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EMERGENCY TOXICOLOGICAL SCREENING FOR DRUGS COMMONLY TAKEN IN OVERDOSE

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SUMMARY

A procedure is described for the screening of body fluids for the presence of drugs commonly involved in poisonings. Separate acidic and basic extracts of the specimen are made with chloroform, followed by concentration and thin-layer chromatography of the two extracts. Identification is based on R_F values, colours produced by spray reagents and spot patterns formed by the drug and its metabolites. Six simple colour tests are carried out concurrently with the preparation of these extracts. When examining urine for the presence of benzodiazepines a separate procedure involving hydrolysis, petroleum ether extraction and subsequent thin-layer chromatography is performed.

INTRODUCTION

The ability to identify barbiturates and salicylates in the body fluids of comatose patients has for many years been adequate to deal with most cases of drug overdose, but increased prescribing of drugs from other groups has resulted recently in a corresponding demand for more comprehensive analysis of body fluids.

Although a number of screening procedures are reported in the literature, the analyst wishing to extend the range of drugs that he can confidently detect finds that these are not usually tailor-made to answer his problem. Hence, it was felt that whilst a number of publications have described the screening for specialised groups of drugs, there was an urgent need for an overall scheme which could be easily introduced into the routine clinical chemistry laboratory and which dealt with the whole range of drugs that are commonly encountered in the overdose situation. Furthermore, although there is no substitute for personal experience, by illustrating the results with colour photographs it is hoped that technicians who only occasionally undertake a toxicological examination will find it more easy to interpret their own thin-layer chromatograms.

Thus we aim in this paper to bring together our more recent developments and the best of previous work in this field so as to present to the clinical chemist a concise, but comprehensive scheme which demonstrates our approach to drug screening and which a routine laboratory could turn to when required. It is not intended to supersede

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standard procedures such as UV determination of barbiturates or colorimetric salicylate assays, but rather as an adjunct whereby the pathology laboratory may easily extend its role to cover the detection of the majority of drugs encountered in poisoning emergencies.

Thin-layer chromatography (TLC) is rapid and ideally suited to drug identification. It is a technique which can be easily introduced for only a small capital outlay and its application to the analysis of urine specimens is particularly commendable since in some cases characteristic spot patterns are produced by the drugs and their metabolites.

EXPERIMENTAL

Apparatus

The minimum essential equipment is: three TLC tanks (Shandon Scientific Co. Ltd., London, Great Britain); general-purpose spray bottles (Scientific Supplies, Vine Hill, London E.C.2); McCartney bottles (28 ml) and polypropylene lids (Gallenkamp, Christopher Street, London E.C.2); a 100-μl syringe (Phase Separations Ltd., Queensferry, Flintshire, Great Britain); TLC plates (silica gel, 250 μ thick; hand-made or commercially available from Anderman Co. Ltd., London); UV lamp (Camlab Ltd., Cambridge Great Britain).

The hand-made TLC plates were prepared by making a slurry of 30 g of silica gel with 60 ml of distilled water and spreading to a thickness of 250 μ with an automatic plate leveller and spreader (Scientific Supplies). The plates were dried for 1 h at 110° before use.

Reagents

All chemicals were A.R. grade and purchased from Hopkin and Williams (Chadwell Heath, Essex, Great Britain), and were used to prepare the following reagents:

Benzodiazepine reagent—Sulphuric acid 9 *M* (solution 1); sodium nitrite 1% w/v, freshly prepared (solution 2); ammonium sulphamate 5% w/v (solution 3); and N-1-naphthylethylenediamine hydrochloride (1 g) dissolved in 80 ml of acetone and 20 ml of water (solution 4).

Dragendorff's reagent—A stock solution was made by mixing: 850 mg of bismuth subnitrate, 40 ml of water and 10 ml of glacial acetic acid with 8 g of potassium iodide and 20 ml of water. The spray reagent was prepared by mixing 10 ml of stock with 20 ml of acetic acid and 100 ml of water.

FPN reagent—5 g of ferric chloride dissolved in 100 ml of water, 20 %v/v perchloric acid and 50% v/v nitric acid were mixed in the proportion 1:9:10 by volume.

Furfural reagent—2 ml of redistilled furfuraldehyde was dissolved in 98 ml of acetone (solution 1); 4 ml of concentrated sulphuric acid were added to 96 ml of acetone (solution 2); solution 1 was sprayed first, then solution 2. These reagents should be prepared immediately before use.

Imipramine reagent—50 mg of potassium dichromate were dissolved in a mixture of 7.5 ml of sulphuric acid, 5 ml of perchloric acid and 12.5 ml of nitric acid and diluted to 100 ml with water.

Iodoplatinate reagent—3 ml of 10% w/v platinum chloride solution, 97 ml of

water and 100 ml of a 6% w/v aqueous potassium iodide solution were mixed together and stored in a dark brown bottle.

Mandelin's reagent—1 g of ammonium vanadate was suspended in 100 ml of concentrated sulphuric acid.

Mercuric chlorided–diphenylcarbazone reagent—Equal volumes of a 0.2% w/v ethanolic solution of diphenylcarbazone and a 2% w/v mercuric chloride ethanolic solution were mixed prior to spraying.

