0.05
Butobarbitone	2mg/Kg.	1 hour	10 ml.	Positive. No Butobarbitone 1 Metabolite Rf 0.05
		5 hours	10 ml.	Strongly Positive. No Butobarbitone 1 Metabolite Rf 0.05

3. Onset of excretion of Amylobarbitone in Urine

In order to determine the minimum time when Amylobarbitone will show in urine, a dog was given a dose of 30 mg/Kg. and urine taken by catheter at intervals. The results are shown in Table 6.

TABLE 6.

Time (mins)	Volume obtained and used.	Result
20	5 ml.	Negative
40	10 ml.	Faintly Postive. Amylobarbitone. No metabolities.
75	4 ml.	Positive. Amylobarbitone. 1 Metabolite (Rf 0-0.02)
120	10 ml.	Strongly Positive. Amylobarbitone. 1 Metabolite (Rf 0-0.02)
255	7 ml.	Strongly Positive. Amylobarbitone. 1 Metabolite (Rf 0-0.02)

Conclusion. With Amylobarbitone, the onset of excretion was about 30 minutes after an oral dose.

4. Extraction using Florisil columns

Stokes [31] recommended the use of Florisil columns as a method of preparing very pure extracts of barbiturates from urine and for separating barbiturates in urines containing salicylates and other interfering substances. In this method, a sample of urine was acidified with dilute hydrochloric acid and extracted with 2 volumes of Chloroform. This extract was passed through a 5 x 1 cm. column of Florisil (60/100 mesh.) previously treated with chloroform. When the solvent was 1 - 2 cms. from the top of the column, the barbiturates were eluted with 25 ml. of a Chloroform/Methanol (9/1) mixture. Urinary pigments were held on the column. Stokes consistently obtained 99% recoveries using this method. All his work was done on samples containing pure added barbiturate and it was of interest to see whether metabolites of barbiturates would be held on the column. A dog was given 6 mg/Kg. of Amylobarbitone and a urine sample collected after three hours. The sample was extracted using Stokes method and after evaporation of the eluant the extract was run on a chromatoplate. A very clear separation of Amylobarbitone and metabolites was obtained with only a trace of urinary pigment present. The metabolites were estimated as

Amylobarbitone	60%	Rf 0.33
Metabolite 1.	5%	Rf 0.26
Metabolite 2.	5%	Rf 0.15
Metabolite 3.	30%	Rf 0.02.

In this experiment and in others where the barbiturate being studied had a low Rf value in Chloroform/Acetone 9/1, a better separation of metabolites could be obtained by adjusting the Chloroform/Acetone ratio. Thus in Chloroform/Acetone 2/1. The equivalent Rf values were

Amylobarbitone	Rf 0.81
Metabolite 1.	Rf 0.62
Metabolite 2.	Rf 0.36
Metabolite 3.	Rf 0.05

5. Doriden.

Two dogs were given oral doses of Doriden in order to determine the excretion rates and metabolites. Urine samples were extracted as described previously for Doriden. The results are shown in Table 7.

TABLE 7.

Dose	Time of Sample	Volume of Sample	Result
8 mg/ Kg.	1 hour	10 ml.	Positive. No Doriden. 5 Metabolites Rf. 0.3, 0.24, 0.20, 0.16, 0.12
	3 hours	10 ml.	Positive. No Doriden. 5 Metabolites Rf. 0.30, 0.24, 0.20, 0.16, 0.12
8 mg/ Kg.	1 hour	10 ml.	Positive. No Doriden. Streak. Rf. 0.30-0.00
	3 hours	10 ml.	Strongly Positive. No Doriden. Streak Rf 0.30-0.00

With the standard method of extraction for barbiturates previously described only one metabolite at Rf 0.20 was extracted.

Conclusion

Pure Doriden has not previously been detected in the urine of dogs after oral administration.(32) Our results confirmed this. The dealkylated derivative α- phenylglutarimido and a mixture of glucuronides have been identified in the urine of dogs.(33)

Cases Received

Samples from 44 cases of suspected suicidal overdosage have been examined for the presence of barbiturate. Twenty eight of these were found to contain barbiturates or related substances. In all cases the drug was completely identified except where Butobarbitone or Amylobarbitone was used, those two being indistinguishable.

The drugs encountered were as shown in Table 3.

TABLE 3.

DRUG	NO. OF CASES
Phenobarbitone	4
Pentobarbitone	4
Cyclobarbitone	1
Quinalbarbitone + Amylobarbitone	1
Butobarbitone or Amylobarbitone	12
Pentobarbitone + Carbromal	4
Amylobarbitone + Chlorpromazine + Alcohol	1
Primidone	1
Negative	16

* Of these cases Tablets found at scene were Amylobarbitone 4 and Butobarbitone 2.

Also using those methods over a thousand pre-race and post-race samples from racing greyhounds have been examined for the presence of barbiturates. The following barbiturates have been encountered.

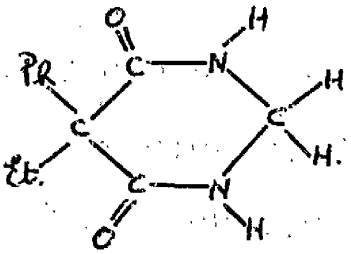
DRUG	NO. OF GREYHOUNDS
Phenobarbitone	22
Phenobarbitone + Chlorpromazine	1
Phenobarbitone + Alkaloid (Unidentified)	15
Pentobarbitone	2
Quinalbarbitone	1
Butobarbitone or Amylobarbitone	1

Primidone

The following fatal case of Primidone overdosage was interesting in that no cases of this kind had previously been recorded.

INTRODUCTION.

Primidone (Mysoline) is an anticonvulsant used in the treatment of epilepsy. Its structure (I) is similar to that of phenobarbitone (II) and is converted to it by normal metabolic processes.



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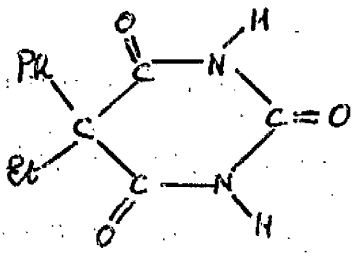


II

Ph – Phenyl Et – Ethyl.

Butler and Waddell[34] first reported this conversion and estimated it to be 15%. In view of the relatively high doses of primidone (0.75 - 1.5 grammes daily) used, they concluded that the phenobarbitone levels were sufficiently high to produce a significant anti-epileptic effect. Plas and others[35] reported three cases of neurotoxic effects in patients under primidone treatment. They measured phenobarbitone levels in blood, using an ultraviolet spectrophotometer and found levels of 4.0, 5.0 and 4 mg. %. These levels were sufficiently high to be the cause of the neurotoxic effects. In the three other non-fatal cases[36,37,38] of primidone overdosage reported, there was no mention of the biotransformation to phenobarbitone. By measuring plasma levels of phenobarbitone after administration of primidone and phenobarbitone, Frey and Hahn[39] concluded that at least 50% of the effect of primidone was due to its conversion to phenobarbitone. They also found that only traces of phenobarbitone are formed when primidone is given to guinea pigs. In this species the anticonvulsant effect was very weak. Radouco-Thomas[40] and others were also unable to obtain protection against convulsion in this species by the use of primidone. Oral administration to mice and rats showed primidone to be less neurotoxic

than phenobarbitone, but also less potent.[41] In cats and rabbits it was much more potent and more neurotoxic.

