[Chem. Pharm. Bull.
21(9)1996—2003(1973)]

UDC 547.233.08 : 543.51 : 543.544

Detection of Amphetamine and Related Amines in Urine by Gas Chromatography and Combined Gas Chromatography- Mass Spectrometry[1]

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(Received January 31, 1973)

A method using gas chromatography and combined gas chromatography-mass spectrometry techniques for the detection of amphetamine and related amines in urine was investigated. The perfluoroacyl derivatives of these amines have excellent gas chromatographic and mass spectrometric properties. Typical fragmentation processes in their mass spectra were also discussed.

Introduction

A number of methods for the detection and identification of amphetamine and related amines in body fluids have been developed in response to biological, clinical, or forensic requirements. Most of the methods are based on the use of gas chromatography (GC)[3-9] or thin-layer chromatography.[9-12] Although the chromatographic techniques have a high sensitivity, they lack somewhat in reliability for confirmatory analysis. A combination of gas chromatography and mass spectrometry (GC–MS) has already been proved to be applicable to a wide variety of analytical problems. This technique has also been applied to the analyses of amphetamine and related amines[6,13,14]; however, few studies have been reported concerning the details of their detection in urine.

We investigated the gas chromatographic and mass spectrometric properties of several derivatives of amphetamine and related amines in order to get basic information on their detection and confirmation in urine. The purpose was mainly to apply a method using GC and GC–MS techniques to the routine analyses of drugs likely to be used in competitive sports.

Experimental

Materials——Trifluoroacetic and heptafluorobutyric anhydrides and N,O-bis-(trimethylsilyl) acetamide were purchased from Tokyo Kasei Kogyo Co., Ltd. Pentafluoropropionic anhydride was prepared from

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1) *a*) This paper constitutes Part V of the series entitled "Studies of the Doping Test by Gas Chromatography–Mass Spectrometry"; Part IV: R. Suzuki, M. Murata, K. Kamei, and A. Momose, *Yakugaku Zasshi*, 93, 942 (1973); *b*) Presented in part before the 92th Annual Meeting of the Pharmaceutical Society of Japan, Osaka, April, 1972.
2) Location: *Kamiyoga, Setagaya-ku, Tokyo*.
3) E. Brochmann-Hanssen and A.B. Svendsen, *J. Pharm. Sci.*, **51**, 938 (1962).
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pentafluoropropionic acid by treatment with phosphoric anhydride.[15] The hydrochlorides of dimethylamphetamine, ethylamphetamine, fenfluramine, and norfenfluramine were kindly donated by Professor Beckett of London University. Methoxyphenamine hydrochloride was given by Nippon Shinyaku Co., Ltd. The other drugs were purchased from the following commercial sources: amphetamine sulfate from Takeda Chemical Industries, Ltd.; methylamphetamine hydrochloride from Dainippon Seiyaku Co., Ltd.; ephedrine hydrochloride from Sanko Seiyaku Co., Ltd.; methylephedrine hydrochloride from Fuji Chemical Industries, Ltd.

Apparatus and Conditions——The gas-chromatographic analyses were carried out on a Hitachi K-53 gas chromatograph equipped with a flame ionization detector. The following packed columns were used in this study:

Column A: 2 m × 3 mm i. d. glass tube with 2% PEG 20 M+5% KOH on Chromosorb G, AW, DMCS (80—100 mesh).

Column B: 1 m × 3 mm i. d. glass tube, similar packing material with Column A.

Column C: 2 m × 3 mm i. d. glass tube with 2% OV-17 on Chromosorb W, AW, DMCS (80—100 mesh). The carrier gas was nitrogen, and the flow rate 50 ml/min.

The mass-spectrometric analyses were carried out on a Hitachi model RMU-7 double-focussing mass spectrometer connected with a model K-53 gas chromatograph. The ionization potential was 70 eV, and the total emission was 80 μA. The temperatures were 200° for both the ion source and the molecular separator (Wattson–Biemann type), and 190° for the tubing. All the sample route in the instrument had previously been silanized by injecting a solution of 5% hexamethyldisilazane in dried toluene. The high-resolution mass spectra were also taken on this mass spectrometer, equipped with a direct heated glass inlet system. All the mass spectral data were converted to digital signs by means of a Hitachi model 002 data processing system, equipped with a mini-computer (4 KW) and a drum (64 KW).