Mercuric nitrate reagent—1 g of mercurous nitrate was dissolved in 100 ml of water to which were added a few drops of concentrated nitric acid so as to obtain a clear solution.

o-Cresol reagent—*o*-Cresol was mixed with water and allowed to stand for a day to saturate the solution.

Salicylate reagent—40 mg of mercuric chloride were dissolved in 850 ml of water. 120 ml of 1 *M* hydrochloric acid and 40 g of hydrated ferric nitrate were added and the volume was made up to 1 l.

TLC standards—The drugs were dissolved in ethanol or chloroform at a concentration of 1 mg/ml. It was sometimes convenient to prepare mixed standards in order to reduce spotting time.

Method

The colour tests were carried out concurrently with the preparation of the TLC extracts, *i.e.* step 13 was started while shaking in step 2. Blood is normally reserved for subsequent quantitative analysis.

Preparation of extracts for TLC

(1) Take two McCartney bottles and half-fill with urine or stomach contents. (If both fluids are available it is wise to analyse each specimen.)

(2) Make one bottle acid with 0.5 *M* H_2SO_4, the second alkaline with 1 *M* NaOH. Fill with chloroform and label A and B, respectively. Screw on plastic lids and shake for 5 min. Centrifuge.

(3) Aspirate off the aqueous phase using a Pasteur pipette connected to a water vacuum pump and if the starting material was urine, omit steps 4–7.

(4) To the acid extract (A) add 3 ml of 0.5 *M* NaOH and to the alkaline extract (B) add 3 ml of 0.25 *M* H_2SO_4.

(5) Shake the bottles again, centrifuge and discard the organic layers.

(6) Re-acidify A with 0.5 ml of 3 *M* H_2SO_4 and re-alkalinise B with 0.5 ml of 6 *M* NaOH. Add 10 ml of chloroform to each bottle.

(7) Shake, centrifuge and discard the aqueous layers.

(8) Filter the extracts through a Whatman No. 90 paper to remove the last droplets of moisture into 10-ml conical tubes.

(9) Evaporate to dryness under a stream of air or nitrogen.

(10) Re-constitute the residue in 0.1 ml of chloroform.

(11) *TLC of barbiturate extract (A).* (a) Divide a TLC plate into eight equal columns by drawing lines with a spatula. Draw a horizontal line 10 cm from the origin. (b) Apply 10-μl spots of the standard solutions and 25-μl spots of the extract as shown in Fig. 1a. (c) Run the plate in a tank containing 100 ml of chloroform–acetone (9:1), until the solvent has reached the horizontal line. Remove the plate from the tank and dry under a stream of air. (d) Cover columns 3 to 8 with a glass

plate and spray columns 1 and 2 with mercuric chloride–diphenylcarbazone reagent. White spots on a lilac background indicate the presence of barbiturates or related compounds. Columns 3 and 4 can then be sprayed with mercurous nitrate to confirm the presence of barbiturates which give black spots initially, but these turn white with heavy spraying. (e) Cover all the columns except 5 and 6 and spray with Dragendorff's reagent, orange spots indicating the presence of either methaqualone or glute-