It appears that a large part, if not all, of the potency and neurotoxicity of primidone is due to its conversion to phenobarbitone. This is supported by the following report of a case of fatal primidone over-dosage.

CASE HISTORY

The subject was a 40-year-old man with a 15-year history of epilepsy. He had not worked for over four years due to illness and was subject to fits of depression. He had been admitted to hospital once before, suffering from an overdose of drugs, and on numerous occasions had threatened to take his own life. The subject was under the care of an epileptic specialist and had been prescribed 500 primidone tablets (0.25 grams). At the time of the incident 150 tablets were available, of which he took about 130. About one hour after ingestion it was noticed that he was unsteady and on questioning admitted taking the tablets. Five hours later he was admitted to hospital in a semi-comatose condition, where his stomach was washed out and he was placed on a drip feed. Two hours later he was deeply unconscious, and thirty-three hours later he died without regaining consciousness.

The principal post-mortem findings were marked oedema of the lungs and congestion of the gastric mucosa - the characteristic end results of massive barbiturate poisoning.

ANALYSIS

Blood, brain, liver and urine specimens were taken post-mortem. Phenobarbitone levels were determined on a recording ultraviolet spectrophotometer, by measuring the difference in absorption at 260 mμ in sodium hydroxide solution (0.4M) and in borate buffer solution. Tests showed that primidone did not interfere in the analysis, even when present in high concentrations. The identity of the phenobarbitone was established by thin layer chromatography and by spot tests on the separated drug. The values found are shown in Table 10 and are typical of those found in fatal barbiturate poisoning. A series of results of barbiturate analysis is given for comparison.

CONCLUSION

The investigation of a case of primidone poisoning showed typical signs of barbiturate poisoning. The amount of phenobarbitone found were such that death was due largely if not wholly to its effects.

PHENOBARBITONE LEVELS (mg.%) IN FATAL POISONING

SOURCE	NO. OF CASES.	URINE	BLOOD	BRAIN	LIVER		COMMENT
Present case	1	6.7	9.3	7.5	27.5		Primidone poisoning
(42) Gonzales et al.	9	10.0 (Single case)	-	4.1	3.2	Average.	Barbiturate not specified.
			-	2.9-	1.8-		
			-	7.2	5.3	Range.	
(43) Schmidt	1	-	4.8	3.8	13.7		
	1	-	54.0	83.3	145.0		
(44) Domitchsen et al.	18	-	10.3	--	14.9	Average.	
		-	6-18	-	6.2-	Range.	

DRUGS FROM AQUEOUS ALKALINE SOLUTION

Alkaloids and Related Bases

The meaning of the term 'alkaloid' is not generally agreed upon. In chemistry an alkaloid is considered to be an organic natural product containing nitrogen.(45) In medicine the term has come to mean any organic nitrogenous compounds exerting physiological activity and is not necessarily a natural product. The medical interpretation of this word is the one most suitable in toxicology. The methods of detection and identification involve a screening test for the presence of any alkaloidal substance and, the assignment of this substance to one of the sub-groups within the alkaloids.

Methods of Detection of Alkaloids

Two general alkaloidal reagents were selected from the large number which were in use as spray reagents in paper chromatography, viz. Dragendorff's reagent and Potassium Iodoplatinate. These reagents were prepared as follows and tested for their efficiency in detecting alkaloids on thin-layer chromatograms.

Dragendorff's Reagent.[46]

One gm. of Bismuth subcarbonate, 6 gms. of Potassium Iodide and 11 ml. of hydrochloric acid (11 N.) were dissolved in water and made up to 100 ml. Alkaloids yield orange spots on a yellow background with this reagent.

Potassium Iodoplatinate.

Potassium Iodoplatinate.

One ml. of plotinum chloride solution (10% w./v.) was added to 25 ml. of potassium iodide solution (4% w./v.) and the resulting mixture made up to 50 ml. This reagent gives blue colours when sprayed on to spots of a large number of the alkaloids.

Potassium Iodoplatinate was the more sensitive and gave much clearer spots than Dragendorff's reagent. It was also found that Dragendorff's reagent sometimes gave 'false positives' with normal urinary extracts making its use unsatisfactory with these samples. Potassium Iodoplatinate also gives much clearer spots on Thin Layer chromatoplates than on paper. Therefore in the initial assessment of thin layer and solvent systems Potassium Iodoplatinate was used for detecting alkaloidal spots. This reagent gives blue-purple spots on a red-brown background with over 90% of the

alkaloids. The few which did not react with this spray could be detected with Dragendorff's reagent.

Thin Layer Chromatographic Separation of Alkaloids.

In toxicology, thin-layer chromatography serves three purposes. (a) The separation of small amounts of toxic substances from biological material. (b) The assignment of a drug to a particular group of drugs by its reaction with a particular spray reagent and (c) the identification of the drug within this group by its Rf values. Since the number of alkaloids is so great that even preliminary identification of a drug is difficult, more than one solvent system would be advantageous and the greater the difference in Rf values between each system the better. To give the widest variation of Rf values from one solvent to another various combinations of acidic, basic and neutral solvents were tried. Initially a mixture of Codeine, Ephedrine, Mescaline and Ritalin was used when examining the suitability of any solvent as a separating medium. Using silica gel plates the following solvents were examined.

Cyclohexane	: Ethanol	9 : 1	All Rf's	0.00
Chloroform	: Acetone	9 : 1	All Rf's	0.00
Chloroform	: Methanol	9 : 1	All Rf's	0.3
Chloroform	: Methanol	3 : 1		Rf
			Codeine	0.46
			Ritalin	0.65
			Ephedrine	0.21
			Mescaline	0.60

This mixture of Chloroform : Methanol 3 : 1 gave good separation of the alkaloids with the majority showing as clear round spots, a few being tailed. This solvent mixture, being neutral is ideal in any screening test since it produces least interference with colours produced by spray reagents. Randerath[47] states that neutral solvents will not produce separation of alkaloids on silica gel plates since they are slightly acidic. This was not found to be the case and from Table 21 the spread of Rf values is good. As a further solvent system, various mixtures of acetone and acids and bases were tried:

Acetone : Water 9:1	Rf.
Codeine	0.1
Ritalin	0.42
Ephedrine	0.10T
Mescaline	0.10T

(T:- Tailed)

Acetone : Acetic Acid 4:1	Rf.
Codeine	0.1
Ritalin	0.3
Ephedrine	0.2
Mescaline	0.1

Acetone : Acetic Acid : Water 70:20:10	Rf.
Codeine	Streak
Ritalin	0.70
Ephedrine	0.45
Mescaline	0.25

Acetone : Acetic Acid : Water 60:30:10	Rf.
Codeine	Streak
Ritalin	0.70
Ephedrine	0.43
Mescaline	0.25

With this solvent spots were slightly clearer than with Acetone : Acetic Acid : Water 70 : 20 : 10.

