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Routine detection and identification in urine of stimulants and other drugs, some of which may be used to modify performance in sport

A. H. BECKETT, G. T. TUCKER AND A. C. MOFFAT

A general procedure for the analysis in urine of basic drugs (and their metabolites), some of which may be misused as stimulants in sport, has been developed. The techniques used include gas-liquid and thin-layer chromatography and linked gas-liquid chromatography—mass spectroscopy. It is recommended that international control of drug-taking in sport be based primarily upon urine analysis by gas-liquid chromatography systems and also derivative formation followed by gas-liquid chromatography. The principles outlined in the procedure can be applied in a much wider forensic context.

IT is now generally accepted that the sensitivity and specificity of analytical procedures based on gas-liquid chromatography (GLC) make this the technique of choice for the detection of many drugs in body fluids. Following general work on the GLC of biologically important amines (Fales & Pisano, 1962; Brochmann-Hanssen & Svendsen, 1962; Parker, Fontan & Kirk, 1962; Vanden Heuvel, Gardiner & Horning, 1964; and others) several specific procedures for the detection of amphetamine or methylamphetamine, or both, the drugs most commonly misused in human sporting activities, have been reported. Of these, the methods most relevant to the problem of control include those of Cartoni & De Stefano (1963), Beckett & Rowland (1965a), Venerando & De Sio (1964), Lebbé & Lafarge (1965, 1966), Kolb & Patt (1965) and Greco, Paolucci & Taponeco (1965).

The purpose of the present communication is to outline a comprehensive analytical procedure for drugs likely to be misused in sport, with emphasis on the confirmation of results obtained from preliminary GLC screening. This procedure has evolved as the result of experience obtained from tests made during several major international sporting events held in the United Kingdom (Tour of Britain Cycle Races, 1965, 1966; World Cup Football Championship, 1966). It relies largely on GLC techniques but also makes use of thin-layer chromatography (TLC) and instrumental methods (e.g. mass spectroscopy) for supporting information on 'positives'. Sampling procedures, which are as important as the analytical procedures in the overall approach to the problem of detecting drug misuse have been reported elsewhere (Beckett, Tucker & James, 1966).

As well as the inherent sensitivity and specificity of the analytical procedures there are also other factors which can influence the successful detection of a 'positive'.

CHOICE OF SAMPLE

Urine is the most convenient biological sample; also, for the drugs examined, urine offers the advantage of a high concentration of drug

From the Department of Pharmacy, Chelsea College of Science and Technology (University of London), Manresa Road, London, S.W.3.

compared with that in a blood sample in which low concentrations may result from extravascular concentration of the drug.

SELECTION OF DRUGS FOR SCREENING

The use of drugs in sporting activities is usually, in man, intended to increase performance. Therefore, in the present procedure, analysis for drugs with stimulant action is emphasized.

Ariëns (1964) has distinguished between long-term and short-term pharmacological conditioning for sporting events. The former implies the use of drugs, such as hormones and anabolic steroids, during the course of training. The effectiveness of these compounds taken during training is questionable; what is certain so far is that they can produce undesirable side-effects. This use of steroids by sportsmen is an insidious form of drug abuse which would be most difficult to control, at least from a sampling point of view. In short-term conditioning, there is a direct relation between the time the drugs are taken and physical effort. A wide variety of drugs, some of which are apparently effective and others which are valueless under the conditions in which they have been taken, have been and are being used. Of these drugs, the amphetamines are certainly the most common and are also amongst the most dangerous (Venerando, 1963). For these reasons their detection is emphasized in the proposed analytical scheme. Strychnine should be included in the analytical scheme, although its misuse in sport is now not prevalent. Like the amphetamines, caffeine has a stimulant effect on the central nervous system but since it is a constituent of normal beverages it is difficult, if not impossible, to distinguish between its normal use and misuse.

Ariëns (1964) also discusses several classes of compounds which may be used with intent to increase performance but these probably offer no advantage over normal physiological compensation mechanisms or metabolic processes. Such compounds include vitamins, natural metabolites and metabolite intermediates, adrenaline and sympathomimetics, analeptics and vasodilator drugs. Of these, the analeptics (e.g. nikethamide) and sympathomimetics with vasoconstrictor and cardiac stimulant actions are the most amenable to routine analysis.

As far as possible, the analytical procedure should also allow for the inclusion of new drugs having actions making their misuse in sport a possibility.

DRUG METABOLISM AND EXCRETION

The existence of a sensitive and specific analytical procedure for a particular drug is no guarantee that it can be used for the detection of that drug in biological fluids. Fundamental information about drug absorption, distribution and elimination, and particularly the time-course of these processes, is required if the analytical scheme is to be realistic. Unfortunately, for many drugs such information is inadequate. Therefore, ideally, the validity of the assay procedure for each drug considered should be tested by analysis of samples from volunteers to whom the drugs have been administered in normal doses.

A further complication is the fact that the elimination of many drugs is markedly dependent on urinary pH and for some drugs on urine volume

(Milne, Scribner & Crawford, 1958; Peters, 1960; Weiner & Mudge, 1964; Braun, Hesse & Malorry, 1963; Beckett & Rowland, 1964, 1965c; Asatoor, Galman & others, 1965; Beckett & Wilkinson, 1965a). Since urinary pH and output fluctuate throughout the day, these parameters, in addition to the time factor, can influence substantially the concentrations of unchanged drug and of its metabolites in urine. For instance, about 30–40% of a dose of amphetamine is excreted in the urine as unchanged drug over 48 hr under normal conditions (fluctuating urinary pH). However if the urine is rendered acidic (pH ca 5·0) for the same period the proportion of unchanged drug excreted increases to 60–70%. If the urine is rendered alkaline (pH ca 8·0) this percentage falls to below 10% (Beckett & Rowland, 1964, 1965b).

Thus the pH of urine samples should be measured as part of the method. Usually, since exercise tends to produce acidosis, the pH of a participant's urine is relatively acidic, although the use of special diets may complicate the situation. Sodium bicarbonate may sometimes be taken to offset the fatigue resulting from this acidosis. In large doses it will produce an alkaline urine of about pH 8·0 which would reduce the likelihood of detecting a basic drug such as amphetamine because the urinary excretion of this drug is accordingly much reduced. At the same time the subject would experience a more prolonged pharmacological effect from the drug.

These observations emphasize the need for sufficient sensitivity in the analytical methods to allow detection of the unchanged drug and, if possible, of drug metabolites under all conditions. The detection of metabolites also affords a means of distinguishing between drug that has passed through the body and drug that for some purpose may have been added directly to urine. The latter situation may arise when the sampling procedures lack security, or when a control sample has been added to a batch of test samples.

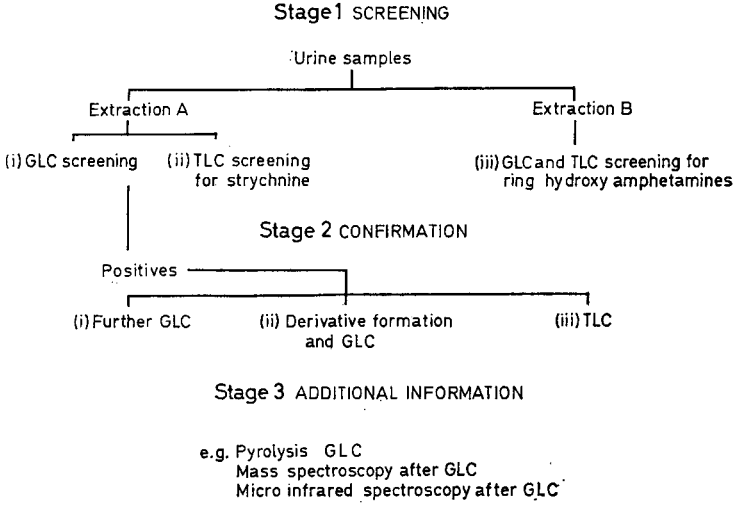


FIG. 1. Scheme of urine analysis.