Preparation of Derivatives——For the amines with primary or secondary amino groups in a molecule, three typical perfluoroacyl derivatives, namely, trifluoroacetyl (TFA)-, pentafluoropropionyl (PFP)-, and heptafluorobutyryl (HFB) derivatives, were prepared in the following way. The amines, as free bases, were dissolved in redistilled ethylacetate and an equal volume of the appropriate perfluoro acid anhydrides was added. After heating for 10 min at 50°, the solvent and the excess reagent were removed by concentration under reduced pressure using a rotatory evaporator. The residue was then dissolved in ethylacetate.

Trimethylsilyl (TMSi) ethers of ephedrine and methylephedrine were prepared, with N,O-bis-(trimethylsilyl) acetamide as the reagent and dried pyridine as the solvent, in the usual manner.[16]

Extraction Procedure——5 ml portion of urine containing a small amount of the amines used was acidified with 1N HCl (pH 2—3) and placed in a separatory funnel, together with 20 ml of freshly-distilled ether. This mixture was shaken by means of a mechanical shaker for 3 min, and then the ether phase was removed. The aqueous phase was alkalified with 2N NaOH (pH 11—12) and extracted with 2 × 15 ml of ether for 5 min. Emulsions may occur at this point, but they can be eliminated by centrifugation. The ether phase was removed from the aqueous phase, dehydrided by freezing,[17] and then transferred to a round-bottom flask. Before concentration, 1 ml of 1% glacial acetic acid in ether was added in this flask to avoid any loss resulting from the evaporation of the volatile amines. The contents of the flask were concentrated until the volume about 2 ml under reduced pressure by using a rotatory evaporator; then they were transferred to a small test tube with a finely-tapered base by using a glass micro pipet, and further evaporated almost to dryness in a water bath at about 50°. The residue was dissolved in 50 μl of redistilled ethylacetate containing one volume of β-phenylethylamine as the internal standard.

Result and Discussion

Gas Chromatography

The gas chromatographic properties of the amines used either as free bases or their derivatives had previously been examined on various packed columns with different selectivities and polarities. On the basis of the results, three packed columns-Columns A and B with the same alkaline packing material, and Column C with OV-17 liquid phase-were chosen for this study.

For the detection of the free bases, the alkaline columns gave chromatographically a number of good peaks, as has been reported by earlier workers.[18,3,6] In the case of somewhat

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less volatile amines, such as ephedrine and methylephedrine, the short (1 m) column, B, is more suitable for their detection than Column A (2 m), because the continuous detection of all the amines used on Column A takes quite a bit of time and the operation of strong alkaline columns under high temperatures may cause some decomposition of the original amines. The retention data on Columns A and B are shown in Table I. β-Phenylethylamine is useful as an internal standard. This standard can be applied against the time of further GC after the formation of the perfluoroacyl derivatives.

TABLE I. Relative Retention Times of Amphetamine and Related Amines on Columns A and B

Compound	Column[a) A]	Column[a) B]
Fenfluramine	0.67	0.19
Norfenfluramine	0.75	0.20
Methylamphetamine	0.79	0.22
Dimethylamphetamine	0.81	0.23
Ethylamphetamine	0.83	0.24
Amphetamine	0.86	0.25
Methoxyphenamine	2.95	0.74
Methylephedrine	5.99	1.46
Ephedrine	7.37	1.79
β-Phenylethylamine[b)]	1.00 (7.5 min)	
Diphenyl[b)]		1.00 (4.2 min)

a) A: 2 m × 3 mm i.d. glass tube, 2% PEG 20M+5% KOH, column temp. 140°
B: 1 m × 3 mm i.d. glass tube, 2% PEG 20M+5% KOH, column temp. 150°
b) internal standard

It is well known that the perfluoroacyl derivatives, such as trifluoroacetamide, pentafluoropropionamide, or heptafluorobutylamide, of the biologically important amines have excellent gas-chromatographic properties.[19–23)] These derivatives were also used in our study. Table II shows the relative retention times of the amines used and the three perfluoroacyl derivatives on Column C. A typical chromatogram of the TFA derivatives is shown in Fig. 1.