Acetone : dil. Ammonia	95 : 5	Rf.
	Codeine	0.19
	Ritalin	0.60
	Ephedrine	0.92T
	Mescaline	0.65

Of these solvents, Chloroform : Methanol 3 : 1 and Acetone : dil. Ammonia 95:5 gave good separations of the majority of alkaloids studied, as shown in Table 21 while solvent mixture Acetone : Acetic Acid : Water 60 : 30 : 10 was useful with sympathomimetic amines.

Separation of Alkaloids from Biological Material

Blood

Blood was precipitated using the Folin-Wu tungstate method as described for barbiturates. The final extract was adjusted to pH 10 with ammonium hydroxide and shaken with 2 volumes of chloroform. The chloroform layer was filtered and evaporated under an infra-red lamp. The residue was taken up in chloroform and spotted on a chromatoplate.

Urine

Urine was adjusted to pH 10 with dil. ammonium hydroxide and shaken with 2 volumes of Ethyl Acetate. The Ethyl Acetate layer was filtered and extracted with 10 mls. hydrochloric acid (10% v/v). The aqueous layer was made alkaline to pH 10 with dil. Ammonium hydroxide and shaken with 2 volumes of Ethyl Acetate. The Ethyl Acetate layer was dried with sodium sulphate and evaporated. The residue was taken up in chloroform and transferred to a chromatoplate.

Stomach Contents

Stomach contents were extracted as for urine.

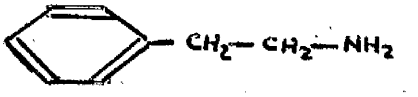
Viscera

Viscera were macerated with a little water. The mixture was then extracted as for urine.

While the purpose of our study was to find suitable methods of screening samples for the presence of drugs, it was convenient to investigate different sprays specific for different sub-groups of alkaloids, the allocation to or exclusion from a particular group making identification easier. The investigation consisted of testing the colour reagents, formerly used for paper chromatograms, on thin-layer chromatoplates.

SYMPATHOMIMETIC AMINES

All the drugs in this group have a basic β- phenylethylamine structure. (I)



I.

Friedhoff and Vin le[48] have reported a system for specifically identifying β- phenylethylamine derivatives on paper chromatograms. They reported that β- phenylethylamines give pink spots with Ehrlich's reagent after spraying with ninydrin. β- phenylethylamines gives no colour with Ehrlich's reagent alone.

Thus a specific test for β- phenylethylamines should be

1. Purple spots with S_1.
2. Negative to S_2 alone.
3. Pink spots with S_2 after S_1.

S_1 0.5% Ninhydrin in n-butanol. Heat at 105°C for 5 minutes.

S_2 Ehrlich's Reagent A solution of Dimethylaminobensaldehyde (2% W./V.) in a 9 to 1 mixture of Acetone and Hydrochloric Acid (11.N.).

The results found using these reagents were as shown in Table II.

TABLE II.

Sympathomimetic Amines	S_1.	S_2.	S_1 followed by S_2.
Amphetamine	Faint Pink	Faint Pink	Faint Pink
Euphenine	-	Faint Pink	Pink-Brown
Ephedrine	Red	-	Brown
Nephentermine	-	-	-
Mescaline	Brick Red	-	Brown
Methylamphetamine	-	Yellow	Yellow
Proludin	Pink	Pink	Pink
Propadrin	Red	-	Orange
Ritalin	-	-	Faint Brown

As can be seen, little agreement is found between these results and those found by Friedhoff on paper chromatograms.

Bromo-Cresol Green (B.C.G.) is an indicator which gives Blue spots on a green background with all the sympathomimetic amines. It is useful as a secondary spray but care must be taken with its use since urine was found to give 'false positives' with this reagent.

Thin-Layer Chromatography also allows corrosive sprays to be used directly on chromatograms and the Sulphuric Acid based alkaloidal reagents can be used directly. Marquis reagent (1 ml. 36N Sulphuric Acid - 1 drop 40% Formaldehyde) can provide further evidence as to the identity of the sympathomimetic amines. The results of the sprays are shown in Table 12.

TABLE 12.

Sympathomimetic Amine	U.V.	P_tI_4	Marquis	D.C.G.
Amphetamine	Adsorbs	- ve	Orange	Blue
Euphenine	"	Blue	Dull Red	"
Ephedrine	"	"	-	"
Nephentermine	"	"	Dull Red	"
Mescaline	"	"	Orange	"
Methylamphetamine	"	"	Orange	"
Preludin	"	"	-	"
Propadrin	"	Yellow	-	"
Ritalin	"	Blue	-	"

The Rf values of the Sympathomimetic Amines were determined and are as shown in Table 13.

TABLE 13.

Sympathomimetic Amines	S_1	S_2	S_3
Amphetamine	0.25T	0.63	0.74
Buphenine	0.56	0.81	0.90
Ephedrine	0.20T	0.54	0.92T
Nephentermine	0.19T	0.57	0.24
Mescaline	1.00T	0.47	0.65
Methylamphetamine	0.56	0.64	0.39
Proludin	0.63	0.68	0.53
Propadrin	0.32T	0.47	0.65
Ritalin	0.65	0.70	0.80

T = Tailed.

S_1 - Chloroform / Methanol 3/1
S_2 - Acetone / Acetic Acid / Water 60/30/10
S_3 - Acetone / dil. Ammonia 95/5.

Detection in Biological Material

Three of the commonest sympathomimetic amines were given to dogs and urine samples collected in order to determine when excretion of the drug began, and the Rf values of any metabolites formed. The results are shown in Table 14.

Discussion

With methylamphetamine, the first metabolite was probably amphetamine and excretion began between 1 hour and 1½ hours from administration. The detection of amphetamine in urine was not satisfactory as normal urine samples when extracted directly give Blue spots with Bromo-Cresol Green with similar Rf values to amphetamine. Any amphetamine present in urine samples would have to be removed by steam distillation before Thin Layer Chromatography could be used. The detection of Ritalin was very good even in the small doses given. Excretion began in less then one hour.

Practical Application

A urine sample was received where the defendant was accused of driving under the influence of drink or drugs. The accused had been taking Preludin for obesity. Pure

Preludin was easily identified in the urine. The drug extracted had Rf 0.63 in Chloroform / Methanol 3/1 and reacted with both Iodoplatinate and Bromo-Cresol Green.

TABLE 14.

