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PENTAFLUOROBENZOYL DERIVATIVES OF DOPING AGENTS

I. EXTRACTIVE BENZOYLATION AND GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION OF PRIMARY AND SECONDARY AMINES*

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SUMMARY

A sensitive and rapid method for the gas chromatographic (with electron-capture detection) confirmation of derivatizable sympathomimetic amines is described. Extractive derivatization with pentafluorobenzoyl chloride is performed on 2-ml urine or plasma samples. Especially for primary amines, the method appears to be very sensitive. Mass spectral data allowed confirmation of the monobenzoylation of all congeners.

INTRODUCTION

Various methods for the identification of doping agents in biological material have been presented during recent years [1—6]. Although it is well known that gas chromatography combined with mass spectrometry (GC—MS) is superior for the identification of drugs, it is relatively simple to prove or disprove the identity of a drug by cumulative analytical data. Therefore positive or suspected cases need to be confirmed on columns of different polarities after derivatization of the analyte. The present work reports on the derivatization

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with pentafluorobenzoyl chloride (PFBCl) of primary and secondary sympathomimetic amines by extractive benzoylation.

EXPERIMENTAL

Materials

The following amines were studied: *l*-amphetamine sulphate (SKF, Philadelphia, PA, U.S.A.), chlorphentermine hydrochloride (Tropon Werke, Köln, G.F.R.), cyclopentamine hydrochloride, dioxadrol (Cutter, Berkeley, CA, U.S.A.), ethylamphetamine hydrochloride, fencamfamine hydrochloride and its metabolite 2-amino-3-phenylnorbornane hydrochloride (Merck, Darmstadt, G.F.R.), fenfluramine hydrochloride (Servier, Orleans, France), mephentermine sulphate (Wyeth, Philadelphia, PA, U.S.A.), methylamphetamine hydrochloride (Merck), methylphenidate hydrochloride (Ciba, Basle, Switzerland), pentorexum (Nordmark Werke, Hamburg, G.F.R.), phacetoporane hydrochloride (Specia, Paris, France), phenmetrazine hydrochloride (Boehringer, Ingelheim, G.F.R.), phentermine hydrochloride and tranylcypromine sulphate (SKF). Stock solutions of these drugs were freshly prepared with double-distilled water.

Pentafluorobenzoyl chloride (PFBCl) was obtained from Aldrich Europe (Beerse, Belgium).

The triethanolamine—cyclohexane (CH—TEA) extraction solvent was prepared by briefly refluxing cyclohexane with small amounts of triethanolamine, cooling and separating the two phases.

The ammonium buffer was a saturated ammonium chloride solution adjusted to pH 9.4 with undiluted ammonia.

All glassware was silanized and the organic solvents (analytical grade) were freshly distilled before use.

Dilutions were made with a Hamilton diluter/dispenser.

Gas chromatography

A Varian 3700 gas chromatograph equipped with a ^{63}Ni detector and connected to a Varian CDS 111 integrator was used. The glass column (200 × 0.25 cm I.D.) was packed with 2% OV-225 on Chromosorb W AW DMCS, 80—100 mesh. Nitrogen was used as carrier gas at a flow-rate of 25 ml min^{-1}. Temperature was programmed from 165°C, 5 min hold-time, to 225°C at a rate of 5°C min^{-1}. The injector and detector temperatures were kept at 240° and 320°C, respectively. Chart speed was 20 cm/h.

Gas chromatography—mass spectrometry

Spectra were obtained with a Hewlett-Packard 5985B quadrupole instrument, equipped with a directly coupled fused-silica open tubular capillary column (OV-101, 10 m × 0.22 mm I.D.). Helium (70 cm sec^{-1}) was used as carrier gas. Injections were made via an all-glass "moving needle" system. Temperature settings were: injection port 290°C; oven temperature programmed from 140° to 240°C at 16°C min^{-1}; transfer line 250°C; source temperature 200°C. Spectra were taken under electron-impact conditions (70 eV).

Methods

Two millilitres of urine (or plasma) were made alkaline with 0.2 ml of NH_4^+/NH_4OH buffer and 6 ml of CH—TEA were added followed by 0.01 ml of PFBCl (0.5% in cyclohexane). Extractive benzoylation was performed during 5 min. The organic phase was evaporated under nitrogen (40°C) and the residue redissolved in 500 or 100 μl of ethyl acetate depending on the starting material (urine or plasma). One microlitre was injected into the gas chromatograph.