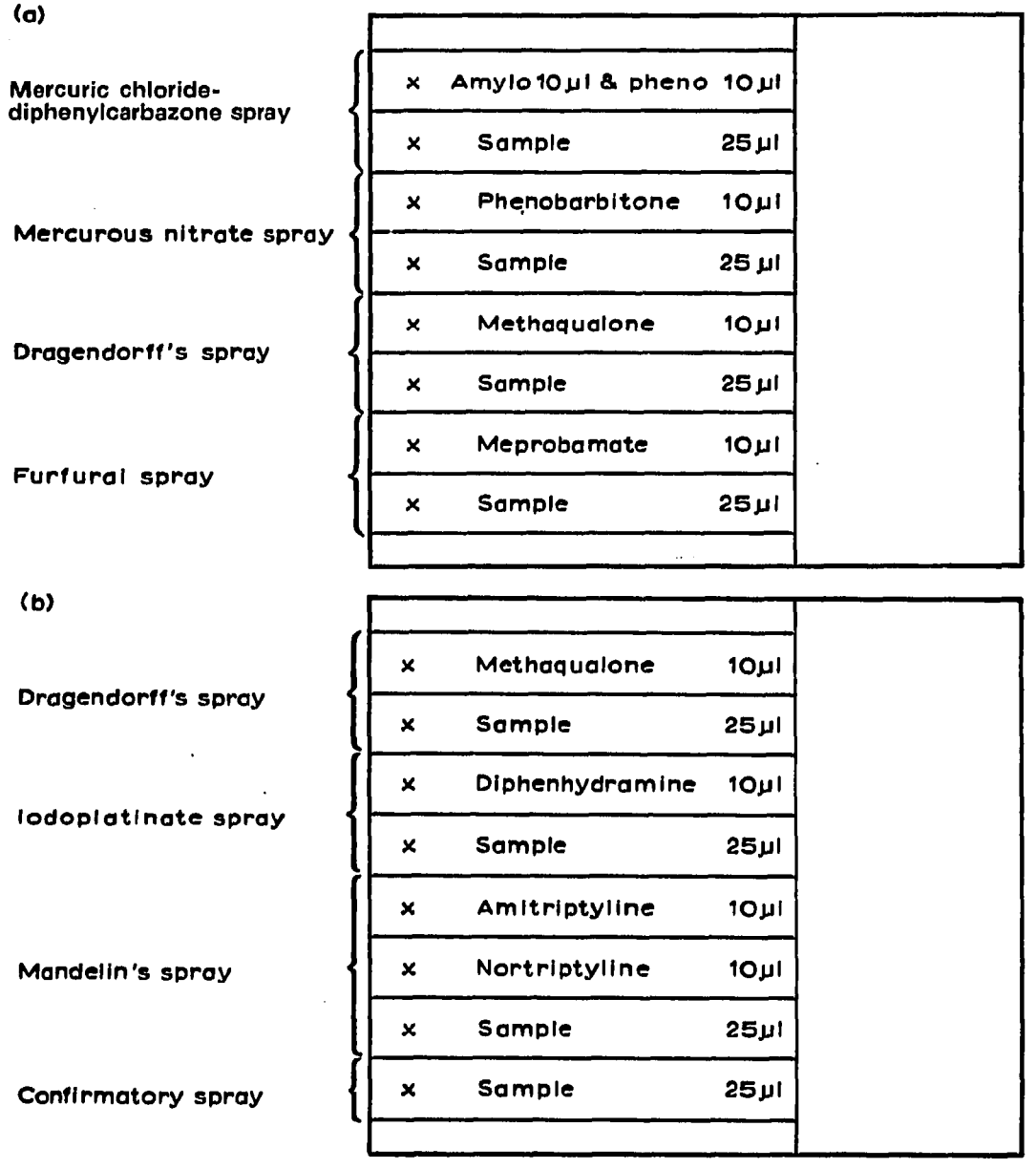

Fig. 1. (a) The arrangement of the spots on a TLC plate for the acidic extract. (b) The arrangement of the spots on a TLC plate for the alkaline extract.

thimide. (f) Finally spray columns 7 and 8 with furfural reagent to detect the presence of carbamates. Meprobamate gives a violet spot.

(12) *TLC of the alkaline extract (B).* (a) Divide another TLC plate into eight columns as described above. Apply standards and samples as shown in Fig. 1b. (b) Develop the plate in a tank containing methanol–12 *N* ammonia (100:1.5) and dry under a cold air-stream. (c) Cover the last six columns and spray columns 1 and 2 with Dragendorff's reagent. This gives orange, red or brown spots with most basic drugs or their metabolites. (d) Cover all but columns 3 and 4 which can then be sprayed with iodoplatinate reagent. Grey-blue colours appear with most basic drugs. (e) Expose columns 5, 6, and 7 and spray with Mandelin's reagent. Inspect the plate under UV light of 254 nm for any fluorescent spots. (f) Measure the R_F value of any spot appearing in the sample extract columns and use the final column to confirm the identity of any suspected drug (see *Reactions of specific drugs*).

(13) *Colour tests on the remaining urine.* Take three test tubes. Pipette 1 ml of urine (or stomach contents) into each. (a) Add 1 ml of salicylate reagent to the first tube. A violet colour indicates the presence of salicylates. (b) Add 1 ml of FPN reagent to the second tube. A red or blue colour indicates the presence of phenothiazines. Confirmation should be sought on the TLC plate of extract B. (c) Add 1 ml of imipramine reagent to the third tube. A greenish blue colour indicates the presence of imipramine or desipramine. Again, this should be confirmed on the TLC plate of extract B.

(14) Mix equal volumes of urine and concentrated HCl in a test tube and heat in a beaker of boiling water for 10 min. An aliquot from a standard urine collected for 24 h after ingestion of 1 g of paracetamol should also be hydrolysed. Cool and dilute 0.1 ml of each solution to 10 ml with *o*-cresol reagent. Add 2 ml of 4 *M* NH_4OH. A deep royal blue is formed with paracetamol or phenacetin ingestion.

(15) Place 1 ml of urine into a test tube and add 1 ml of 10 *M* NaOH and 1 ml of pyridine. Stand in a beaker of boiling water for 1 min. A water blank and an aliquot of standard urine from a subject who has taken a therapeutic amount of chloral hydrate should be carried through the procedure simultaneously. A red colour in the pyridine layer is formed with metabolites of chloral hydrate and related drugs.

(16) Place 2 ml of urine in a test tube and sprinkle a spatula-full of diphenylamine sulphate crystals onto the surface. Incline the tube and trickle in 1 ml of concentrated sulphuric acid. A red colour on the surface of the crystals is formed in the presence of ethchlorvynol. A standard early-morning urine from a volunteer who had taken 0.5 g of ethchlorvynol should be used as a comparison.

(17) *Identification of the benzodiazepines.* Place 10 ml of urine in a McCartney bottle together with 3 ml of concentrated HCl. As a reference 1 ml of a solution containing 10 mg of both oxazepam and nitrazepam in 100 ml of 1 *M* HCl should be added to a separate 9-ml blank urine and acidified with 3 ml of acid. Screw on the plastic tops of the bottles and heat in an autoclave or pressure cooker for 15 min, at 15 p.s.i. Cool the solutions and extract with 10 ml of petroleum spirit of b.p. 40–60°. Centrifuge, remove the organic layer and evaporate to dryness. Redissolve the residue in 0.1 ml of methanol.