DRUG	DOSE mg/Kg	TIME OF URINE SAMPLE	VOLUME OF SAMPLE USED	RESULT	Rf VALUE IN CHLOROFORM/ METHANOL	REACTION TO $Pt_e I_4$	REACTION TO B.C.C.
Methylamphetamine	0.5	60 mins.	10 ml.	Negative			
		95 mins.	5	Faintly Positive			
		140 mins.	5	Positive. Methylamphetamine	0.60	+	+
				Metabolite 1.	0.24	-	+
				Metabolite 2.	0.05	+	+
		270 mins.	7	Positive. Methylamphetamine	0.60	+	+
				Metabolite 1.	0.24	-	+
				Metabolite 2.	0.05	+	+
Amphetamine	0.5	1 hour	20	Positive. Amphetamine	0.25 - 0	-	+
		3 hour	20	Positive. Amphetamine	0.25 - 0	-	+
Amphetamine	0.5	1 hour	20	Positive. Amphetamine	0.25 - 0	-	+
		3 hour	20	Positive. Amphetamine	0.25 - 0	-	+
Amphetamine	0.5	1 hour	20	Positive. Amphetamine	0.25 - 0	-	+
		3 hour	20	Positive. Amphetamine	0.25 - 0	-	+
Ritalin	1.0	1 hour	40	Positive. Ritalin	0.65	+	+
				Metabolite 1.	0.20 - 0	+	+
		4 hour	40	Positive. Ritalin	0.65	+	+
				Metabolite 1.	0.20 - 0	+	+

Phenothiazines

The phenothiazines have grown in popularity in the last few years and are frequently encountered in toxicology mainly by their use as sedatives. This class of drugs has received considerable attention and much is known about their metabolism.[49-53] The principle excretion route is in the urine as the "free" form, the sulfoxide or to some extent as the glucuronid

Preparation of sulfoxides

Some of the sulfoxides were prepared from the parent compound by the method of Turner.[54]

Glacial acetic acid (0.5 ml) and hydrogen peroxide solution (0.02 ml ; 30 per cent) were added to the base (50 mg.) and the mixture heated at 45°C for 30 minutes. The mixture was evaporated under reduced pressure and the residue dissolved in water. This solution was made alkaline with sodium hydroxide (10%) and extracted with chloroform. The chloroform was filtered and evaporated. Turner reported a 95% yield of the sulfoxide.

Discussion

Detection of the phenothiazines and their sulfoxides as a group could be accomplished by treatment

with sulphuric acid or other oxidising agent when reddish-pink colours,[59] were produced.

This reaction was very sensitive and a spray of Sulphuric Acid / Ethanol 1/2 was found to be suitable for use directly on chromatograms. The reactions of the phenothiazines and their sulfoxides were as shown in Table 15.

TABLE 15.

Drug	Sulphuric Acid	Iodoplatinate
Chlorpromazine	Reddish Pink	++
Methotrimeprazine	Violet	"
Prochlorperazine	Reddish Pink	"
Promethazine	Pink	"
Thioproperazine	Reddish Pink	"
Thiopropazate	Reddish Pink	"
Trifluoperazine	Reddish Pink	"
Chlorpromazine Sulfoxide	Reddish Pink	"
Methotrimeprazine Sulfoxide	Violet	"
Promethazine Sulfoxide	Reddish Pink	"
Trifluoperazine Sulfoxide	Red	"

The Rf values of the Phenothiazines are given in Table 21. The Rf values of the sulfoxides are given in Table 16.

TABLE 16.

SULFOXIDE	Rf values S_1	Rf values S_2
Chlorpromazine Sulfoxide	.43	.17
Methotrimeprazine Sulfoxide	.43	.22
Promethazine Sulfoxide	.41	.17
Trimeprazine Sulfoxide	.43	.25

S_1 - Chloroform / Methanol 3/1

S_2 - Acetone / dil. Ammonia 95/5.

Detection in Biological Material

Chlorpromazine

Chlorpromazine is one of the commonest non-barbiturate sedatives and has been studied extensively. The principle metabolites are the mononsthyl and completely demthylated sulfoxides. (56) To investigate the detection of chlorpromazine, two dogs were given chlorpromazine and urine samples taken. The results are shown in Table 17.

TABLE 17.

Dose mg/Kg.	Time of Sample	Vol of Sample used	Result	Rf value in Chloroform/ Methanol	Approx. % of Total
1	1 hour	20	Positive.		
			Chlorpromazine	0.57	20%
			Metabolite 1.	0.30	40%
			Metabolite 2.	0.18	30%
	3 hours	20	Strong Positive		
			Chlorpromazine	0.57	20%
			Metabolite 1.	0.30	40%
			Metabolite 2.	0.18	30%
2	1 hour	20	Strong Positive		
			Chlorpromazine	0.55	20%
			Metabolite 1.	0.30	35%
			Metabolite 2.	0.18	35%
			Strong Positive		
			Chlorpromazine	0.55	20%
			Metabolite 1.	0.30	35%
			Metabolite 2.	0.18	35%

Discussion

Chlorpromazine and the metabolites of chlorpromazine were detected with ease at these low dosages using normal alkaloid extraction with Ethyl Acetate. This was surprising since Kotionis[57] has reported that phenothiazines and their metabolites are not extracted from chloroform into acidic solution since they form soluble hydrochlorides. As well as the two main metabolites a number of smaller metabolites were observed making up approximately 10% of the excreted alkaloidal material.

Promethazine

A dog was given Promethazine and urine samples taken in order to determine the excretion and metabolites of this drug. The results are shown in Table 18.

TABLE 18.

Dose	Time of Sample	Vol. Used	Result
1mg/Kg.	1 hour	20	Negative
	3 hours	20	Positive. No promethazine 1 Metabolite Rf 0.40 (Chloroform/ Methanol)

Discussion

No pure promethazine was found in the urine. One metabolite was found which was probably the sulfoxide.

Practical Applications

1. A urine sample (10 ml.) from a post-mortem dissection was found to contain a low concentration of an alkaloid. This gave a reddish-pink colour with sulphuric acid and was found to be Thiopropazate Amylobarbitone was also identified in the urine and there was an alcohol concentration of 213 mgms / 100 ml. urine.

2. A post-race urine sample (30 ml) from a greyhound was found to contain an alkaloid. From the excretion pattern, the colours produced by sulphuric acid and from Rf values, the phenothiazine was identified as chlorpromazine.

Antihistamines

The antihistamines have widely varying structures and give no specific reactions as a group. Benadryl and Dramamine were found to give very specific and sensitive yellow colours with Marquis' reagent and were easily separated and identified from other alkaloids. Anthisan was also unique in that it gave a deep purple colour with Marquis' reagent and as such was the only one of the alkaloids which was liable to be confused with the opiates. The colour reactions and Rf values of the antihistamines are given in Tables 21 and 22.

Detection in Biological Material

Two of the most frequently used antihistamines, Tripelennamine ('Pyribenzamine') and Chlordiazepoxide ('Librium') were given to dogs and urine samples taken in order to determine the excretion products. The results are shown in Table 19.

TABLE 19.