METHODS

A suitable scheme of analysis, summarized in Fig. 1, involves three consecutive stages; not all stages are required routinely. Stage 1 is a routine screening procedure in which the emphasis is placed on GLC; the assay, by one worker, of 16 urine samples can be completed within a few hours of receipt. In general, implementing Stage 2 is sufficient to identify a drug giving a positive result in Stage 1. Stage 3 provides further support to the identification should this be required.

Stage 1: SCREENING

(i) GAS-LIQUID CHROMATOGRAPHY

The method is based on previously reported procedures for the determination of amphetamines (Beckett & Rowland, 1965a) and ephedrines (Beckett & Wilkinson, 1965b) in urine, and is capable of detecting these drugs for periods of up to 48 hr after normal therapeutic doses.

Extraction procedure A. Urine (1 to 5 ml) is pipetted into a glass-stoppered centrifuge tube together with 0·5 ml 20% sodium hydroxide solution. The urine is then extracted with 2 × 2·5 ml freshly distilled Analar diethylether using a mechanical tilt-shaker, centrifuged, and the ether extracts transferred to a 15 ml Quickfit test tube with a finely tapered base. The extract is then concentrated to about 50 μl on a water bath at 40°.

TABLE 1. GLC SYSTEMS USED IN STAGES 1 AND 2

System	Tubing	Liquid phase	Solid support	Oven temp. (°C)	N_2 (lb. in $^{-2}$)	H_2 (lb. in $^{-2}$)	Air (lb. in $^{-2}$)
A	3 m ss $\frac{1}{8}$ in. o.d.	5% Carbowax 6000 5% KOH	Chromosorb G A/W DMCS treated 80–100 mesh	155	20	20	25
B	1 m ss $\frac{1}{8}$ in. o.d.	2% Carbowax 20M 5% KOH	,,	140 & 180	15	15	25
C	2 m glass $\frac{1}{4}$ in. o.d.	2·5% SE-30	,,	120 & 160	20	24	30
D	2 m ss $\frac{1}{8}$ in. o.d.	10% Apiezon L 10% KOH	,,	155	20	20	25

Perkin-Elmer F11 gas-chromatographs with hydrogen flame-ionization detectors and Leeds & Northrup Speedomax type G (0–5mV) and Hitachi 159 (0–2·5mV) recorders were used.
Stream splitters (ratios approx. 1 : 5) were used with systems A, B and D.
Columns were conditioned at their operating temperatures for 24 hr before use and silanized with hexamethyldisilazane.

Approximately 3 to 5 μl of the concentrate is injected into each of two gas-chromatographic systems designated A and B (see Table 1). This combination detects most of the more commonly used central nervous system stimulants which, in general, are relatively small molecules based on the amphetamine structure. Systems B, C or D operated at higher temperatures can be used, if required, to screen many compounds of larger

molecular weight (unpublished observations) although modification of the extraction procedure may be necessary; for example, examination for analgesics like codeine and methadone, local anaesthetics like cocaine and lignocaine, caffeine and many antihistamines.

The retention times of the compounds screened using systems A and B are listed in Table 2; composite chromatograms are represented in Figs 2 and 3.

A 'positive' result at this point would be shown by the presence of a chromatographic peak, obtained on analysis of a test sample, with a retention time comparable to that of one of the drugs listed in Table 2. For instance, the chromatogram obtained on analysis of a urine sample from a competitive racing cyclist and reproduced in Fig. 4 indicates the presence of methylamphetamine and its metabolite amphetamine; confirmation requires the preparation of some of the derivatives listed in Table 2.

Discussion. In general, as little as 0·1 μg drug base per ml of urine may be determined by the above method, without interference from normal urinary constituents. Possible interference from breakdown products derived from such constituents on prolonged storage is minimized by refrigeration. Amphetamine itself is stable in refrigerated urine (4°) for at least three months.

Only basic compounds would be eluted from the alkaline columns used in systems A and B, and no interference is encountered from larger molecular weight molecules such as alkaloids and tranquillizers.

It was convenient to use separate isothermal systems for the present work since several gas chromatographs were available. However, a single 2 metre column as used in system B of Table 1, together with a temperature programming unit, could be used to detect the compounds listed in Table 2 with reasonable retention times for each. [Although the column materials used in systems A and B are essentially similar, the latter is more flexible since it employs Carbowax 20M as the liquid phase, which has a much higher operating temperature limit (ca 200°) than the Carbowax 6000 used in system A (limit 175°)]. Alternatively, a dual column instrument fitted with a 3 metre and a 1 metre type A or B column, and operated under isothermal conditions (oven temperature 150–160°) could be used. The retention times reported in Table 2 using systems A and B were reproducible over a period of at least six months.

Since some athletes are tobacco smokers, nicotine has been included in Table 2 so that a peak due to this compound is not confused with those from other materials being screened.

Most of the compounds investigated gave a single, symmetrical peak using systems A and B. Only phenoxypropazine gave more than one major peak; diethylpropion, methylphenidate and pyrovalerone gave single major peaks with small shoulders indicating decomposition. The possibility of enolization in these latter compounds on the potassium hydroxide-coated support material could explain these effects. Single peaks were obtained using system C.

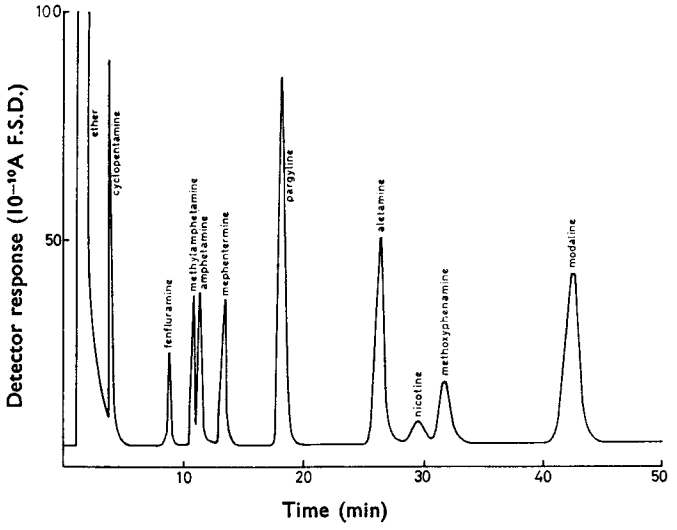


FIG. 2. Composite chromatogram of some stimulants and related compounds on Column "A".

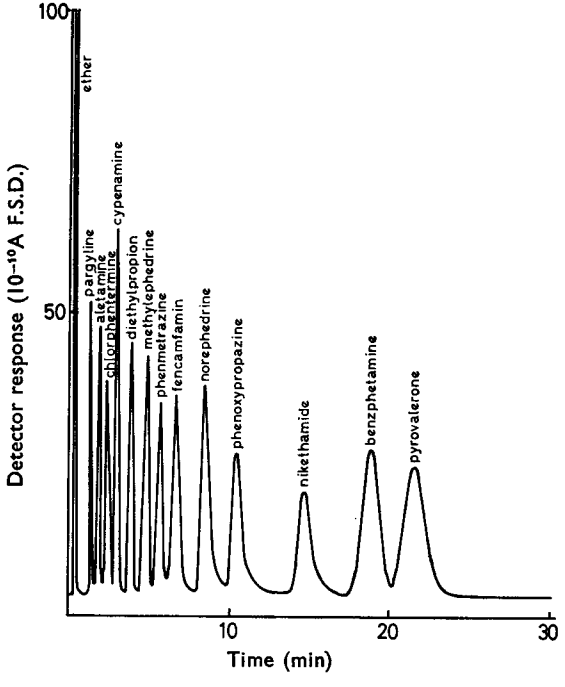


FIG. 3. Composite chromatogram of some stimulants and related compounds on Column "B".