TLC of benzodiazepine extract

Divide a plate into two equal columns and spot 50 μl of an extract into each

column. Develop the plate in benzene–acetic acid (97:3) and then carefully dry under a cold-air blower. Spray successively with solutions 1, 2, 3, and 4 (see *Reagents*, Benzodiazepine reagent) drying the plate between sprays with warm air.

DISCUSSION

Preparation of extracts

The extraction procedures were carried out in McCartney bottles. These have the advantage of being cheap and robust, withstanding both centrifuging and autoclaving. Another important factor was the plastic screw tops which prevented solvent escaping, as so often happened with the more common metal caps.

Chromatography

The division of plates into separate columns, thus ensuring a greater reproducibility of R_F values across a plate has already been discussed by Berry and Grove[1]. Since it is unwise to rely on R_F values alone we spot reference samples on every plate and the R_F values in Tables I–III are simply to indicate the order of running. A considerable emphasis has been placed on the arrangement of the plates, hence the novice should find interpretation of results quite easy. By using the sprays sequentially in the order described, with reference standards in adjacent columns to samples, the unknown drug may soon be identified.

Tables I–III show the R_F values obtained using unsaturated tanks and handmade plates. The virtues of employing unsaturated tanks for emergency toxicology

TABLE I

COLOUR REACTIONS AND R_F VALUES OF SOME ACIDIC AND NEUTRAL DRUGS IN CHLOROFORM–ACETONE (9:1)

Drug	*R_F value**	*Mercuric chloride–diphenylcarbazone*	*Mercurous nitrate*	*Remarks*
Meprobamate	0.3	–	–	Lilac with furfural
Primidone	0.3	+	+	
Phenytoin	0.8	+	+	
Phenobarbitone	1.0	+	+	
Barbitone	1.0	+	+	
Cyclobarbitone	1.2	+	+	
Butobarbitone	1.3	+	+	
Heptabarbitone	1.3	+	+	
Pentobarbitone	1.3	+	+	
Amylobarbitone	1.4	+	+	
Quinalbarbitone	1.5	+	+	
Glutethimide	1.6	+	+	Weak reaction with Dragendorff's reagent
Methaqualone	1.6	–	–	Positive reaction with Dragendorff's reagent. Metabolites also react

* Relative to phenobarbitone.

TABLE II

COLOUR REACTIONS AND R_F VALUES OF SOME BASIC DRUGS IN METHANOL–12 *N* AMMONIA (100:1.5)

Drug	R_F *value**	*Dragendorff's reagent*	*Iodoplatinate reagent*	*Mandelin's reagent*	
				Daylight examination	UV_{254} *examination*
Desipramine	0.43	+	+	Deep blue	—
Nortriptyline	0.57	+	+	Pale mauve	Mauve centre, yellow rim
Codeine	0.71	+	+	—	—
Morphine	0.71	+	+	—	—
N-Desmethylorphenadrine	0.72	+	+	Pale yellow	Pale blue
Imipramine	0.86	+	+	Deep blue	—
Chlorpromazine	0.93	+	+	Red	—
Thioridazine	0.93	+	+	Blue	—
Amitriptyline	1.0	+	+	Pale mauve	Mauve centre, yellow rim
Diphenhydramine	1.0	+	+	Pale yellow	Pale yellow
Orphenadrine	1.07	+	+	Pale yellow	Pale blue
Quinine	1.14	+	+	—	Intense blue
Methaqualone	1.57	+	—	—	—

* Relative to amitriptyline.

are obvious, indeed, we have not found it necessary to employ saturated tanks in our routine determinations. In laboratories occasionally employing TLC procedures ready-made plates are undoubtedly advantageous. They do not affect the order of running or R_F values to any great extent, although in our experience the sprays are sometimes less sensitive.

Standard urines

Because sensitivity is high we recommended in several procedures that urine from volunteers, after therapeutic dosing with the drugs sought, be analysed alongside the sample. This is important, since one can compare the colour obtained from the standard urine with that of the sample and perhaps judge from the intensity whether the amount of drug present is, excessive, *e.g.*, a positive reaction will be obtained for five days after therapeutic dosing with chloral hydrate. Similarly the Indophenol Blue coupling reaction with *p*-aminophenol in the test for paracetamol can be formed very strongly after ingestion of only two paracetamol tablets. Once obtained, these urines can be deep-frozen and used as reference standards for many assays.

Back-extraction of the stomach aspirate

If a direct organic extract of a stomach wash-out is evaporated to dryness and TLC attempted, one invariably finds that fat and co-extractable materials produce difficulty in spotting and an impaired TLC separation. It is imperative therefore to carry out a back-extraction when dealing with this type of specimen. However, when

employing this procedure neutral drugs such as glutethimide and meprobamate (normally sought in the acid extract) remain in the chloroform layer and are lost when this is rejected. It is better therefore to look for these drugs in urine.