Drug	Dose	Time of Sample	Vol of Sample	Result
Tripelennamine	2mg/Kg	1 hour	20 ml.	Strong Positive. No tripelennamine. 1 Metabolite at Rf 0.1.
		6 hours	20 ml.	"
Chlordiazepoxide	1mg/Kg	1 hour	20 ml.	Positive. Chlordiazepoxide Rf 0.90. No metabolites.
		5 hours	20 ml.	"

Discussion

No pure tripelennamine was found in the urine. A metabolite which was easily found had Rf value 0.1 in chloroform/methanol 3/1. With chlordiazepoxide, the drug was excreted unchanged and was easily detected in this low dosage.

Practical Application

A urine sample was received from a woman who had taken an overdose of 'Librium' (Chlordiazepoxide) tablets. Pure chlordiazepoxide could be identified easily in as little 0.5 ml. of urine. No metabolite were found.

Morphine Alkaloids

In colour tests the Morphine alkaloids give strong colours with sulphuric acid reagents. Umberger[42] lists six of these but states that they all duplicate each other and as much information can be gained from two (Marquis' and Mecke's) as from the others. Thin-Layer chromatography allows the direct application of corrosive reagents and these two reagents were tested for their usefulness in detecting opiates on chromatoplates. Marquis' reagents gave much clearer spots than Mecke's. The colours with Sulphuric Acid alone were also noted Blue and Blue-Purple colours with Marquis' reagent are quite specific for the opiates with the exception of Anthisan, one of the antihistamines.

Marquis reagent can be used after a sulphuric spray without any diminution in sensitivity. Thus this spray can be used after a test for phenothiazine Routinely in a test for alkaloids, two spots are made. If the first reacts with iodoplatinic acid, the second is sprayed, first, with sulphuric acid to detect phenothiazines and, secondly, with Marquis' reagent. The allocation to or exclusion

from either of these drug groups is a major step forward in an analysis. The reactions of alkaloids to these sprays is given in Table 22.

Practical application

Autopsy sample was received and an alkaloid was isolated which reacted positively with Potassium Iodoplatinate and gave a purple spot with Marquis' reagent. The alkaloid had an Rf value of 0.26 in Chloroform / Methanol 3/1. The deceased had been receiving dihydrocodeinone. On running dihydrocodeinone with the unknown identical Rf values were obtained. No metabolites were present.

Death was attributed to Pentobarbitone (0.5 mgs / 100 ml. blood), Alcohol (201 mgs. / 100 ml. blood) and Dihydrocodeinone.

Other Alkaloids

From these studies on specific methods of identifying groups of alkaloids, a method of screening samples was evolved to provide the maximum information from small amounts of material. With the extract, two spots were made on each chromatoplate. After elution, one spot was covered and the other sprayed with Potassium Iodoplatinate. If this spot reacted positively the second spot was sprayed with sulphuric acid. Reddish-pink colours identified the alkaloid as a phenothiazine derivative. If no colours were observed this same spot was sprayed with Mequis reagent to identify the opiates and a few antihistamines. If the first spot failed to react with Potassium Iodoplatinate, the second was sprayed with Dragendorff's reagent. An orange spot with this reagent allocates the alkaloid to the group shown in Table 22 which did not react with Potassium Iodoplatinate.

The Detection of Quinine and Strychnine in Biological Material

Easton's syrup in pill form, a mixture of Quinine and Strychnine, was one of the commonest 'stoppers' used in the doping of animals. Although used as a stimulant in man, these pills upset the coordination of the animal and had the net result of slowing it. A mixture of Quinine and Strychnine as in these tablets, was given to find out if they could be detected in urine. The results are shown in Table 20.

Discussion

In the dose given, strychnine was not identified. Quinine could be identified as well as a number of metabolites. Excretion of Quinine begins 1 to 1½ hours after administration. No difficulties were experienced in the identification of this drug.

PAGE 20.

Dose	Time of Sample	Vol. of Sample (ml.)	Result
6 mg/Kg Quinine 1/10 mg/Kg Strychnine	55 mins.	8	Negative.
	90 mins.	5	Positive. Quinine Rf 0.60. Metabolites indistinguishable. No strychnine identifiable.
	155 mins.	5	Positive. Quinine Rf 0.60. 1 strong Metabolite Rf 0.23. No strychnine.
	240 mins.	10	Strong Positive. Quinine Rf 0.60 1 Strong Metabolite Rf 0.23. Number of other lesser metabolit Rf 0.60 - 0.23
6mg/Kg Quinine 1/15 mg/Kg Strychnine	8 hours	10	Positive. Quinine Rf 0.56. 1 Strong Metabolite Rf 0.26. No strychnine.
6mg/Kg Quinine 1/15 mg/Kg Strychnine	5 hours	10	Positive. Quinine Rf 0.50. 1 Strong Metabolite Rf 0.23.

TABLE 21.

Rf values of Alkaloids

ALKALOID	S_1	S_2
Antistan	.43	.50$_g$
Antistin	.11	.23
Atropine	.07	.25$_g$
Benadryl	.47	.62
Benzocaine	.85	.91
Butacaine	.69	.57
Caffeine	.56$_g$	.76
Chlorpromazine	.44	.55
Chlorprothixene	.61	.64
Cocaine	.71	.63
Codeine	.19	.46
Dihydrocodeinone	.17	.30
Dihydromorphinan	.15	.26
Diptpanone	.67	.68
Dramamine	.42	.54
Heroin	.54	.65
Histantin	.35	.57
Hydroxyzine	.61	.72
Hyoscine	.54	.63

Leptazol	.68	.65
Levorphanol	.16	.26
Ethrium	.60	.92
Maratine	.42	.81
Methadone	.49	.55
Methotrimeprazine	.62	.62
Morphine	.12	.29
Narcotine	.74	.84
Nikethamide	.58	.80
Papaverine	.74	.90
Procaine	.65	.46
Prochlorperazine	.24	.64
Promethazine	.56	.69
Pyrtheneamine	.44	.54
Quinine	.30	.58$_p$
Reserpine	.80	.88
Ritalin	.60	.65
Strychnine	.09	.40
Thioproperazine	.60	.92
Trimeprazine	.54	.55$_p$

S_1 - Acetone / Ethyl Acetate 95/5

S_2 - Chloroform / Methanol 9/1.

TABLE 22.