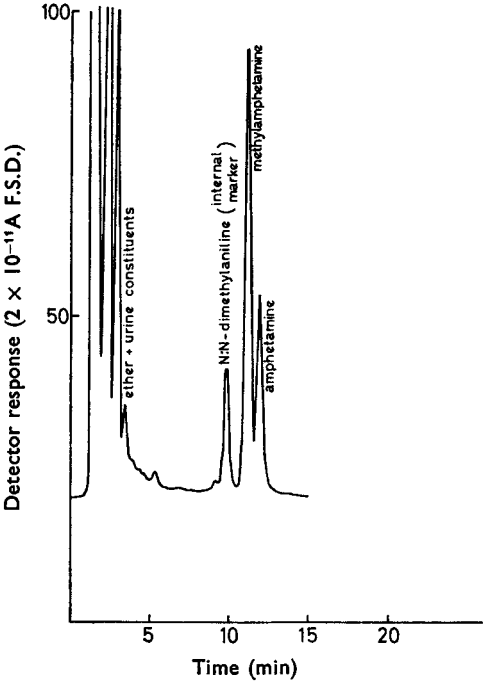


FIG. 4. Reproduction of a chromatogram obtained on analysis of a racing cyclist's urine showing the presence of methylamphetamine and its metabolite amphetamine in the urine.

(ii) THIN-LAYER CHROMATOGRAPHIC SCREENING FOR STRYCHNINE

Urine (10–20 ml) is taken through extraction procedure A (p. 276), using 2 × 5–10 ml ether. Portions of the final ether concentrate are applied to thin-layer plates which are then developed using at least two different solvent systems (see Table 4). In each instance reference strychnine is run on the same plates. A 'positive' result is recorded if, in each system, a spot is obtained with an Rf value comparable to that obtained for the reference compound.

The method was evaluated on 20 ml of a 1–3 hr urine sample obtained from a volunteer who took 4 mg strychnine hydrochloride. TLC systems IV and VI were used (see Table 4). The interpretation of the chromatograms was not complicated by the presence of normal urinary constituents, and a positive result was recorded.

(iii) GAS-LIQUID AND THIN-LAYER CHROMATOGRAPHIC SCREENING FOR RING-HYDROXY AMPHETAMINES

This group of compounds includes *p*-hydroxyamphetamine, *p*-hydroxymethylamphetamine, phenylephrine and metaraminol. Although all reports on the clinical use of these drugs emphasize the absence of central stimulation (Goodman & Gilman, 1965) their potential misuse in the present context cannot be excluded.

TABLE 2. GLC AND URINARY EXCRETION DATA FOR SOME STIMULANTS AND RELATED COMPOUNDS

Compound	Formula No. (p.283)	Retention time (min)** System A (155°)	B (140°)	C (120°)	D (155°)	Excretion in urine: Unchanged	Metabolite(s)¶	Derivative retention time (min)§ System A (155°) Acetone	B (140°) Acetone	B (180°) N-Acetyl	B (180°) N-Propionyl	C (120°) Acetone	C (160°) N-Acetyl	C (160°) N-Propionyl
2-Aminoheptane	1	2·2	—	0·9	ND	NT		2·8	—	0·75	0·75	1·7	1·7	2·1
Methylaminomethylheptane	2	2·4	—	1·4	ND	NT		S	S	0·75	0·75	S	2·8	3·5
Isomethepten	3	3·1	—	1·8	ND	NT		S	S	1·0	1·0	S	3·2	3·9
Cyclopentamine	4	3·9	—	1·8	4·6	NT		S	S	1·3	1·3	S	4·0	5·0
Isopropylhexedrine	5	5·2	—	2·9	8·2	NT		S	S	1·7	1·7	S	5·6	7·1
Propylhexedrine	6	5·2	—	2·9	ND	NT		S	S	1·7	1·7	S	5·6	7·1
Fenfluramine	7	8·8	—	3·9	ND	Yes	N	S	S	2·1	2·1	S	5·5	6·9
Norfenfluramine	8	10·0	—	2·8	ND	Yes		9·0	—	3·2	3·3	4·3	3·7	4·4
Methylamphetamine*	9	10·8	—	3·4	8·2	Yes	A	S	S	3·1	3·2	S	5·6	6·9
Dimethylamphetamine*	10	10·8	—	4·2	ND	Yes	MA + A	T	T	T	T	T	T	T
N-Ethylamphetamine*	11	11·0	—	4·4	10·2	Yes	A	S	S	3·0	3·0	S	6·4	8·2
Phentermine	12	11·2	—	2·8	7·5	Yes		Not formed	Not formed	2·8	2·8	Not formed	3·8	5·3
Amphetamine*	13	11·4	—	2·4	6·1	Yes		12·5	—	4·0	4·1	4·7	4·0	4·9
Mephentermine	14	13·4	—	4·3	ND	Yes		S	S	2·5	2·6	S	5·6	7·6
Pargyline	15	18·4	1·4	3·6	9·1	Yes		T	T	T	T	T	T	T
Aletamine	16	26·2	1·9	5·2	ND	NT		21·8	1·6	5·6	5·6	8·8	7·0	8·8
Tranylcypromine	17	28·6	2·0	3·8	ND	NT		43·0	3·4	15·6	16·2	9·5	7·6	9·6
Nicotine	18	29·4	2·1	7·1	17·7	Yes		T	T	T	T	T	T	T
Methoxyphenamine	19	31·7	2·4	7·7	ND	NT		S	S	7·0	7·1	S	11·4	13·6
Chlorphentermine	20	32·6	2·4	6·5	21·7	Yes		Not formed	Not formed	7·4	7·4	Not formed	8·1	11·0
Modaline†	21	42·4	3·1	10·2	ND	NT		T	T	T	T	T	T	T
Cypenamine†	22	42·4	3·1	7·5	ND	NT		56·3	3·3	13·5	12·7	15·4	11·1	14·0
Diethylpropion*	23	—	3·6	13·6	43·0	Yes	D	T	T	T	T	T	T	T
Phendimetrazine*	24	—	3·6	11·6	35·7	Yes	PH	T	T	T	T	T	T	T
Prolintane	25	—	3·7	29·2	ND	NT		T	T	T	T	T	T	T
Methylephedrine*	26	—	4·7	9·5	25·2	Yes	E + NE	T	T	T	T	T	T	T
Phenmetrazine*	27	—	5·7	10·5	31·3	Yes		S	S	11·2	11·5	S	12·6	16·3
Ephedrine*	28	—	6·3	8·4	19·4	Yes	NE	—	2·7	17·8	19·2	11·6	9·9	13·1
Pseudoephedrine	28	—	6·3	8·4	ND	Yes	NP	—	2·5	16·0	16·0	10·4	10·0	13·1
Ethylephedrine*	29	—	6·5	13·0	ND	Yes	E + NE + DM	T	T	T	T	T	T	T
Fencamfamin	30	—	6·7	31·3	ND	NT		S	S	16·8	16·8	S	37·4	47·0
Norpseudoephedrine	31	—	8·4	6·9	ND	Yes		—	3·0	29·5	30·8	7·8	8·3	10·7
Norephedrine	31	—	8·6	6·9	16·1	Yes		—	2·8	28·6	30·0	8·8	8·6	10·5
Encyprate†	32	—	9·5	26·5	ND	NT		T	T	T	T	T	T	T