As expected, free bases gave relatively tailing peaks on the OV-17 column, but the perfluoroacyl derivatives all produced single, sharp, and symmetrical peaks with the exception of the derivatives of methylephedrine. The retention times and the relative molar responses with a flame-ionization detector among their derivatives were approximately the same. The perfluoroacyl ethers of methylephedrine were less stable and gave some decomposed peaks in the gas chromatographic analysis; however, the TMSi ether was very stable. Fig. 2 shows the gas chromatogram of the TMSi ethers of methylephedrine and ephedrine.

Mass Spectrometry

Mass spectra for TFA-, PFP-, and HFB derivatives of amphetamine, methylamphetamine, ethylamphetamine, fenfluramine, norfenfluramine, methoxyphenamine, and ephedrine were measured by means of GC–MS. The TMSi ethers of ephedrine and methylephedrine were also measured. The major fragmentation processes in these derivatives were postulated by

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TABLE II. Relative Retention Times of Some Perfluoroacyl Derivatives of Amphetamine and Related Amines on Column C[a]

Compound	Free base	Derivative		
		TFA	PFP	HFB
Methylephedrine	3.28	0.60	0.64	0.71
Dimethylamphetamine	1.09	—	—	—
Norfenfluramine	1.08	0.88	0.88	0.88
Amphetamine	0.91	0.89	0.89	0.89
Ephedrine	3.09	1.17	1.12	1.21
Fenfluramine	0.89	1.51	1.51	1.51
Methylamphetamine	0.66	1.64	1.67	1.64
Ethylamphetamine	1.03	1.85	1.82	1.77
Methoxyphenamine	2.84	4.85	4.59	4.39
β-Phenylethylamine[b]	1.00 (3.2 min)	1.00 (5.8)	1.00 (4.9)	1.00 (5.2)

a) 2 m × 3 mm i.d. glass tube, 2% OV-17, column temp. 120°
b) internal standard

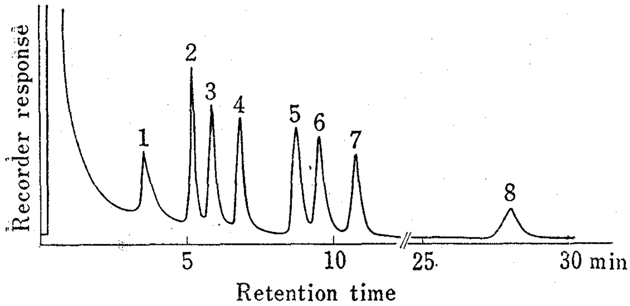

Fig. 1. Typical Gas Chromatogram of Trifluoroacetyl Derivatives of Amphetamine and Related Amines on Column C[a]

peak 1: methylephedrine, 2: amphetamine, 3: β-phenylethylamine (internal standard), 4: ephedrine, 5: fenfluramine, 6: methylamphetamine, 7: ethylamphetamine, 8: methoxyphenamine
a) 2 m × 3 mm i.d. glass tube, 2% OV-17, column temp. 120°

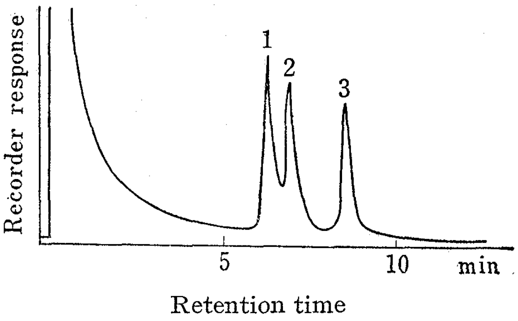

Fig. 2. Gas Chromatogram of Trimethylsilyl Derivatives of Ephedrine (1) and Methylephedrine (2) on Column C

peak 3: diphenyl (internal standard) column temp. 120°

a comparison of the obtained spectra and by studying the high-resolution mass spectra. Some typical fragments in these derivatives are summarized in Table III.