Reaction of Alkaloids to various Sprays on Silica Gel

Group	Compound	PtI_4.	H_2SO_4	Marquis' Reagent
Phenothiazines	Chlorpromazine	Blue	Reddish-Pink	Reddish-Pink
	Methotrimeprazine	Blue	Violet	Violet
	Prochlorperazine	Blue	Reddish-Pink	Reddish-Pink
	Promathazine	Blue	Pink	Pink
	Thiopropazate	Blue	Lt. Orange	Lt. Orange
	Trimeprazine	Blue	Lt. Orange	Lt. Orange
	Thiopropazate	Blue	Reddish-Pink	Reddish-Pink
Antihistamines	Antazan	Blue	-	Dp. Purple
	Antistin	Blue	-	-
	Benadryl	Blue	-	Yellow
	Chlorprothixene	Blue	Red-Brown	Red
	Dramamine	Blue	-	Yellow
	Histantin	Blue	-	-
	Hydroxyzine	Blue	-	-
	Librium	Blue	-	-
	Marazine	Blue	-	-
	Pyribenzamine	Blue	Lt. Brown	Lt. Brown

Opiates	Codeine	Blue	–	Dp. Purple
	Dihydrocodeinone	Blue	–	Purple
	Dihydromorphinan	Blue	–	Purple
	Heroin	Blue	–	Dp. Purple
	Morphine	Blue	–	Purple
	Narceine	Blue	–	Yellow-Green
	Narcotine	Blue	–	Yellow-Green
	Papaverine	Blue	–	Blue
Local Anaesthetics	Benzocaine	Yellow	–	–
	Butacaine	Blue	–	–
	Cocaine	Blue	–	–
	Procaine	Blue	–	–
Non-Opiate Analgesics	Diphenone	Blue	–	–
	Methadone	Blue	–	–
Analeptics	Leptazol	White	–	–
	Nikethamide	Yellow	–	–
Other Alkaloids	Atropine	Blue	–	–
	Hyoscine	Blue	–	–
	Quinine	Blue	–	–
	Reserpine	Blue	Yellow	Dk. Green
	Ritalin	Blue	–	–
	Strychnine	Blue	–	–
Phenothiazine Sulphoxides	Methotrimeprazine Sulph.	Blue	Violet	Violet
	Trimeprazine Sulph.	Blue	Red	Red
	Chlorpromazine Sulph.	Blue	Reddish-Pink	Reddish-Pink
	Promethazine Sulph.	Blue	Reddish-Pink	Reddish-Pink

DRUGS EXTRACTABLE FROM NEUTRAL SOLUTION

Drugs extractable from neutral solution

The main groups of drugs extracted in the neutral fraction are the barbituro - derivatives and the carbamates. The phenothiazines, now an important group in toxicology, and caffeine form organic soluble hydrochlorides and must be looked for in this fraction. The phenothiazines are detectable in the alkaline fraction and it is necessary to spray the neutral fraction with a general alkaloidal reagent. If an alkaloid is detected in the alkaline fraction, the neutral fraction is also tested for alkaloids. If the greater part of the alkaloid has been extracted in the neutral fraction, the alkaloid will probably prove to be a phenothiazine. Although the phenothiazines and caffeine are found in this fraction, their physical data are included with the alkaloids.

Trichlorobutanol (Chlorobutanol)

Introduction

Chlorobutanol ('Chloretone'), 2, 2, 2, - Trichloro 1 - 1 - dimethylethanol is a sedative with a similar action to chloral hydrate, but with a less irritant action on the gastric lining. It is a common 'doping' agent being easily obtained without prescription. No previous studies have been made on the metabolism of chlorobutanol.

The halogenated hydrocarbon most frequently encountered in toxicology, chloroform, chloral hydrate and carbon tetrachloride are commonly detected by the Fujiwara[58] reaction. In this reaction an aliquot of the solution to be tested is added to a mixture of 1 ml. pyridine and 2 ml. Sodium Hydroxide (10% w./v.). The mixture is placed on a boiling water bath for two minutes and the upper pyridine layer is observed. A pink colour indicates halogenated hydrocarbons. Chlorobutanol was found to give this test. This was unexpected as 2, 2, 2 trichloroethanol, a metabolite of chloral hydrate (Von Mering),[59] does not give the Fujiwara test (Butler T.C.).[60] The limit of visible detection of chlorobutanol was found to be similar to that for Chloralhydrate.

A further method of detecting Chlorobutanol is described by Rehms and Mader.[61] They say that 'little or no work has been done on colorimetric methods of detecting chlorobutanol.' They describe a method using hydroxylamine / ferric chloride. This method was found to be much less sensitive than the Fujiwara test and also was open to interference by amides, imines, barbiturates and many other groups commonly encountered in toxicology.

Detection in Biological Material

Four dogs were given doses of 15 mgms / Kg. of chlorobutanol and the urine collected after 1 hour and after 3 hours. None of the urine samples gave positive results by directly adding urine to the Fujiwara reagents. Steam distillation and testing of the distillates also produced negative results. Either chlorobutanol is not excreted, in this period, in sufficiently high concentrations or it is excreted in a form which does not give the Fujiwara reaction.

Discussion

Trichloroethanol, the principle metabolite of chloral hydrate is excreted almost wholly as the glucuronide, urochloralic acid, in dogs and to a lesser extent in humans.[62] Butler[60] oxidises urochloralic acid with dichromate to give a Fujiwara positive product. It was thought that the excretion of 2, 2, 2 - trichloroethanol and chlorobutanol would be similar and 10 ml. of each urine sample were oxidised with dichromate solution (0.5 gms. potassium dichromate; 25 mls. sulphuric acid; 20 mls water,) but again proved negative when tested directly. 10 ml samples of urines when incubated at 37°C for 24 hours with β- glucuronidase also gave negative results.

Babgood and Powell[63] steam distil biological material containing halogenated hydrocarbons and extract the distillate with a small volume of toluene. 10 ml aliquots of the urine samples were steam distilled and the distillate extracted with 2 mls. of toluene. Two of the 1 hour samples and one of the 3 hour samples from the four dogs showed positive. After incubation for 24 hours at 37°C with β - glucuronidase, the urine samples were again tested. On adding the toluene layer to the Fujiwara reagents all the samples showed positive.

Method

The urine samples, after incubation with β - glucuronidase, were extracted directly with 2 ml. of toluene. After settling 1 ml. of the toluene layer was pipetted into the Fujiwara reagents and the mixture heated at 100°C for 2 minutes. On shaking gently the toluene does not absorb any chromogeneous material and when tested alongside the extracts from the equivalent steam distillate gave equally intense colours.

Function Tests

Two dogs were given doses of 10 mg./Kg, two 15 mg./Kg. and two 20 mg/Kg of chlorobutanol. Urine samples were collected after 3 hours and after 6 hours 10 ml. portions of each sample were analysed. The 3 hour and 6 hour samples of one of the dogs receiving 15 mg/Kg and the 3 hour sample of the dog receiving 10 mg/Kg showed positive. All the others showed negative. When 10 ml portions of each sample were incubated overnight with β - glucuronidase and the test repeated all the samples showed positive.

Carbamates

Meprobamate, the most important of the carbamate group, is one of the commonest non-barbiturate sedatives at present being used in medicine. Moss & Jackson[64] described a reagent which was specific for the detection of this and other carbamates on paper chromatograms. The method involved spraying with freshly redistilled furfuraldehyde and after the paper dried respraying with concentrated hydrochloric acid. The resultant black spots were characteristic of carbamates.