TABLE 2—*continued*

Compound	Formula No.	Retention time (min)** System A (155°)	B (140°)	C (120°)	D (155°)	Excretion in urine: Unchanged	Metabolite(s)¶	Derivative retention time (min)§ System A (155°) Acetone	B (140°) Acetone	B (180°) *N*-Acetyl	B (180°) *N*-Propionyl	C (120°) Acetone	C (160°) *N*-Acetyl	C (160°) *N*-Propionyl
Furfurylamphetamine	33	—	10·2	26·0	ND	NT		S	S	16·8	17·2	S	24·0	28·7
Furfurylmethylamphetamine	34	—	10·2	35·0	ND	Not detected	M+A	T	T	T	T	T	T	T
Phenoxypropazine	35	—	10·1	12·4	ND	NT		—	7·4	13·0	13·8	15·5	13·0	14·6
Nikethamide*	36	—	17·6	16·0	33·7	NT		T	T	T	T	T	T	T
Benzphetamine	37	—	19·0	74·0	ND	Yes (small amounts)	BA+MA+A	T	T	T	T	T	T	T
Methylphenidate*	38	—	20·9	46·4	ND	Yes		S	S	31·0	32·0	S	40·0	49·6
Pyrovalerone†	39	—	21·6	87·0	ND	NT		T	T	T	T	T	T	
Leptazol*	40	—	52·0	15·8	ND	NT		T	T	T	T	T	T	T

* Stimulant drugs most likely to be used as doping agents.

† Examples of newer drugs with central stimulant actions. Compounds listed also include other amphetamine analogues mainly used for their vascular and bronchial effects or as anoretics, reputedly with much less or no central stimulant action. Pargyline, tranylcypromine and phenoxypropazine are listed as examples of the monoamine oxidase inhibitor type of stimulant.

S = secondary amine. T = tertiary amine. ND = not determined. NT = not taken by volunteer(s)

** Determined with reference to amphetamine (system A) and to ephedrine (systems B and C).

§ Determined with reference to the amphetamine acetone derivative (system A); the ephedrine acetone derivative (system B, 140° and system C, 120°) and the acyl-amphetamine derivatives (system B, 180° and system C, 160°).

¶N norfenfluramine; A amphetamine; MA methylamphetamine; P phentermine; D de-ethylated analogue(s); PH phenmetrazine; E ephedrine; NE norephedrine; NP norpseudoephedrine; DM demethylated analogue of ethylephedrine; BA benzylamphetamine.

TABLE 3. GLC DATA FOR SOME AMPHETAMINE DERIVATIVES (Retention times in min.)

System	Oven temp (°C)	Free amphetamine	Reagent A	MEK	MIK	MPK	IMK	CP	CHX	CHT	BMK	AA	HFB	CS
A	155	11·4	12·3	14·6	14·9	19·6	21·9	47·9	66·9	—	—	—	—	24·9
B	140	0·85	0·95	1·1	1·2	1·5	1·8	3·6	5·0	8·2	26·0	24·1	1·9	6·1
C	120	2·4	4·3	6·5	8·2	9·8	12·9	16·8	21·5	44·6	—	—	6·3	11·2

A acetone; MEK methyl ethyl ketone; MIK methyl isopropyl ketone; MPK methyl n-propyl ketone; IMK isobutyl methyl ketone; CP cyclopentanone; CHX cyclohexanone; CHT cycloheptanone; BMK benzyl methyl ketone; AA acetyl acetone; HFB heptaflurobutyric anhydride; CS carbon disulphide.

The presence of the phenolic group in these compounds necessitates modification of both extraction and chromatographic procedures; a suitable procedure for the detection of *p*-hydroxyamphetamine is as follows.

Extraction procedure B. Urine (10 ml) is rendered alkaline (pH 9–10) by the addition of solid sodium carbonate and then extracted with 3 × 5 ml portions of freshly distilled Analar diethyl ether. The combined ether extracts are concentrated as in extraction procedure A (p. 276). The concentrate is then analysed by GLC and TLC. The extracted urine is then neutralized and heated at 80–100° with 2 ml of 6N hydrochloric acid for 1 hr, to hydrolyse the conjugated drug (glucuronide or ethereal sulphate). After cooling, the pH is adjusted to 9–10 by the addition of 2 ml of 6N sodium hydroxide and solid sodium carbonate. The urine is then extracted as before and analysed by GLC.

GLC *analysis.* 1 μl of the final concentrated ether extracts from the unhydrolysed and hydrolysed urine is analysed using GLC system C at 160° (the use of an alkali-coated support in systems A and B prevents the elution of compounds containing acidic or phenolic groups). A 'positive' result is recorded if a peak is obtained with a retention time comparable to that of reference *p*-hydroxyamphetamine (t_R = 3·3 min).

Confirmation is obtained by chromatography of the acetyl derivative (t_R = 18·0 min) and a trimethylsilyl (TMS) derivative (t_R = 4·3 min) (see Stage 2 for methods of preparation).

TLC *analysis.* Portions of the concentrated ether extract from the unhydrolysed urine are applied to thin-layer plates which are then developed using at least two different solvent systems (see Table 4). In each instance, reference *p*-hydroxyamphetamine is run on the same plates. A 'positive' result is recorded if, in each system, a spot is obtained with an Rf value comparable to that obtained from the reference compound. Interpretation of chromatograms obtained on running the concentrated ether extract from hydrolysed urine is complicated by interference from normal urinary constituents. This is less of a problem in the GLC analysis.