The lowest concentration which allowed comfortable detection ("routine detection limit") was determined by adding different volumes (0—100 μl) of several aqueous amine solutions to 2 ml of horse urine or plasma. The extraction was done as described and a 5-ml aliquot of the organic phase was used for evaporation.

The effect of the reaction time and the stability of some PFB derivatives was measured using tetrachlorodiphenylethane (TDE) as internal standard.

RESULTS AND DISCUSSION

After extraction, PFB derivatives of amines are generally made with trimethylamine as catalyst [7] or at elevated temperatures [8—10] during periods varying from 20 min to 2 h [7]. Moreover, after derivatization it is often necessary to remove the strongly interfering hydrolysis product (pentafluorobenzoic acid) of PFBCl by supplementary washings with alkali. We found that most of this reagent interference could be diminished by using minute amounts of PFBCl.

The extractive benzoylation with PFBCl as presented here combines extraction, derivatization and consumption of the reagent.

The addition of triethanolamine to the extraction solvent results in higher recoveries in contrast with cyclohexane alone.

Identity of the postulated derivatives was investigated by GC—MS under electron-impact (70 eV) conditions. Although only for some components was a weak molecular ion observed, the presence of diagnostic ions allowed confirmation of the monobenzoylation of all congeners.

The stability of the pentafluorobenzyloxonium ion (*m/z* 195), through electron sharing with the non-bonding orbital of the oxygen atom, was characteristic for all derivatives. This ion was either the base peak or very abundant for all derivatives. The ion at *m/z* 167 originated from further fragmentation with loss of neutral CO molecule.

The strong electron-donating ability of the nitrogen atom also provided abundant ions by α-cleavage with loss of the benzyl moiety, $[M - 91]^+$. For the piperidyl congeners this reaction was even more important; for example, phacetoperane gave a base peak at *m/z* 278.

Another typical fragment included McLafferty rearrangement with charge migration to the alkyl side-chain, the intensity reflecting the stability of the formed ion. For tranylcypromine this ion was the base peak (*m/z* 116), while for pentorexum this ion yielded only a relative intensity of 1.3% (*m/z* 146). Also for fencamfamine this type of fragmentation gave rise to an abundant ion (*m/z* 170, 86.1%).

Other ions were indicative of the cyclic structure of the congeners, e.g. *m/z* 91, *m/z* 77 for benzyl and *m/z* 115 for bicyclic congeners. Mass spectra and the fragmentation of tranylcypromine and chlorphentermine are given as illustrative examples in Figs. 1—4. Mass spectral data of all congeners are summarized in Table I, according to the major fragmentation pathways discussed.

The effect of the reaction time on the formation and stability of the PFB

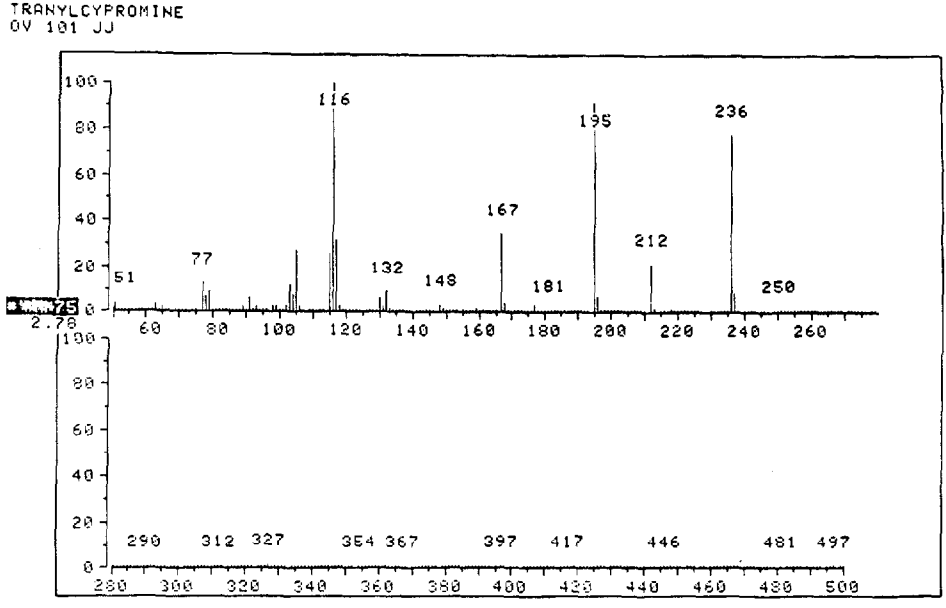

Fig. 1. Electron-impact mass spectrum of N-pentafluorobenzamide of tranylcypromine.