It was found that hydrochloric acid vapour was sufficient to develop the black colours. The chromatoplates were sprayed with furfuraldehyde, allowed to dry and then suspended in a gas jar which contained a little concentrated hydrochloric acid. Clear black spots developed in less than a minute. Meprobamate and its congener Ethinamate were eluted in the solvents already in use for the separation of barbiturates and alkaloids. Rf values in Chloroform/Acetone 9/1 and Chloroform/Methanol 9/1 are shown below. Clear round black spots which were stable for

a number of months were obtained with both solvents

	Chloroform/Acetone 9/1 9/1	Chloroform/Methanol 3/1 3/1
Meprobamate	0.08	0.58
Ethinamate	0.39	0.67

Chloroform/Methanol 3/1 was the solvent of choice in routine analyses.

Separation from Biological Material

Blood

5 ml. of blood were shaken directly with 50 ml. of chloroform. The chloroform layer was filtered and evaporated to dryness. The residue was dissolved in a few drops of chloroform and spotted on a chromatoplate.

Urine

5 ml. of urine were shaken with 10 mls of ethyl acetate. The ethyl acetate layer was then dried with anhydrous sodium sulphate, filtered and evaporated to dryness. The residue was dissolved in a few drops of chloroform and spotted on a chromatoplate.

Stomach Contents

The samples were treated in the same manner as the urine samples.

Viscera

The viscera were macerated with an equal volume of water and extracted in the same manner as blood samples.

Detection in Biological Material

Ethinamate

In order to determine the excretion patterns of ethinamate in dogs and to determine the Rf values of any metabolites, two dogs were given doses of ethinamate and urine samples taken. The results are shown in Table 23.

TABLE 23.

Dose	Time of Sample	Volume of sample used	Result
18 mg/kg	1 hour	10 mls	Positive No pure ethinate found. 1 Metabolite Rf 0.52
	6½ hours	10 mls	Negative
18 mg/kg	1 hour	10 mls	Strongly Positive No pure ethinamate found. 1 Metabolite Rf 0.52
	6½ hours	10 mls	Positive. No pure ethinamate found. 1 Metabolite Rf 0.52.

Conclusion

In man the main metabolic pathway of ethinate (1 - ethynylcyclohexylcarbamate) is by hydroxylation to hydroxyethinamate (1-ethynyl-4-hydroxycyclohexyl-carbamate).[65] Unchanged ethinamate has been found in the blood, liver and urine of humans after overdosage of

the drug.[66]

From our experiments it would appear that dogs do not excrete any unchanged ethinamate at low dosages. The single metabolite found is likely to be hydroxyethinamate.

Meprobamate

In order to determine the excretion patterns of meprobamate, the Rf values of any metabolites, and the relative concentrations of each metabolite, 4 dogs were given meprobamate. The urine samples collected and analyzed giving the results shown in Table 24.

Conclusion

Berger [67] found that man and rats excreted equal amounts of unchanged meprobamate and several conjugates which have not been identified. In dogs it would appear that there are two main metabolites. The excretion began about one hour after oral doses.

TABLE 24.

DOSE	TIME OF SAMPLE	VOLUME OF SAMPLE USED	RESULT	APPROXIMATE RELATIVE CONCENTRATIONS
15mg/Kg	1 hour	10 mls.	Strongly Positive.	
			Pure Meprobamate	30%
			Metabolite 1. (Rf 0.55)	10%
			Metabolite 2. (Rf 0.42)	60%
	6 hours	10 mls.	Positive.	
			Pure Meprobamate	30%
			Metabolite 1.	10%
			Metabolite 2.	60%
15mg/Kg	1 hour	10 mls.	Negative.	
	6 hours	10 mls.	Strongly Positive.	
			Pure Meprobamate	50%
			Metabolite 1.	10%
			Metabolite 2.	40%
15 mg/Kg.	1 hour	10 mls.	Negative	
	6 hours	10 mls.	Strongly Positive.	
			Pure Meprobamate	40%
			Metabolite 1.	20%
			Metabolite 2.	40%
15 mg/Kg	1 hour	10 mls.	Faint Positive.	
			Pure Meprobamate	50%
			Metabolite 2.	50%
	6 hours	10 mls.	Strong Positive.	
			Pure Meprobamate	40%
			Metabolite 1.	20%
			Metabolite 2.	40%

C O N C L U S I O N

Conclusion

Thin-Layer Chromatography has been shown to be a useful method for the detection and identification of drugs in body fluids. Methods have been found for the detection of the majority of toxicologically important drugs. Where a complete identification of a drug is not possible Thin-Layer Chromatography provides an easy means of purification and preliminary identification which is necessary for most of the other techniques used in toxicological investigation. A scheme has been devised for the rapid screening of samples for the presence of drugs. This scheme which can be completed in less than one hour has found widespread use in the pre-race testing of racing animals for 'dope.' A number of laboratories now use these methods exclusively. These rapid methods of detection in racing animals can enable a 'doped' animal to be withdrawn prior to a race and obviate all the difficulties which can arise from the detection of 'dope' in an animal subsequent to racing.

Also medico-legal experts when investigating deaths for the Crown Authorities frequently require rapid results in possible poisoning cases. If these are not available then unnecessary stress may be caused to the relatives and the investigation of the case may be impeded. Thin-Layer Chromatography has been found most useful in these cases.

Although the object of rapid tests with reasonably wide coverage has been achieved the following points arose and require further investigation.

1. Too little work has been done on metabolites of drugs. In most cases encountered in toxicological investigation, a gross overdosage of drug is encountered and there is always a great amount of unchanged drug present. However in investigations into 'doping' of racing animals or humands frequently the only foreign substances present are the products of metabolism and the drug has to be identified from these.

2. The complete identification of alkaloids is difficult unless they can be assigned to the opiates or the phenothiazines. Also two of the

commonest alkaloidal stimulants, caffeine and amphetamine, are not readily identifiable.

3. The actual effect of drugs on racing animals needs to be measured. There is some doubt whether the use of stimulants in racing animals is effective. Certainly strychnine, used as a stimulant in humans, has the not effect of slowing greyhounds and is used to this end. Greyhounds are relatively good animals for assessing the effect of drugs since they run with remarkable consistency making a 1% variation in standard immediately obvious.