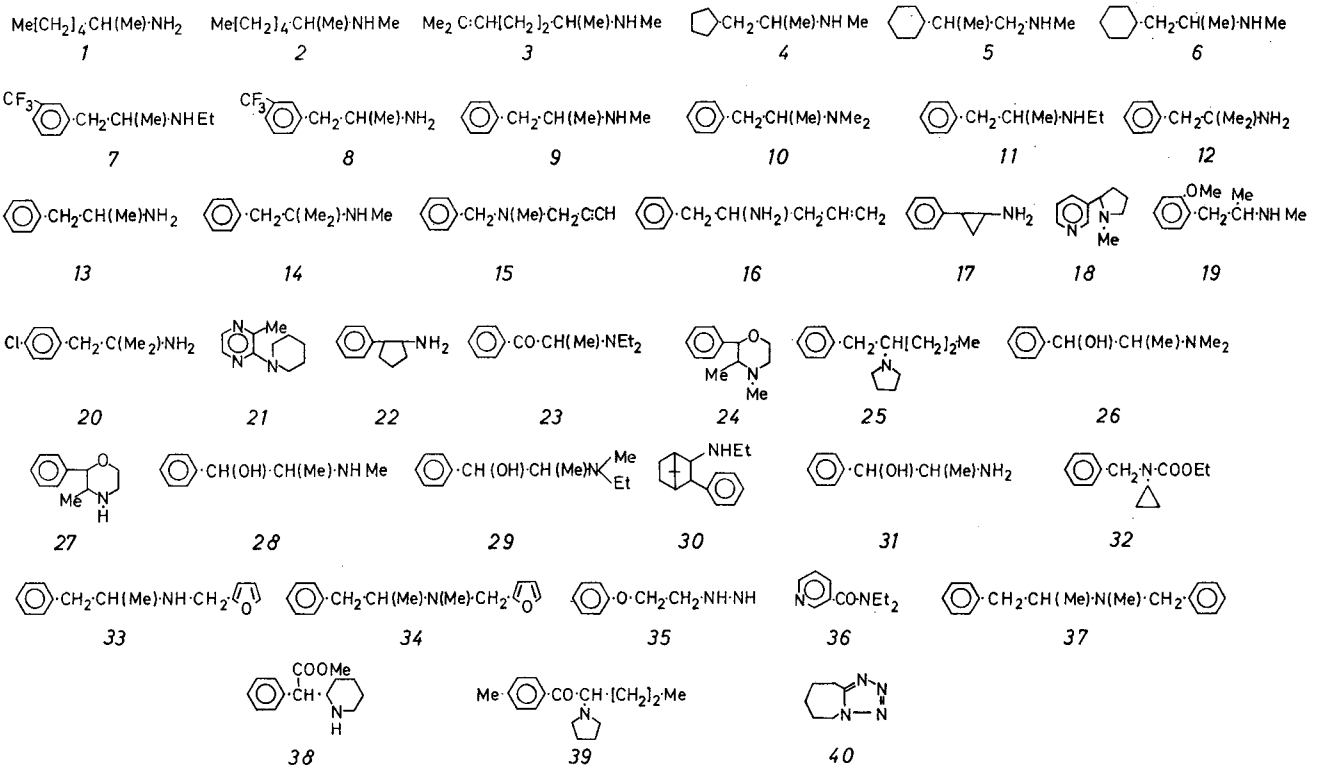
The method was evaluated on 10 ml of a 2–4 hr urine sample from a volunteer who had ingested 60 mg *p*-hydroxyamphetamine hydrobromide. A large amount of the drug was detected in the urine and the results indicated that a considerable proportion was present in a conjugated form.

Stage 2: CONFIRMATION

In this stage, conclusive identification is obtained of 'positives' recorded in Stage 1. This confirmation is based primarily on the results of further GLC, i.e. the use of different systems, and the preparation and chromatography of simple derivatives. TLC is also used as an auxilliary technique although in general it is less sensitive and specific than the GLC methods.

(i) FURTHER GLC

(a). Portions of the concentrated ethereal extract obtained in Stage 1 are injected into GLC systems C and D and the chromatograms obtained



Structural formulae of compounds listed in Table 2.

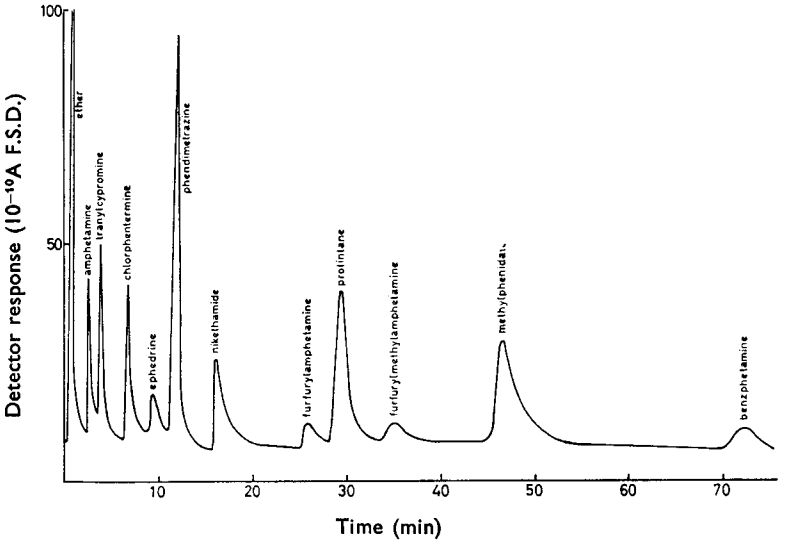


FIG. 5. Composite chromatogram of some stimulants and related compounds on Column "C".

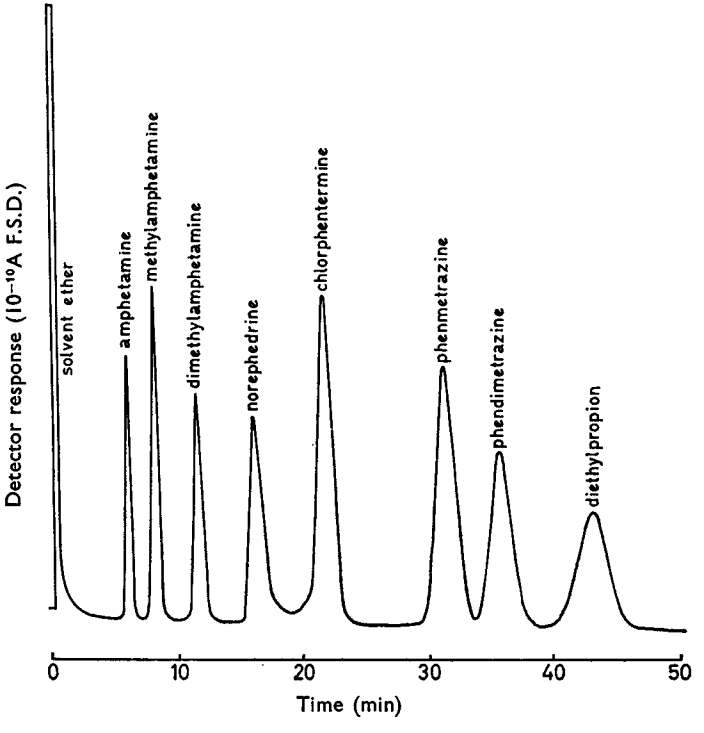


FIG. 6. Composite chromatogram of some stimulants and related compounds on Column "D".

are compared with reference chromatograms (see Figs 5 and 6). Preliminary identification, supporting the evidence obtained in Stage 1, is made by comparing retention times of the peak(s) obtained with those given by the reference compounds (see Table 2).

(b). The *relative* retention time of a compound, giving rise to a peak noted in (a), is determined with respect to an appropriate internal marker, using systems A or B, and D. (Any reference compound giving a peak with a retention time close to, but distinct from, the peak due to the compound under investigation can be used.) Identification of the compound is made by comparing its relative retention time with that determined under the same conditions using known reference compounds.

(c). The quantity of drug, identified in (b), in a 'positive' sample may be determined using the internal standard technique (see Beckett & Rowland, 1965a; Beckett & Wilkinson, 1965b).

The use of systems C and D allows a different and in many instances a greater separation of the compounds screened in Stage 1. The order of elution of several pairs of compounds (e.g. amphetamine and methylamphetamine) is the reverse of that obtained using systems A or B. A limitation of system C is that the time between sample injection and peak maximum increases and the peak tends to broaden, as the sample size is decreased. For example, 1 μg of nicotine injected in 1 μl of ether has a retention time of 7·1 min, but this is increased to 7·6 min when the concentration is decreased to 0·2 μg/μl. Therefore, it is necessary to compare retention times of the reference compounds with that of a suspected 'positive' at similar peak heights. The retention times listed in Table 2, using system C, were determined with solutions containing approximately 1 μg base per μl of ether. Retention times determined using system D (see also Lebbé & Lafarge, 1965, 1966) are independent of sample size and the use of this system is preferable, for the confirmation of 'positives'.