In the mass spectra of amines with a structure like amphetamine, the molecular ion does not appear or has a very low abundance in most cases.[6,24] Other peaks arise mainly from a strong cleavage of the C-C bond at the β-position to the nitrogen. These peaks are, however, present in the relatively low mass region or have too low an abundance to be confirmed. Therefore, it is desirable to convert some suitable derivatives.

The comparative spectra among the parent amines and the three perfluoroacyl derivatives of amphetamine and methylamphetamine are shown in Fig. 3 and Fig. 4, respectively. The abundant peaks in the spectra of the parent amines are very poor in comparison with those of the derivatives. The molecular ions did not appear in the derivatives, either, but many characteristic peaks with a high abundance were observed in the relatively high mass region.

In the spectra of all the perfluoroacyl derivatives of these amines except those with ephedrine and methylephedrine, three prominent fragments resulting from two main cleavages at the α- and β-positions appear (see Chart 1 and Table III). In most cases, the base peak is an

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Table III. Mass Spectral Data of Some Derivatives of Amphetamine and Related Amines by GC–MS

Compound	Deriv.	mol. wt.	m/e values of characteristic fragments (% relative height)							
			By β-cleavage		By α-cleavage					
Amphetamine	TFA	231	140(Bp)[a]	91(63)	118(92)		70(10),	45(27)	
	PFP	281	190(97)	91(64)	118(Bp)		70(8),	45(35)	
	HFB	331	240(91)	91(60)	118(Bp)		70(7),	45(33)	
Methylamphetamine	TFA	245	154(Bp)	91(12)	118(23)		110(38),	42(48)	
	PFP	295	204(Bp)	91(16)	118(25)		160(36),	42(38)	
	HFB	345	254(Bp)	91(28)	118(23)		210(25),	42(58)	
Ethylamphetamine	TFA	259	168(Bp)	91(19)	118(26)		140(38),	70(10),	45(8)
	PFP	309	218(Bp)	91(30)	118(38)		190(35),	70(9),	45(16)
	HFB	359	268(Bp)	91(44)	118(50)		240(36),	70(10),	45(18)
Fenfluramine	TFA	327	168(Bp)	159(10)	186(6)		308(3),	140(32),	70(9)
	PFP	377	218(Bp)	159(20)	186(8)		358(4),	190(38),	70(7)
	HFB	427	268(Bp)	159(24)	186(8)		408(4),	240(34),	70(10)
Norfenfluramine	TFA	299	140(Bp)	159(10)	186(22)		280(4),	70(12),	45(13)
	PFP	349	190(Bp)	159(17)	186(36)		330(4),	70(13),	45(20)
	HFB	399	240(Bp)	159(21)	186(34)		380(4),	70(8),	45(15)
Methoxyphenamine	TFA	275	154(Bp)	121(14)	148(50)		110(26),	91(21),	42(26)
	PFP	325	204(Bp)	121(19)	148(68)		160(31),	91(31),	42(43)
	HFB	375	254(89)	121(30)	148(Bp)		210(26),	91(58),	42(75)
Ephedrine	TFA	261	154(43)		243(68),	201(13),	110(Bp),	105(14),	56(70)
	PFP	311	204(16)		293(39),	251(5),	160(Bp),	105(8),	56(72)
	HFB	361	254(7)		343(20),	301(3),	210(Bp),	105(6),	56(88)
	TMSi	237	58(Bp)		222(2),	146(2),	45(5),	44(6),	43(5)
Methylephedrine	TMSi	251	72(Bp)		236(2),	106(6),	105(10),	71(6),	56(6)
Dimethylamphetamine	—	163	72(Bp)	91(21)	160(2),	148(5),	70(11),	65(9),	58(6),
					57(6),	56(15),	44(19)		