The use of sulphuric acid rather than hydrochloric acid is also pertinent in this context. Some basic drugs, *e.g.* tricyclic antidepressants, form hydrochlorides which are soluble in chloroform. If hydrochloric acid were employed these could be lost in a back-extraction.

Reactions of specific drugs

Salicylates. The violet colour produced with this reagent has a visual sensitivity in urine of about 20 mg%. If only blood is sent for screening, it is still possible to carry out a qualitative test for salicylates on one drop of plasma. It should be noted that the reaction does not work with stomach contents unless these are first hydrolysed since the colour is not formed with acetylsalicylic acid. Phenothiazines produce a red colour with the reagent but since they are usually present in much lower concentrations false positives are unlikely.

Phenothiazines. The most common phenothiazine drug taken in overdose is chlorpromazine. It is prescribed in high doses, so the red colour obtained with FPN reagent is seldom missed. However, some phenothiazines are taken in much lower doses of 2 to 5 mg and insufficient drug or metabolites appear in the urine to give a positive reaction. In these instances the best fluid for analysis is the gastric washing. We have not included in Table II any phenothiazines other than chlorpromazine and thioridazine but, if others are suspected, reference to the work of Zingales[2,3] should be made.

Phenothiazines and their metabolites produce a large number of spots in the sample extract on the alkaline plate. They react with Dragendorff's, iodoplatinate and Mandelin's sprays. The TLC procedure is much more sensitive than the urine spot test, which produces transient colours, probably due to the instability of the free radicals formed in dilute acid solution[4]. If confirmation of phenothiazines is still required after the Mandelin spray, the final column may be sprayed with either FPN or 18 *N* H_2SO_4, the latter spray producing stable colours for many hours.

Some slight reaction may occur in the urine spot test if either salicylates or imipramine group drugs are present. The confirmation test on TLC plate can then be used in cases when a questionable reaction is observed.

Imipramine and desipramine. The reagent described gives a characteristic blue colour with the urine of most overdoses from this drug group. However, the TLC procedure is more sensitive and acts as a confirmatory test. The drugs react with Dragendorff's and iodoplatinate sprays and give blue colours with Mandelin's reagent which do not fluoresce under UV light (see Fig. 3, columns 6 and 8). Column 6 has standards of imipramine and desipramine and column 8 is an extract from an imipramine overdose, showing the unchanged drug and its metabolite. If desipramine is ingested only one spot is seen. When the R_F values of the spots in the sample indicate the presence of these drugs they can be confirmed by spraying column 8 with imipramine reagent. Although the blue colour produced on the TLC plate is characteristic, it should be remembered that less common, but related compounds such as trimipramine and clomipramine also react. The phenothiazine drug thioridazine will also give a blue colour with this reagent.

Trichloro compounds. This test is based on work described by Fujiwara[5]. We have a 48-h urine sample collected from a volunteer who took 800 mg of chloral hydrate and we use 1 ml as a reference standard.

One must ensure that the urine specimens undergoing the test have not had chloroform added as preservative, since this solvent gives a strong positive response because it contains the reacting $-CCl_3$ group. High backgrounds from $CHCl_3$ vapour in the laboratory can also give false positives if care is not taken.

Paracetamol and phenacetin. The hydrolysis of the urine converts both of these drugs to *p*-aminophenol which, when coupled with *o*-cresol gives an Indophenol Blue. A sample of urine collected for 24 h after ingesting 1 g of paracetamol should be kept as a reference. The impression that *p*-aminophenol is a metabolite of paracetamol[6] is erroneous, being only found free in urine after bacterial breakdown[7]. It is essential to carry out a hydrolysis, therefore, in order to form *p*-aminophenol from free paracetamol and its conjugates.

Ethchlorvynol. The colour test described is based on the reaction of diphenyl amine sulphate with ethchlorvynol. The test is quite sensitive and after ingestion of a therapeutic dose (0.5 g) a positive reaction is obtained with 2 ml of an early-morning urine. The reactive group postulated by Fiorese and Carella[8] is $-C\equiv CH$ and it would therefore be expected that compounds incorporating this moiety in their chemical structure would interfere with the test. This is not so in our experience since the drugs methypentynol and ethinamate, both of which contain this grouping, failed to react even at a concentration of 100 mg/100 ml. Neither chlorpromazine nor imipramine, which give slight reactions with sulphuric acid, interfere at the 2 mg/100 ml level and normal urinary constituents did not produce any false reactions.

Barbiturates. The majority of laboratories in Great Britain routinely estimate barbiturates by UV spectrophotometry of a blood extract without specifying the barbiturate taken. However, interferences can occur from some other drugs[9] and one purpose of this paper is to encourage further investigation after UV analysis has been performed. Emphasis, therefore, is placed on TLC of an acid extract from urine, for not only does it serve to confirm the presence of a barbiturate, but it simultaneously identifies drugs such as glutethimide, meprobamate, methaqualone, primidone, and phenytoin.

The long-acting barbiturates phenobarbitone and barbitone run at lower R_F values in chloroform–acetone (9:1) than the others that are commonly prescribed in Great Britain. Hence, while one cannot state categorically that a patient has ingested a particular barbiturate, to be able to indicate whether or not the drug is long-acting is often all the clinician needs to know.