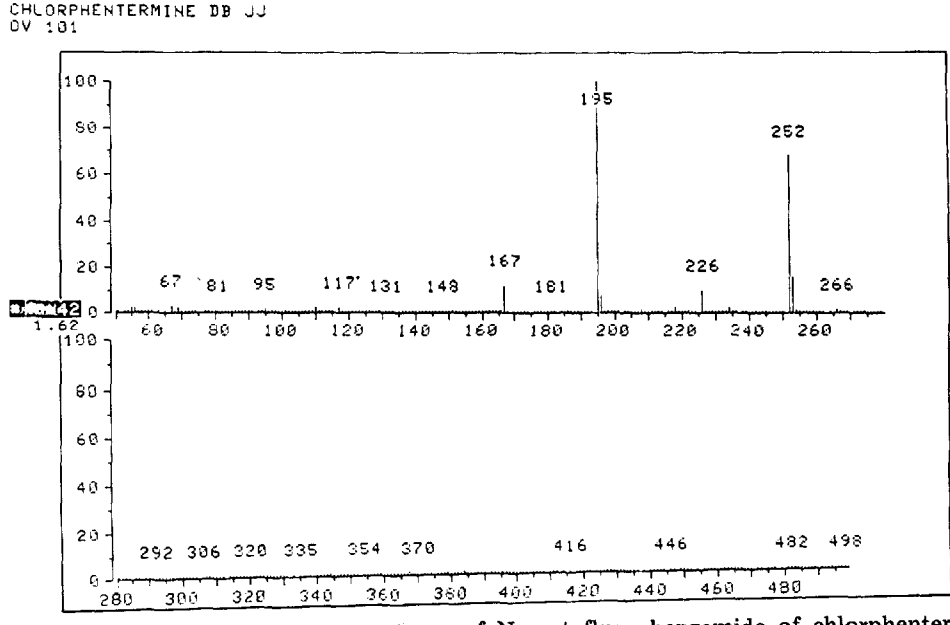

Fig. 2. Electron-impact mass spectrum of N-pentafluorobenzamide of chlorphentermine.

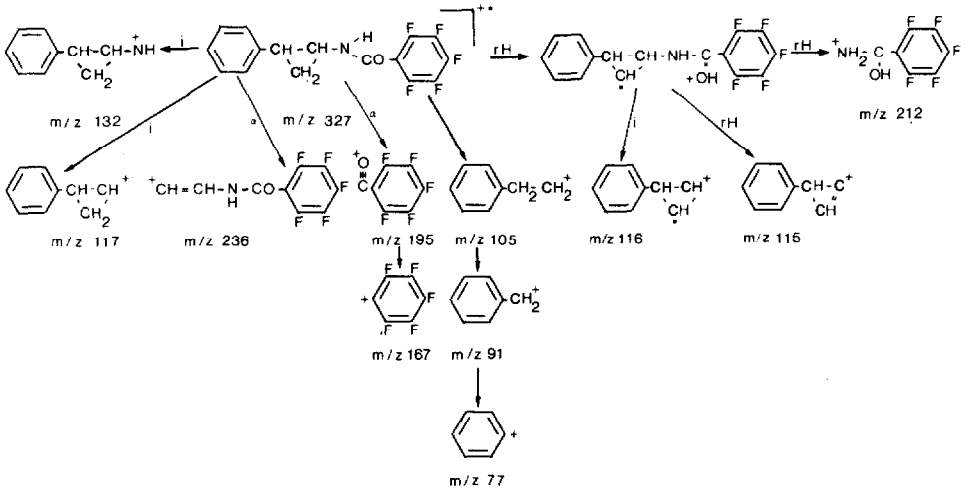

Fig. 3. Fragmentation scheme of N-pentafluorobenzamide of tranylcypromine.