[a] Bp: base peak

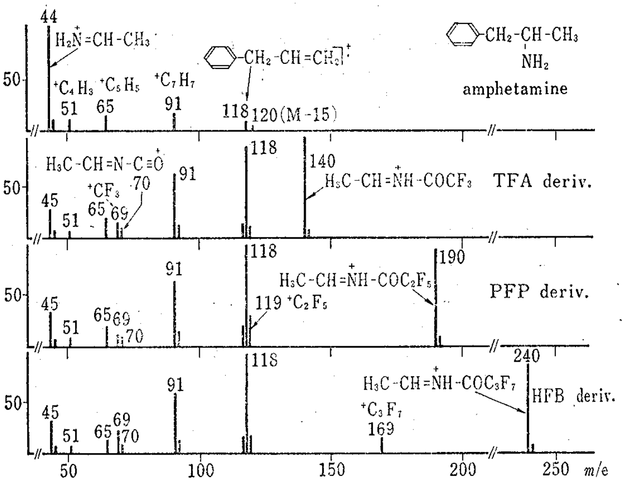


Fig. 3. Mass Spectra of Amphetamine and Its Perfluoroacyl Derivatives

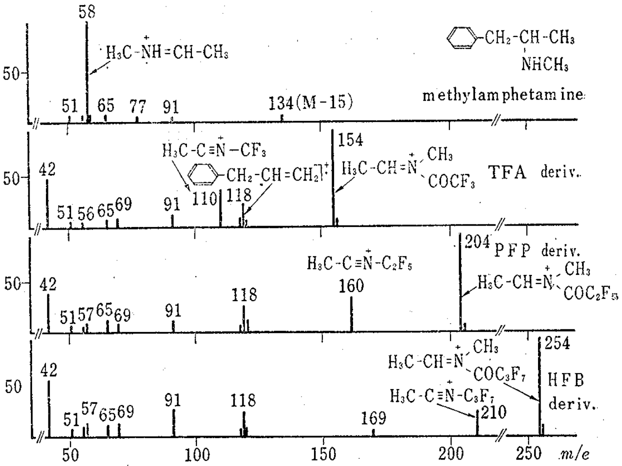


Fig. 4. Mass Spectra of Methylamphetamine and Its Perfluoroacyl Derivatives

alkylamine fragment (b) arising from the simple β-cleavage. When a substituted group is placed on the benzene ring as in the derivatives of fenfluramine, norfenfluramine, and methoxyphenamine, the group retains fragments of both the benzyl residue (c) and the phenylpropenyl residue (d).

Another important feature of these derivatives is the further deconposition of the alkylamine fragment (b), as is shown in Chart 2. With all the compounds with a -NH-COC$_n$F$_{2n+1}$ group, such as the perfluoroacyl derivatives of amphetamine and norfenfluramine, a peak at

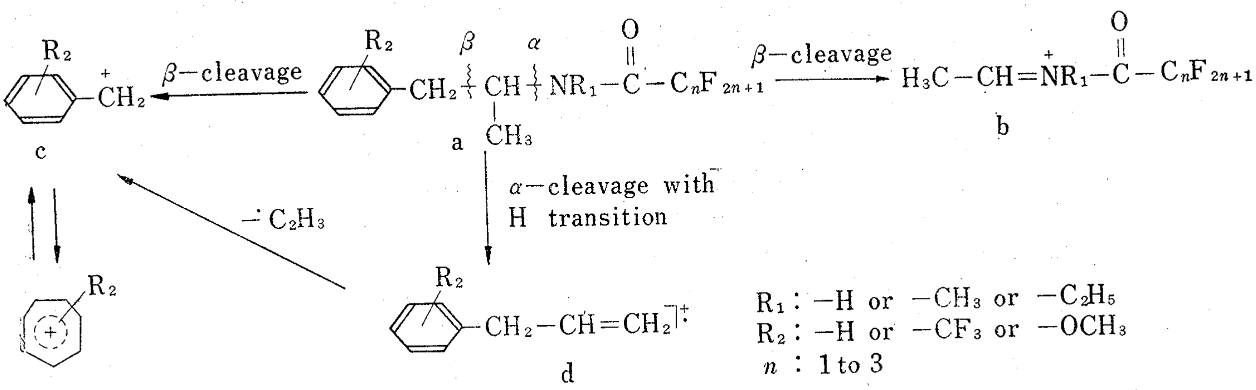
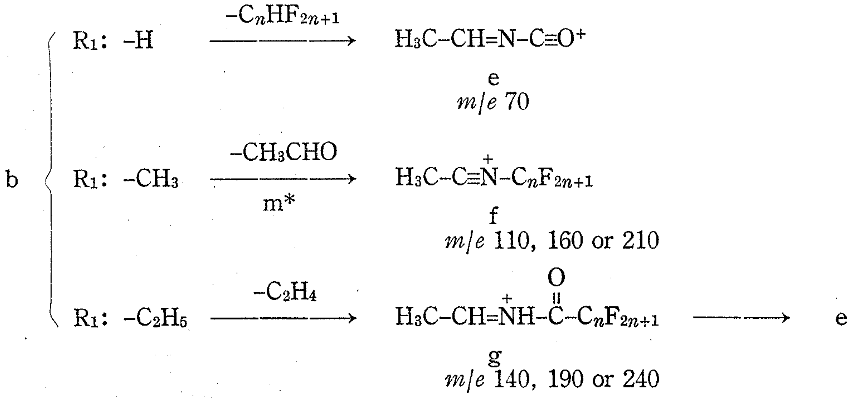