(ii) DERIVATIVE FORMATION AND GLC

The retention times of the primary and secondary amines, listed in Table 2, may be shifted by the formation of simple derivatives using selective reagents. Thus, having treated concentrated ethereal urine extracts from a Stage 1 'positive' with appropriate reagents, as described below, aliquots of the reaction mixtures are then injected into the GLC systems. Identification of the 'positive' is made on the basis of a comparison between the chromatograms produced with those obtained for derivatives of the reference compounds (see Table 2). Retention times relative to an appropriate internal marker can be used as the basis of identification.

This 'peak-shift technique' (Langer & Pantages, 1961) has received much attention in recent years (Brochmann-Hanssen & Svendsen, 1962; Anders & Mannering, 1962; Brooks & Horning, 1964; Vanden Heuvel, Gardiner & Horning, 1964; Capella & Horning, 1966; and others). To be of value a derivative must be formed easily and in good yield, and have a retention

TABLE 4. TLC OF SOME STIMULANT DRUGS AND RELATED COMPOUNDS

Support		Solvent v/v	Rf values × 10²											
			A	MA	*p*OHA	E	ME	NE	P	D	N	S	C	Nic
Alumina	I	$CHCl_3$–MeOH (50:50)[1]	57	81	37	69	84	13	89	92	87	93	93	91
Silica Gel	II	$CHCl_3$–MeOH (50:50)[2]	29	20	16	23	30	19	52	81	89	21	86	72
,,	III	$CHCl_3$–diethylamine (9:1)[3]	74	79	25	35	64	23	67	97	89	70	79	84
,,	IV	$CHCl_3$–acetone–diethylamine (5:4:1)[3]	84	70	46	33	69	83	60	92	82	61	71	81
,,	V	n-butanol–acetic acid–water (5:4:1)[4]	60	49	57	50	41	55	51	37	56	15	57	22
,,	VI	MeOH–acetone (50:50)[5]	59	24	51	30	36	67	45	79	75	12	74	56
,,	VII	MeOH–acetone–NH_3 (35%) (47·5:47·5:5)[5]	85	56	81	78	67	92	77	97	87	49	85	79
,,	VIII	MeOH–acetone–triethanolamine (1:1:0·03)[6]	63	27	49	28	42	71	53	79	80	14	76	63
,,	IX	Isopropanol–NH_3 (5%) (10:1)[7]	46	32	40	29	39	44	51	82	70	24	57	65
,,	X	Dimethylformamide/ethylacetate/+3 drops *n-octanol* (1:9)[8]	21	16	9	20	38	18	26	75	67	9	68	59

[1] Noirfalise, 1966; [2]Noirfalise, 1965, 1966; [3]Waldi, 1964; [4]Debackere & Massart-Leen, 1965; [5]Moerman, 1964; [6]Baumler & others, 1964; [7]Ristic & Thomas, 1962; [8]Eberhardt & Debackere, 1965.

A, amphetamine; MA, methylamphetamine; *p*OHA, *p*-hydroxyamphetamine; E, ephedrine; ME, methylephedrine; NE, norephedrine; P, phenmetrazine; D, diethylpropion; N, nikethamide; S, strychnine; C, caffeine; Nic, nicotine.

DRUG SOLUTIONS. Ethereal solutions of the basic forms of each reference compound were prepared by extraction of alkaline solutions of the salts. Approximately 20–30 μg of each drug was applied to thin-layer plates using a 10 μl Hamilton syringe. A standard solution of (+)-amphetamine sulphate in methanol was used for the determination of spray sensitivities.

PREPARATION OF PLATES. Alumina G (Merck) or Silica Gel G (Merck) (30 g) was mixed with water (60 ml), containing sodium fluorescein (0·04%), by stirring for 2 min in a mortar and spread on to 20 × 20 cm glass plates in a layer 0·25 mm thick. The plates were allowed to dry in the air for 15 min and then for 2 hr in an oven at 80°, after which they were placed in a desiccator to cool before use. Plates were run at ambient room temperature.

DETECTION OF SPOTS. Initially spots were located using ultraviolet light (254 and 350 mμ). All the reference compounds could be visualized in this way. The following spray reagents were used:

(*a*) Iodoplatinate reagent: 3 ml 10% solution of chloroplatinic acid treated with 97 ml water and 100 ml aqueous 6% KI solution added. Stored in a brown glass bottle.
Reference compounds appeared as pale yellow or brown spots except strychine which showed as a distinctive deep violet spot and *p*-hydroxyamphetamine and caffeine which were not detected.
Sensitivity (amphetamine) about 5 μg.

(*b*) i. Freshly diazotized *p*-nitroaniline (Wickström & Salvesen, 1952). ii. 0·5 N NaOH in ethanol.
Reference compounds appeared as yellowish spots except amphetamine which showed as a distinctive pink spot and *p*-hydroxyamphetamine which gave a brown spot. Strychnine, nicotine and caffeine were not detected.
Sensitivity (amphetamine)—about 5 μg.

(*c*) Ninhydrin reagent (Dole & others, 1966): 0·4% ninhydrin in acetone prepared within 30 min of use. After warming under the ultraviolet lamp the reference compounds appeared as pale blue spots except amphetamine, ephedrine and norephedrine which showed as distinctive violet spots. Caffeine and strychnine were not detected.
Sensitivity (amphetamine) about 1 μg.

Note: plates without added fluoroscein were used to determine the above spot colours.

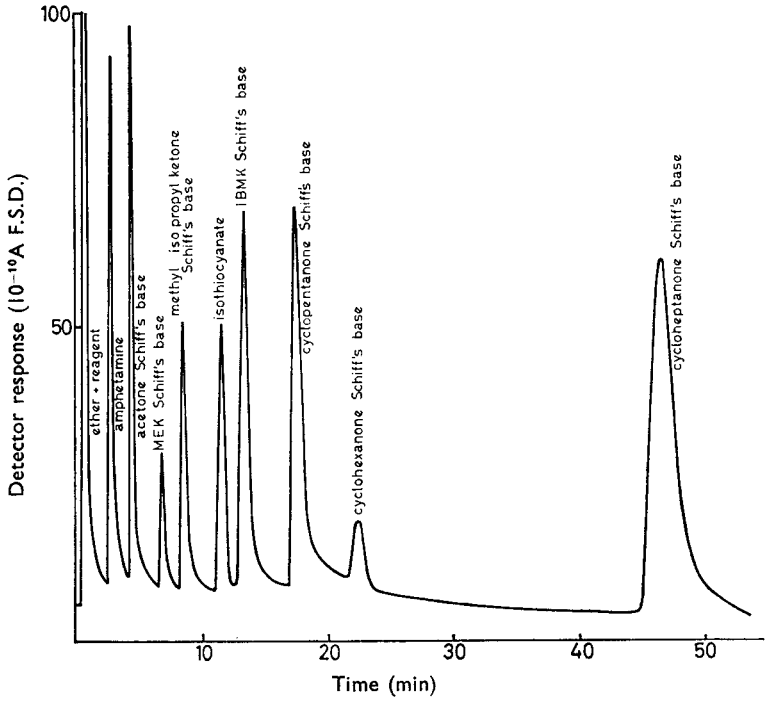


FIG. 7. Composite chromatogram of amphetamine and some of its derivatives on column "C".

time distinct from the parent compound. The following are examples of suitable derivatives.

1. *Acetone Schiff's bases and isothiocyanate derivatives.* (Acetone derivatives are only formed by primary amines. Both primary and secondary amines will react with CS_2 to give dithiocarbamates, but only in the former case will this derivative be converted to the isothiocyanate which will give a peak by GLC.)

The reagent (0·5 ml), acetone or CS_2, is added to the 50 μl concentrated ethereal urine extract. The whole is evaporated to 50 μl on a 60° water bath. In both instances, about 90% conversion is usual under these conditions.