The separation of phenytoin and primidone is included in Table I. They give similar reactions with mercuric chloride–diphenylcarbazone and mercurous nitrate to those of the barbiturates. When their presence is suspected, a second plate in ethyl acetate–methanol–0.88 ammonia (85:10:5) can be run. In this system they have R_F values relative to phenobarbitone of 1.9 and 2.1, respectively, although the sensitivity of primidone to the sprays is poor, needing 40 μg before it can be seen.

Glutethimide runs near the solvent front in chloroform–acetone (9:1) and reacts with both mercury salt sprays (Fig. 2, column 4). It also gives an orange colour with Dragendorff's reagent, although this has poor visual sensitivity, 30 μg being needed to obtain a reaction (Fig. 2, column 9). Methaqualone also has the same R_F

value, but only reacts with Dragendorff's reagent and not with the mercury salt sprays.

Fig. 2 demonstrates the colours obtained with the sprays used on the acid extract plate. The sample columns 3 and 5 also show spots at R_F 0.3 and 0.1 due to barbiturate metabolites. These have very low solubility in chloroform but since their urine concentration is often many times that of the unchanged drug they sometimes appear on the plate. Although primidone and phenytoin also run behind phenobarbitone, the second system of ethyl acetate–methanol–0.88 ammonia can be used to differentiate these anticonvulsants from barbiturate metabolites.

Meprobamate. The furfural spray reagents react with meprobamate to give a lilac colour (Fig. 2, column 10), its sensitivity with freshly prepared reagents being 10 μg. It may be necessary to redistill the furfural since it slowly discolours, resulting

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Fig. 2. A plate run in chloroform–acetone (9:1) showing the reactions of acidic and neutral drugs. Columns 1–3 have been sprayed with mercuric chloride–diphenylcarbazone reagent. 1 = Amylobarbitone + phenobarbitone mixed standard; 2 = quinalbarbitone standard; 3 = urine extract from a patient who ingested an overdose of Tuinal (= amylobarbitone + quinalbarbitone from Eli Lilly and Co.). Columns 4–6 have been sprayed with mercurous nitrate reagent. 4 = Glutethimide standard; 5 = same urine extract from a patient who ingested an overdose of Tuinal; 6 = amylobarbitone + phenobarbitone mixed standard; 7 = methaqualone standard; 8 = urine extract from a patient who ingested an overdose of Mandrax; 9 = glutethimide standard; 10 = meprobamate standard sprayed with furfural reagent.

Fig. 3. A plate run in methanol–12 *N* ammonia (100:1.5) showing the typical reactions after an alkaline extraction. Columns 1–2 have been sprayed with iodoplatinate reagent. 1 = Urine extract from a patient who ingested an overdose of Mandrax; 2 = diphenhydramine standard. Columns 3–4 have been sprayed with Dragendorff's reagent. 3 = Urine extract from a patient who ingested an overdose of Mandrax; 4 = methaqualone standard. Columns 5–8 have been sprayed with Mandelin's reagent. 5 = Amitriptyline + nortriptyline mixed standard; 6 = imipramine + desipramine mixed standard; 7 = urine extract from a patient who ingested an overdose of amitryptyline; 8 = urine extract from a patient who ingested an overdose of imipramine.

Fig. 4. A plate showing the effect of increase in concentration on the fluorescent appearance of amitriptyline under UV light of 254 mn after spraying with Mandelin's reagent. Columns 1, 2, 3, and 4 have 1, 5, 10 and 20 μl of the amitriptyline standard, respectively. Column 5 shows a urine from a patient who took an overdose of amitriptyline and column 6 an extract from a patient who was receiving 25 mg of amitriptyline *t.d.s.* Column 7 has 10 μl of a nortriptyline standard, the major metabolite of amitriptyline.

Fig. 5. Colours of the diazonium compounds formed from benzodiazepine standards and urine extracts, after spraying as described in the text. Columns 1, 2, 5, and 6 have extracts from 10 ml of urine from volunteers taking 10 mg of librium®, valium®, mogadon® and nobrium® (from Roche Products Ltd., Herts., respectively. Columns 3, 4, and 7 have extracts from standards of oxazepam, mogadon and valium that have been carried through the hydrolysis procedure.

Fig. 2.

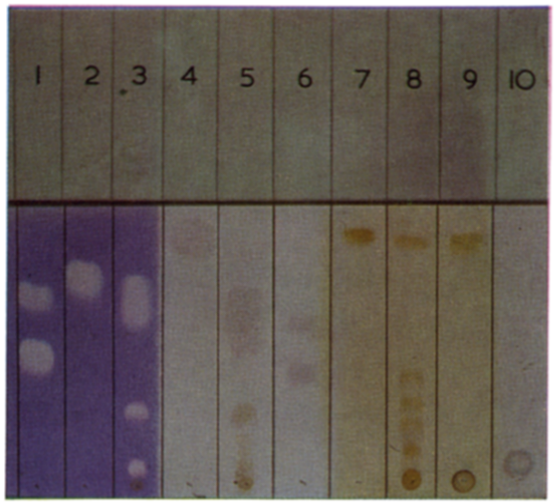

Fig. 3.