Chart 1



$$\text{b}\left\{\begin{array}{lcl} \text{R}_1\text{: -H} & \xrightarrow{-\text{C}_n\text{HF}_{2n+1}} & \text{H}_3\text{C-CH=N-C}\equiv\text{O}^+ \quad \underset{m/e\ 70}{\text{e}} \\ \text{R}_1\text{: -CH}_3 & \xrightarrow[\text{m}^*]{-\text{CH}_3\text{CHO}} & \text{H}_3\text{C-C}\equiv\overset{+}{\text{N}}\text{-C}_n\text{F}_{2n+1} \quad \underset{m/e\ 110,\ 160\text{ or }210}{\text{f}} \\ \text{R}_1\text{: -C}_2\text{H}_5 & \xrightarrow{-\text{C}_2\text{H}_4} & \text{H}_3\text{C-CH=}\overset{+}{\text{N}}\text{H-}\overset{\text{O}}{\overset{\|}{\text{C}}}\text{-C}_n\text{F}_{2n+1} \longrightarrow \text{e} \quad \underset{m/e\ 140,\ 190\text{ or }240}{\text{g}} \end{array}\right.$$

Chart 2

m/e 70 is observed. This peak can be explained by the loss of CHF_3 from the (b) fragment to give the (e) fragment. In the case of the compounds with a $\text{-N}\left\langle\begin{matrix}\text{CH}_3\\ \text{COC}_n\text{F}_{2n+1}\end{matrix}\right.$ group, such as the same derivatives of methylamphetamine, methoxyphenamine, and ephedrine, a common fragment (f) appears upon the loss of CH_3CHO from the (b) fragment. This process is supported by the presence of the appropriate metastable ions in all of the compounds. For the compounds with a $\text{-N}\left\langle\begin{matrix}\text{C}_2\text{H}_5\\ \text{COC}_n\text{F}_{2n+1}\end{matrix}\right.$ group, such as the derivatives of ethylamphetamine, fenfluramine, and norfenfluramine, a fragment (g) appears upon the loss of C_2H_4; this fragment proceeds further to give a fragment (e) described above. The structural compositions of these fragments (e, f, and g) were also confirmed by the measurements of the high-resolution mass spectra (see Table IV). The appearance of these characteristic fragments is very convenient for deciding the types of substituted groups in the alkylamine moiety of parent amines.