2. *Other Schiff's bases.* (Formed by primary amines.) The ketone reagent (50 μl) is added to the concentrated ethereal urine extract and incubated in a closed evaporating tube for 10 min on a 50° water bath. In general, the yield of derivative is smaller with larger molecular weight ketones. In these instances the reaction may be allowed to proceed for a longer time to increase the yield of the derivative.

3. *Benzyl methyl ketone Schiff's bases.* (Formed by primary amines.) Larger ketones give chromatographic peaks which may interfere with those of the simpler amphetamines. Therefore, method 2 is modified when using

higher molecular weight ketones as reagents. The derivative is formed by dissolving 100 mg of the ketone in 1 ml of ether and using 50 μl of this solution for the reaction as in 2.

4. *Oxazolidines.* [Secondary amines with β-hydroxy groups (e.g. ephedrine) form oxazolidines with ketones (Capella & Horning, 1966).] The ketone (0·5 ml) is added to the concentrated ethereal urine extract, left at room temperature for 2 hr, and then evaporated to 50 μl.

5. *Carbinolamines.* [Formed by reaction of secondary amines with cyclic ketones (Capella & Horning, 1966).] Prepared as in method 2.

6. *Acetyl, propionyl and heptafluorobutyryl derivatives.* (Formed by primary and secondary amines.) The appropriate anhydride (5 μl) is added to the concentrated ethereal urine extract, and 5 μl of the mixture is injected into the gas chromatograph. Immediate 100% acylation is usually achieved.

7. *Trimethylsilyl derivatives.* (Formed when the compound contains an aromatic ring-hydroxy group.) An equal volume of reagent (2 parts hexamethyldisilizane, 1 part trimethylchlorosilizane and 10 parts dry distilled pyridine) is added to the concentrated ethereal urine extract and mixed thoroughly. A quantitative reaction is usually achieved after allowing the mixture to stand for 5 min.

Retention times of the acetone, acetyl and propionyl derivatives of appropriate reference compounds, using three of the GLC systems, are listed in Table 2, while Table 3 and Fig. 7 record the retention times of many derivatives which could be prepared, if necessary, from a single compound, i.e. amphetamine.

The value of derivative formation as a method of resolving compounds with similar retention times on the screening columns is shown by a comparison of the data for norfenfluramine, methylamphetamine and dimethylamphetamine (see Table 2). Norfenfluramine (a primary amine) can be separated from the others by the formation of its acetone derivative. Both norfenfluramine and methylamphetamine (a secondary amine) can be acylated, while dimethylamphetamine (a tertiary amine) does not give either of these simple reactions.

All the primary amines studied readily formed Schiff's bases, with the exception of phentermine and chlorphentermine. The two α-methyl groups in these compounds may hinder the reaction with ketones.

Under the conditions used for the formation of acyl derivatives, only the *N*-acyl derivatives would be produced since traces of water in the concentrated ethereal urine extracts would hydrolyse any *O*-acyl groups formed. Thus, when norephedrine base in dry ether was treated with acetic anhydride and chromatographed using system C at 160°, two derivative peaks were obtained at 8·6 and 10·0 min. On addition of water to the reaction mixture and further chromatography, the second peak (presumably either the *N*-acetyl, *O*-acetyl and/or the *O*-acetyl compound) disappeared.

The different rates of reaction of ephedrine and pseudoephedrine with acetone may be used to distinguish between them (Brochmann-Hanssen &

Svendsen, 1962); using method 4, approximately 80–90% of pseudoephedrine is converted to the oxazolidine, but only about 5–10% conversion is achieved with ephedrine.

(iii) THIN-LAYER CHROMATOGRAPHY

Portions of the final ethereal concentrate from 20 ml urine (extraction procedure A, p. 276) are applied to thin-layer plates which are then developed using at least two different solvent systems. Appropriate reference drugs are applied to the same plates. A 'positive' result is recorded if a spot is obtained, in each system, with a comparable Rf value and colour to that obtained from the relevant reference compound. TLC systems and reference drugs are chosen according to the suspected identity of the 'positive'.

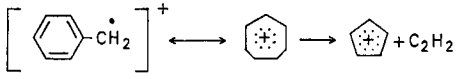
Ten TLC systems, reported in the literature, for the identification of the commonly used amphetamines were investigated using several reference compounds (see Table 4). It is essential to run solutions of reference drugs on each plate since Rf values are not very reproducible. The values quoted in Table 4 serve only to indicate the general order of separation achieved with each system.

Urine extracts from volunteers who had received normal doses of some of the reference drugs were run on all systems (amphetamine and methylamphetamine); on systems II and IX (ephedrine); on systems IV and VI (strychnine) and on systems II and VI (*p*-hydroxyamphetamine). A 'positive' result was recorded in each instance with no interference from normal urine constituents.

Stage 3: ADDITIONAL INFORMATION

The use of the two previous stages in all examples to date has led to the unequivocal identification in human urine of most of the drugs listed in Table 2. However, should additional information be required, techniques such as mass spectroscopy and micro infrared spectroscopy can be used.

For instance, Fig. 9 shows the mass spectra of amphetamine and methylamphetamine (extracted from urine), determined by combined GLC—mass spectroscopy. Ion abundance peaks which were absent from, or significantly greater than, those in the "background" spectrum are labelled in mass units. Although the spectra do not show significant amounts of parent molecular ions at masses of 135 (amphetamine) and 149 (methylamphetamine), differentiation of these closely related compounds is relatively simple by examination of their fragmentation patterns. For example, cleavage pattern b (see Fig. 8) produces an abundant ion, $[Me_3{\cdot}CH{\cdot}NH_2]^+$, of mass 44 in the spectrum of amphetamine, whereas similar cleavage of methylamphetamine gives rise to a large peak at mass 58, due to $[Me{\cdot}CH{\cdot}NH{\cdot}Me]^+$. The removal of the α-methyl group of amphetamine and methylamphetamine (cleavage pattern c in Fig. 8) is indicated by relatively abundant ions of masses 120 and 134 respectively. The peaks, in both spectra, at masses 77 and 91 are due to $[Ph]^+$ and $[Ph{\cdot}CH_2]^+$ respectively (see cleavage patterns a and b in Fig. 8), while the presence of an abundant ion of mass 65 may be accounted for as follows:



mass 91 produced by cleavage pattern b. tropylium ion mass 65

(see Grubb & Meyerson, 1963).

The mass spectrum of β-phenethylamine, on the other hand, does not have a large peak at 58 mass units, which distinguishes it from methylamphetamine, while a peak at 30 mass units (cleavage pattern b in Fig. 8) readily distinguishes it from amphetamine. The spectrum of methyl amphetamine also shows a significant peak at 30 mass units, probably resulting from cleavage d (see Fig. 8), to give $[NH{\cdot}Me]^+$. Unlike the spectra of the two drugs, the spectrum of β-phenethylamine contains a significant peak for the parent molecular ion, at 121 mass units. Spectra obtained after direct introduction of samples into the ionization chamber of the mass spectrometer are qualitatively identical to those obtained after GLC of the bases extracted from urine.