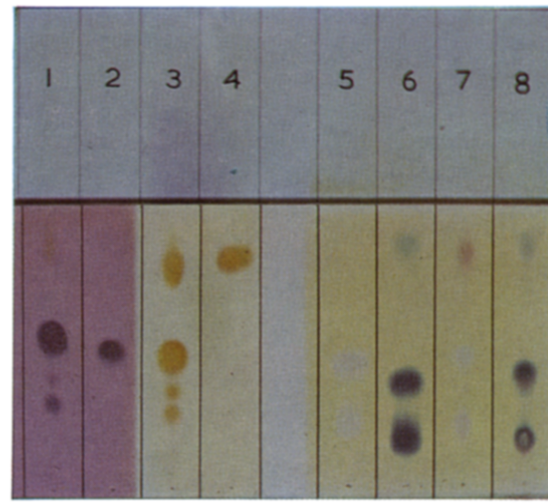

Fig. 4.

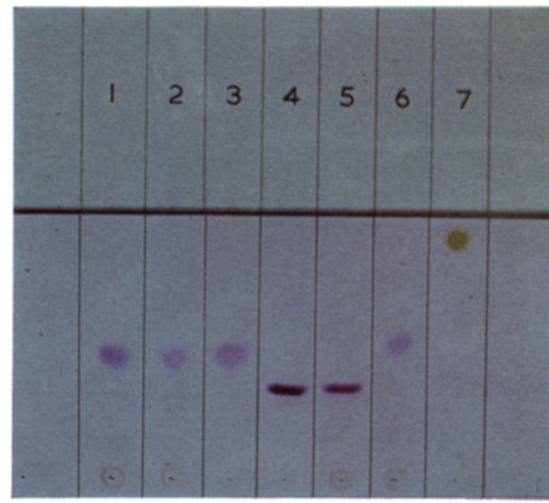

Fig. 5.

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in darker backgrounds with considerable loss in sensitivity. If water is allowed to contaminate the spray, a similar effect is produced. Other carbamates, *e.g.* tybamate, react with furfural reagent, but are not frequently encountered.

Methaqualone. In Great Britain methaqualone is generally prescribed in combination with diphenhydramine as Mandrax® (Roussel Laboratories Ltd., Middlesex, Great Britain), each tablet containing 250 mg of methaqualone and 25 mg of diphenhydramine hydrochloride. The combination is very useful since it gives rise to the characteristic "Mandrax pattern" on the alkaline plate. Fig. 3, columns 1–4 show this arrangement. Columns 1 and 2 have been sprayed with *neutral* iodoplatinate reagent and show the reactions produced with a diphenhydramine standard and a urine extract from a patient who ingested an overdose of Mandrax. Columns 3 and 4 are sprayed with Dragendorff's reagent and show a methaqualone standard and extract from the same overdose. It is clear from a comparison of columns 1, 3 and 4 that the methaqualone fails to react with neutral iodoplatinate spray, but gives an orange spot with Dragendorff's reagent. The appearance of a spot at R_F 0.9 which reacts with Dragendorff's reagent only and iodoplatinate reacting spots opposite the diphenhydramine is characteristic of the Mandrax overdose. The iodoplatinate reaction occurs between diphenhydramine and its metabolites and normally the analyst will see only two spots depending on the amount taken and the time of ingestion relative to the collection of the urine sample. In Fig. 3, column 1, a faint third spot presumably due to another metabolite can be seen, but this is not always apparent.

A useful confirmatory test is the reaction produced when the acid plate is sprayed with Dragendorff's reagent (see Fig. 2, columns 7 and 8). Methaqualone can be extracted with chloroform from urine over the pH range 2–13, while diphenhydramine will only be found in the alkaline fraction. Fig. 2, column 7 has a methaqualone standard and column 8, 25 μl of an extract (A) from the overdose urine. The overdose shows a methaqualone spot (R_F 0.9) and five additional spots between R_F 0.0 and 0.45, due to methaqualone metabolites. Again all of the metabolite spots may not always be visible, but since the only other Dragendorff reacting drug which moves in this solvent system is glutethimide, which also reacts with the two barbiturate sprays, no confusion can arise. Some authors[10,11] have hydrolysed urine to identify methaqualone after therapeutic ingestion, but we have not found this necessary even for minor overdoses.

Amitriptyline and nortriptyline. Overdose ingestion of these drugs is becoming more frequent and, for the majority of laboratories, their identification is best performed by TLC. They are extracted from alkalinised urine and on the plate react with Dragendorff's, iodoplatinate, and Mandelin's reagents. The latter spray produces only a pale mauve colour in daylight (Fig. 3, columns 5 and 7), but a characteristic fluorescence under UV light of 254 nm which is an excellent means of identifying these tricyclic antidepressant drugs. The colours vary with the amount of drug present, a small dose of amitriptyline gives pale yellow fluorescent spots of amitriptyline and its metabolite nortriptyline. At greater concentrations the spots appear as mauve fluorescent centres surrounded by a yellow rim. Fig. 4 shows the effect of increasing concentrations on the appearance of the amitriptyline spots under UV light of 254 nm, together with some urine extracts following amitriptyline ingestion and demonstrates that two spots are produced which are due to the parent drug and its metabolite nortriptyline. Of course nortriptyline overdoses would only have one spot on the plate.