TABLE IV. High-Resolution Mass Measurements

Fragment ion	Original compound	Composition	Measured mass	Calcd. mass
e	amphetamine–TFA	C_3H_4ON	70.0282	70.0293
e	ethylamphetamine–TFA	C_3H_4ON	70.0298	70.0293
e	fenfluramine–TFA	C_3H_4ON	70.0288	70.0293
f(n=1)	methylamphetamine–TFA	$C_3H_3NF_3$	110.0203	110.0218
f(n=1)	methyoxyphenamine–TFA	$C_3H_3NF_3$	110.0224	110.0218
f(n=1)	ephedrine–TFA	$C_3H_3NF_3$	110.0228	110.0218
g(n=1)	ethylamphetamine–TFA	$C_4H_5ONF_3$	140.0346	140.0323
g(n=1)	fenfluramine-TFA	$C_4H_5ONF_3$	140.0351	140.0323

The spectra of the perfluoroacyl derivatives of ephedrine are characterized by the fragments occurring through the β-cleavage, and by the loss of H_2O from the molecular ions. The most abundant peak is the fragment (f) arizing from the loss of CH_3CHO from the alkylamine fragment (b). The benzoyl ion (*m/e* 105) is observed in the spectra of all the derivatives instead of the benzylic ion (*m/e* 107) which may be expected to be formed by the β-cleavage.

Although the TMSi ethers of ephedrine and methylephedrine give good peaks for the gas chromatographic analyses, the mass spectra are fairly poor compared with those of the other perfluoroacylated amines (see Table III). The most abundant peaks are those of the alkylamine fragments (*m/e* 58 and *m/e* 72) arising from the β-cleavage. With the ethers of both ephedrine and methylephedrine, the $[M-15]^+$ fragment appears, but only to the extent of 2% of each of the base peaks. The other peaks lack specificity or have a very low abundance for the confirmation purposes.

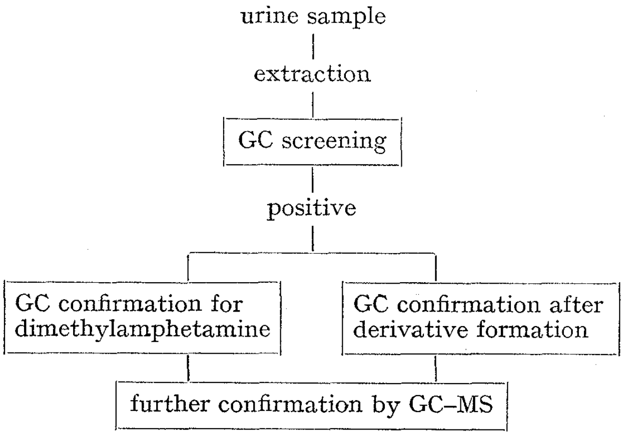


Fig. 5. Analytical Scheme of Urine Samples

Application to Urine Analysis

Various amounts, in the 0—20 μg range, of the amines used were added to 5 ml of normal urine and then extracted in the manner described above. The urine extracts were assayed by the following tests: (i) preliminary screening by the GC of the free bases; (ii) subsequent confirmatory test by GC after trifluoroacetylation or trimethylsilylation (for methylephedrine only); and (iii) further confirmation by the GC–MS of the derivatives. The analytical route is summarized in Fig. 5.

The recovery values of the extraction procedure were obtained by comparing the peak-height ratios of the amines and β-phenylethylamine as the internal standard. For the measurement of ephedrine, diphenyl was used as the standard. The results are shown in Table V.

Table V. Recovery of Some Amines added to 5 ml of Normal Urine

Compound	Amount added (μg)	Amount recovered[a] (μg)	Recovery (%)
Amphetamine	5	4.4	88
	10	9.4	94
	15	14.0	93
	20	17.9	90
Methylamphetamine	5	4.6	92
	10	9.0	90
	15	13.8	92
	20	18.8	94
Fenfluramine	5	4.4	88
	10	9.1	91
	15	13.9	93
	20	17.9	90
Ephedrine	5	3.9	78
	10	7.9	79
	15	12.9	86
	20	16.2	81

a) Each value represents the average of four different samples.

The lower limits of detection in this method were approximately 0.3—0.5 μg in one ml of urine. At these amounts, the complete gas chromatograms and mass spectra were obtained for all of the amines without any interference by urine components or other compounds.

These results show that the method is satisfactory for the routine detection of amphetamine and related amines in a usual doping test.

Acknowledgement The authors are indebted to Professor Beckett, London University, for his generous gifts of dimethylamphetamine, ethylamphetamine, fenfluramine, and norfenfluramine hydrochlorides.