Although the spectra shown in Fig. 9 were obtained on analysis of approximately 10–20 μg of drug (extracts of 5 ml aliquots of urine containing 4 μg drug base per ml were used, i.e. concentrations of the order likely

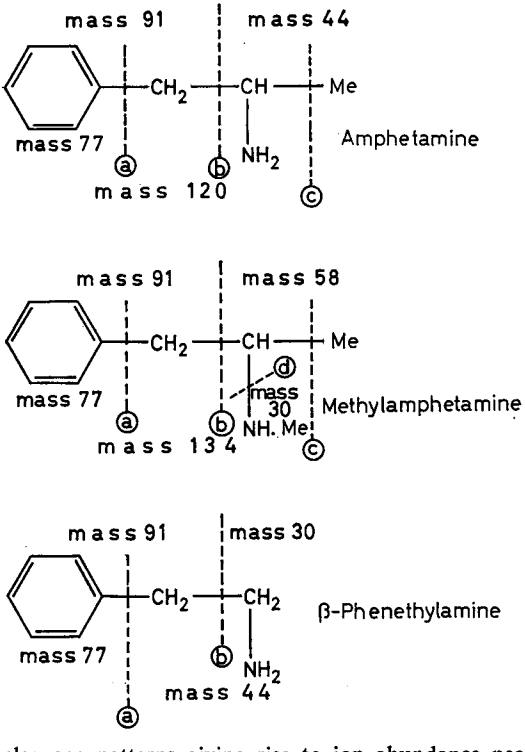


FIG. 8. Main cleavage patterns giving rise to ion abundance peaks in the mass spectra of amphetamine, methylamphetamine and β-phenethylamine.

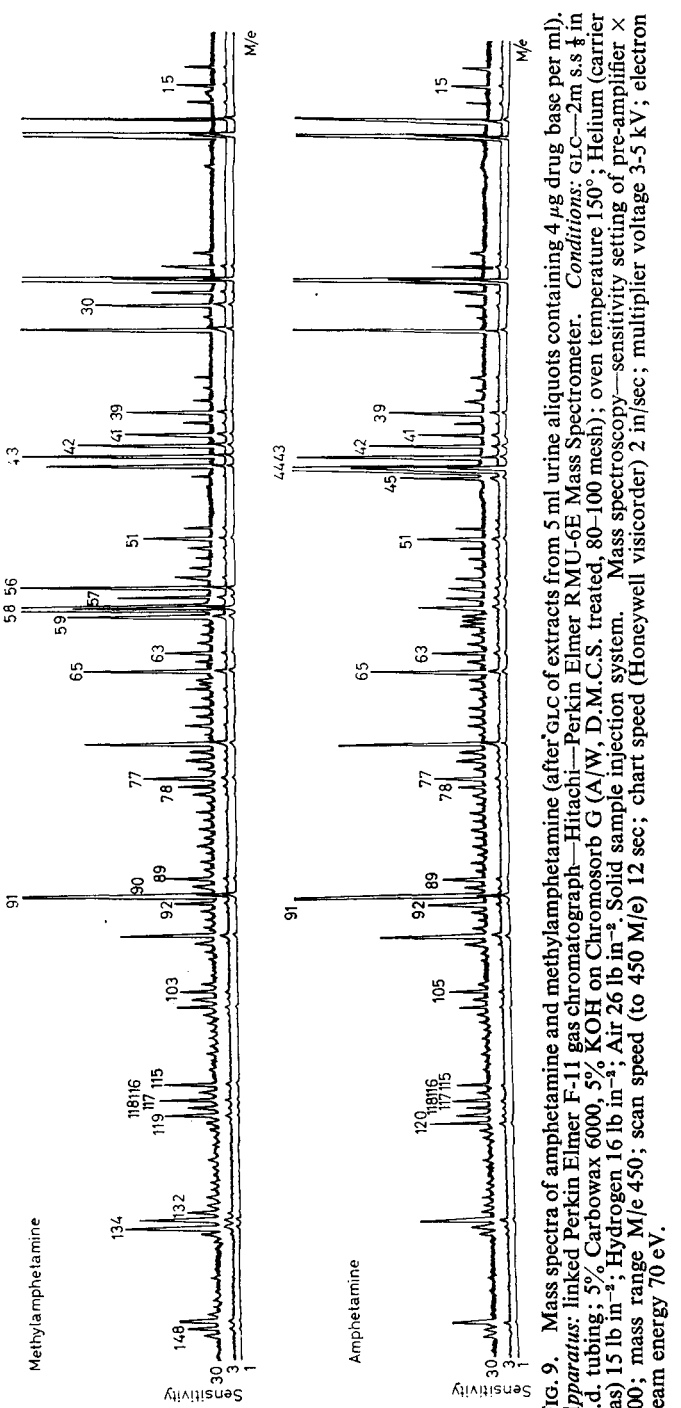


FIG. 9. Mass spectra of amphetamine and methylamphetamine (after GLC of extracts from 5 ml urine aliquots containing 4 μg drug base per ml). *Apparatus:* linked Perkin Elmer F-11 gas chromatograph—Hitachi—Perkin Elmer RMU-6E Mass Spectrometer. *Conditions:* GLC—2m s.s ¼ in o.d. tubing; 5% Carbowax 6000, 5% KOH on Chromosorb G (A/W, D.M.C.S. treated, 80–100 mesh); oven temperature 150°; Helium (carrier gas) 15 lb in^{-2}; Hydrogen 16 lb in^{-2}; Air 26 lb in^{-2}. Solid sample injection system. Mass spectroscopy—sensitivity setting of pre-amplifier × 100; mass range M/e 450; scan speed (to 450 M/e) 12 sec; chart speed (Honeywell visicorder) 2 in/sec; multiplier voltage 3-5 kV; electron beam energy 70 eV.

after drug taking), much smaller amounts may be detected by this method. At present the limits of detection using linked GLC–infrared systems or micro-preparative GLC followed by micro–infrared analysis are somewhat higher (50–100 μg of compound).

Discussion

The scheme of analysis described is effective in distinguishing between the amines of Table 2, some of which are, and some of which may be misused in sport. Constituents in the urine do not interfere with this scheme of identification.

It has been held by some analysts that the presence of a drug is not proved unless the drug is identified by more than one type of analytical technique. We have therefore, included TLC in this scheme of analysis although, in general, we have found it to be much less sensitive than GLC for the detection of the amines examined. We recommend that international control of drug taking in sport be based upon the adoption of urine analysis involving GLC with different systems combined with derivative formation followed by GLC. The range of derivatives is capable of extension and it should thus be possible to draw up a list of retention times of agreed derivatives for each drug, and metabolites where applicable, relative to those for standard compounds, which would constitute acceptable proof of identity of an administered drug; the application to amphetamine (see Table 3) illustrates such an approach to the problem.

When, as well as the drug, its metabolites are excreted in urine, the GLC characteristics of the metabolites and derivatives of the metabolites using different systems, afford additional proof of the presence of the ingested drug, e.g. administered methylephedrine yields not only this compound in the urine but also ephedrine and norephedrine (Beckett & Wilkinson, 1965c) which can be characterized by the use of various columns and derivatives (see Table 2). The above scheme is capable of progressive refinement as more information is acquired about the elimination of drugs. Furthermore it is unlikely that new stimulants will now be marketed in the absence of information on their metabolism in man.

The sensitivity of the GLC method, in general, makes it possible to detect the drug and its metabolites in urine for as long as 48 hr after the ingestion of a normal dose. Thus such a drug can be detected in a participant's urine on one day and then, if necessary, a further urine sample can be requested on the following day to remove any doubt about the sampling procedure. Analysis of this sample will also furnish a check against a challenge that deliberate addition of a drug to a participant's urine has occurred.

Although the scheme was devised to detect the misuse of stimulants in sport, it is equally applicable to their detection in other circumstances. Thus it is now possible by objective tests to establish the extent of the problem of abuse of stimulants (and narcotics, which can be determined by similar techniques) provided urine collection from subjects is authorized.

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