After spraying with Mandelin's reagent the pale reddish mauve spots, visible on the photograph (Fig. 3, columns 5 and 7) are not always evident with therapeutic doses and therefore the plate should always be examined under UV light when looking for these drugs. The spot at R_F 0.9 is always seen even in blank urine extracts.

Fike and Sunshine[12] have reported the reactions of antihistamines with Mandelin's reagent and, although these are not often encountered, the group member orphenadrine is occasionally taken in overdose. This reacts with Mandelin's reagent, giving a pale blue fluorescence under UV light, but does not produce at high concentration the yellow rim characteristic of the amitriptyline group. In overdose urine extracts two blue spots appear on the plate due to the parent drug and its N-demethyl metabolite.

The narcotics. Occasionally the clinician may encounter the drug addict in coma and then the laboratory may be asked to find drugs such as morphine (metabolised from heroin), methadone, codeine, cocaine, amphetamine, methylamphetamine and quinine. Methanol–12 *N* ammonia fails to separate morphine and codeine but by running a second plate in benzene–dioxan–ethanol–0.88 ammonia (50:40:5:5) these now have R_F values of 0.33 (codeine) and 0.18 (morphine). The merits of these two solvent systems and suitable extraction procedures for screening the urine of drug addicts have been discussed elsewhere[1].

The benzodiazepines. When present in stomach contents the benzodiazepines can be extracted from alkaline media and separated by TLC procedures using chloroform–acetone (9:1) and spraying with Dragendorff's reagent (Table III).

TABLE III

R_F VALUES OF THE BENZODIAZEPINE DRUGS RELATIVE TO NITRAZEPAM IN CHLOROFORM–ACETONE (9:1)

Drug	*R_F value*
Chlordiazepoxide	0.44
Oxazepam	0.68
Nitrazepam	1.0
Diazepam	1.7
Medazepam	2.0

The amount of free drug found in the urine after taking an overdose of a benzodiazepine is usually too small to be identified in this way. However, on ingestion each drug is metabolised to compounds which will undergo hydrolysis to primary aminobenzophenones.

Thus, for urine we hydrolyse with hydrochloric acid and after extraction and TLC, couple the resultant benzophenones with N-naphthylethylenediamine to form an azo dye. The method is so sensitive that we can detect a therapeutic ingestion. Hence, the presence of a member of the group can be detected, although it is only possible to distinguish nitrazepam from the other group members, oxazepam, chlordiazepoxide, diazepam, and medazepam, since on hydrolysis these last four drugs or their metabolites produce a common benzophenone, 2-amino-5-chlorobenzophenone. Pure samples of the drugs, diazepam and medazepam do not form primary amino-

benzophenones and therefore do not give colours in the Bratton–Marshall diazotisation reaction, whereas chlordiazepoxide and oxazepam do.

Nitrazepam hydrolyses to give 2-amino-5-nitrobenzophenone, which is separated from the chlorobenzophenone derivative by our TLC solvent system. It gives a different colour in the Bratton–Marshall reaction, thus enabling this drug to be distinguished from other members of the group (see Fig. 5, columns 4 and 5). This plate also demonstrates the sensitivity of the technique for the detection of therapeutic ingestion of these drugs.

Column 7, which was a diazepam standard, does not react, although we have noticed a faint yellow spot formed at about R_F 0.9 which is probably due to the 2-methylamino-5-chlorobenzophenone formed on hydrolysis.

The analgesic, mefenamic acid, is the only drug we have found to interfere with this procedure. After carrying through a urine from a patient taking therapeutic quantities of this drug one finds three green-blue spots after spraying with the acid nitrite only at R_F 0.4, 0.5, and 1. The spot on the solvent front corresponds to hydrolysed pure drug and the others are presumably metabolites.

Another useful indicator of nitrazepam ingestion is to be found on the plate that has been run in methanol–ammonia. If large quantities of this drug have been taken, a blue fluorescent spot will be seen running just in front of amitriptyline when viewing the plate under UV light. This fluorescent compound is the 7-acetamido metabolite as reported by Rieder[13], which extracts directly under alkaline conditions.

CONCLUSIONS

The approach to toxicological screening described here has been developed for routine use in the hospital clinical chemistry laboratory. It enables current screening procedures to be extended to cover drugs commonly encountered in overdose. We have already investigated the reactions of newer compounds such as clomipramine, doxepin, dothiepin, and medazepam and these may be incorporated into the procedures should the need arise. In this way, by screening for a limited number of common drugs, the biochemist or technician, who is occasionally called upon to identify an unknown drug, can rapidly provide valuable assistance to the clinician.

It should, however, be emphasised that TLC identification in one solvent system is not ideal. If time is available, further confirmation in at least another TLC system together with gas chromatographic or UV examination should be carried out.

Finally, the use of TLC in the laboratory is no longer a novelty so that once the reagents have been purchased the scheme may be operated immediately. For those pathology laboratories not regularly using TLC the additional purchase of a box of prepared plates, together with tanks and sprays, still constitutes only a modest expenditure.

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