REFERENCES

1. TSWETT, M. Proc. Warsaw Soc. Nat. Sci. Biol. Section 14, Minute No. 6 1903.

2. KUHN, R. & LEDERER, E. Ber. Dtsch. Chem. Ges. 64, 1349, 1931.

3. KUHN, R., WINTERSTEN, A., & LEDERER, E. Hoppe-Seylers Z. Physiol. Chem. 197, 141, 1931.

4. MARTIN, A.J.P., & SYNGE, R.L.M. Biochem. J. 35, 1358, 1941.

5. CONSDEN, R., GORDON, A.H. & MARTIN, A.J.P., Biochem. J. 38, 224, 1944.

6. WILLIAMS, T.J. Introduction to Chromatography, Glasgow p. 36, 1947.

7. KIRCHNER, J.G., MILLER, J.M., & KELLER, G.D. Anal. Chem. 23, 420, 1951.

8. STAHL, E. Pharmazie 11, 633, 1956.

9. STAHL, E. Chemiker - Ztg. 82, 323, 1958.

10. STAHL, E. Parfumerie U. Kosmetik 39, 564, 1958.

11. STAHL, E. Pharmazie - Rdsch. 2, 1, 1959.

12. STAHL, E. Angew. Chem. 73, 646, 1961.

13. PARRI, F. Bull. Chim. Farm. 63, 4011, 1924.

14. KOPPANYI, S. J. For Med. 4, 65, 1957.

15. SWICKER, E. Pharm. Week Blad. 68, 975, 1931.

16. SWICKER, E. Pharm. Week Blad. 69, 1170, 1932.

17. SCHMIDT, G., 'Methods of Forensic Science' Edit. Lundquist p 454, 1963.

18. DEININGER, R., Arzneimittel-Forsch. 5, 472, 1955.

19. SCHMIDT, G., Deut. Z. Ges. Gerichtl. Med. 49, 259, 1959.

20. ALGERI, E.J. & VALKEN, J.T. Am. J. Clin. Pathol. 22, 37, 1952.

21. CURRY, A.S., Acta. Pharmacol. Toxicol. 13, 357, 1957.

22. MAYNERT, E., and WASHBURN, R. J. Amer. Chem. Soc. 75, 700, 1953.

23. BROUGHTON, P.M.G., Biochem. J. 63, 207, 1956.

24. BROUGHTON, P.M.G., HIGGINS, G., & O'BRIEN, J.M.P. Lancet. 1956, 2, 180.

25. GOLDBACH, H.J. Deut. Z. Ges Gerichtl. Med. 40, 368, 1951.

26. KOPPANYI, J., DILLE, J.M., MURPHY, W.S., & HOP, E. J. Am. Pharm. Assoc. 23, 1074, 1934.

27. VALOV, P., Ind. Eng. Chem. Anal. Ed. 18, 456, 1946.

28. CURRY, A.S. 'Toxicology' II. Ed. Stewart & Stolman p. 150, 1963.

29. SCHMIDT, G. Deut. Z. Ges. Gerichtl. Med. 49, 259, 1959.

30. CURRY, A.S. 'Toxicology' II Ed. Stewart & Stolman p. 165, 1963.

31. STOKES, D.M., Comp., W.J.R., & KIRCH, E.R. J. Pharm. Sci. 51, 379, 1962.

32. SHEPPARD, H., D'ARARO, D.S. & PLUMMER, A.J. J. Am. Pharm. Assoc. 45, 681, 1956.

33. KEMPE, J., HOFFMANN, K., Experientia 12, 21, 1956.

34. BUTLER, T.C. & VADDELL, W.J. Proc. Soc. Exp. Bioc. Med. 93, 544, 1956.

35. PLAA, G.L., FUJOMOTO, J.M., & HINE, G.H. J. Am. Med. Ass. 168, 1769, 1958.

36. ARNOLD, O.H. & GERANIC-HOFBERGER, S. Wien. Med. Wochr. 103, 692, 1953.

37. MORLEY, D. & WYNNE, N.A. Brit. Med. J. 1, 90, 1957.

38. SCIARRA, D., CARTER, S., VIGALE, C.T., & HERBUTT, H.H. J. Am. Med. Ass. 154, 827, 1954.

39. FREY, H. - H. & HAHN, I. Arch. Exper. Path. Pharmacol. 240, 20, 1960.

40. RADOUCO-THOMAS, C., GOLD, P., & RADOUCO-THOMAS, S. Arch. Ital. Sci. Farmacol. 4, 468, 1954.

41. GOODMAN, L.S., SWINYARD, E.A., BROWN, W.C., SCHIFFMAN, D.G., GREWAL, H.S. & BLISS, E.L. J. Pharmacol. 108, 428, 1953.

42. GONZALES, T.A., VANCE, M., HELPERN, M., & UMBERGER, C.J. Legal Medicine, Pathology & Toxicology, 2nd Ed. Appleton - Century - Crofts, Inc., New York.

43. SCHMIDT, G., Methods of Forensic Science I. Ed. Lundquist. Interscience, London.

44. BONNICHSEN, R., MARLEY, A.C., & FRANK, A., J. for Sci. 6, 411, 1961.

45. MANSER, R.N.F. Toxicology II Ed. Stewart & Stolman p. 209, 1963.

46. LUDY-TENGER, F., MIKROCHEMIE Ver. Mikrochimie Acta. 38, 460, 1952.

47. RANDERATH, K. 'Thin-Layer Chromatography' p. 74, Academic Press 1963.

48. FRIEDHOFF, A.J., and WINKLE, E.V. J. Chrom. 11, 272, 1963.

49. FLANAGAN, T.L., REYNOLDS, L.V., NOVICK, W.J., LIN, T.H., HONDIUS, J., & VAN LOON, W.J. J. Pharm. Sci. 51, 833, 1962.

50. FLANAGAN, T.L., NEWMAN, J.H., HAAGS, A.M., & VAN LOON, E.J. J. Pharm. Sci. 51, 996, 1962.

51. DUBOST, P. and PASCAL, S. Ann. Pharm. Franc. 11, 615, 1953.

52. SALZMAN, N.P., and BRODIE, B.B. J. Pharmacol. Exptl. Ther. 118, 46, 1956.

53. BERTI, T. and GIMA, L. Farmaco Pavia Ed. Sci. 12, 159, 1957.

54. TURNER, L.K. J. For Sci. Soc. 4, 39, 1963.

55. AUTENRIETH H. Arch. Pharm. 284, 123, 1951.

56. GOLDENBERG, H., and FISHMAN, V. Proc. Soc. Exptl. Biol. Med. 108, 178, 1961.

57. SOTIONIS, A.Z.K. Arzneimittel - Forsch. 11, 108, 1961.

58. FUJIWARA, K. Sitzber. Naturforsch. Ges. Rostock. 6, 33, 1914.

59. VON HERING, F. Ztschr. F. Physiol. Chem. 6, 480, 1882.

60. BUTLER, T.C. J. Pharmacol. 92, 49, 1948.

61. REINS, C.R. and HADER, W.J. J. Am. Pharm. Assoc. 46, 621, 1957.

62. MARSHALL, W.R. and OWENS, J. Bull. Johns Hopkins Hospital. 95, 1, 1954.

63. HAGGOOD, S. and POWELL, J.F. Brit. J. Ind. Med. 2, 30, 1945.

64. MOSS, M. and JACKSON, J. J. Pharm. & Pharmacol. 13, 261, 1961.

65. McMAHON, J. J. Org. Chem. 24, 1894, 1959.

66. PREISS, H. and MAYER, E. Arch. Toxicol. 18, 243, 1960.

67. BERGER, F.M. J. Pharmacol. Exptl. Ther. 112, 413, 1954.