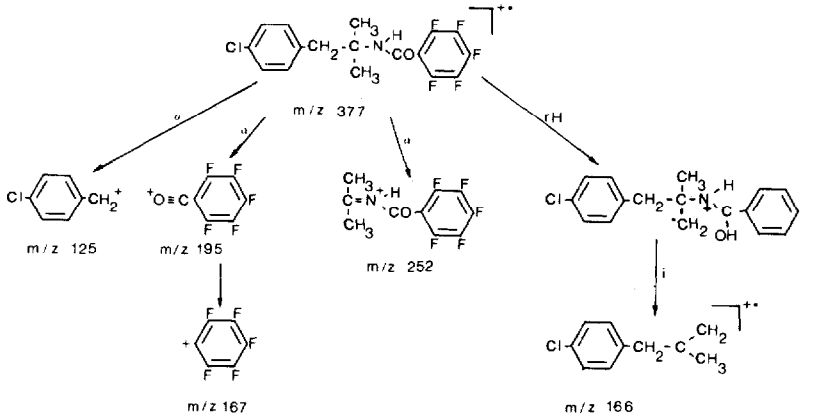

Fig. 4. Fragmentation scheme of N-pentafluorobenzamide of chlorphentermine.

derivatives of the primary amine amphetamine and the secondary amine phenmetrazine was measured in quadruplicate for each time period. As shown in Fig. 5, the amount of both derivatives did not increase after 1 min of extractive benzoylation. However, cleaner chromatograms were obtained after 5 min reaction time, probably due to the complete removal of the unreacted PFBCl.

For PFB-amphetamine no decrease was found after 1-h period, contrary to PFB-phenmetrazine which slowly decomposed after 30 min in the two-phase system.

Moreover, a solution of PFB-amphetamine in ethyl acetate was stable for at least two weeks (room temperature) while the PFB derivative of phenmetrazine was already decomposed after 48 h.

A reference chromatogram of N-pentafluorobenzamides of doping agents is given in Fig. 6.

The detection limits for some derivatives starting with 2 ml of urine or plasma are given in Table II and illustrated in Figs. 7 and 8.

TABLE I

TABULATION OF PRINCIPAL FRAGMENTATION PATHWAYS OF SOME PFB DERIVATIVES

Tranylcypromine	$M^{+\cdot}$ 327 (0.9)	$M-91^+$ 236 (40.8)	$M-115^+$ 212 (14.5)	$M-182^+$ 195 (60.9)	$M-120^+$ 160 (26.5)
Phentermine	$M^{+\cdot}$ 343 (—)	$M-91^+$ 252 (61.1)		$M-148^+$ 195 (100.0)	$M-176^+$ 167 (15.1)
Chlorphentermine	$M^{+\cdot}$ 377 (—)	$M-125^+$ 252 (56.0)		$M-182^+$ 195 (100.0)	$M-210^+$ 167 (15.9)
Pentorexum	$M^{+\cdot}$ 357 (—)	$M-105^+$ 252 (69.2)		$M-162^+$ 195 (100.0)	$M-190^+$ 167 (13.5)
Amphetamine	$M^{+\cdot}$ 329 (—)	$M-91^+$ 238 (32.8)		$M-134^+$ 195 (100.0)	$M-162^+$ 167 (14.5)
Mephentermine	$M^{+\cdot}$ 357 (—)	$M-91^+$ 266 (52.6)		$M-162^+$ 195 (100.0)	$M-190^+$ 167 (14.3)
Methylamphetamine	$M^{+\cdot}$ 343 (0.1)	$M-91^+$ 252 (36.9)		$M-148^+$ 195 (100.0)	$M-176^+$ 167 (18.4)
Ethylamphetamine	$M^{+\cdot}$ 357 (0.2)	$M-91^+$ 266 (32.7)		$M-162^+$ 195 (100.0)	$M-210^+$ 167 (16.2)
Fenfluramine	$M^{+\cdot}$ 425 (—)	$M-159^+$ 266 (41.5)		$M-230^+$ 195 (100.0)	$M-258^+$ 167 (21.0)
Cyclopentamine	$M^{+\cdot}$ 335 (1.2)	$M-83^+$ 252 (57.6)		$M-140^+$ 195 (100.0)	$M-168^+$ 167 (12.6)
Methylphenidate	$M^{+\cdot}$ 427 (—)	$M-149^+$ 278 (67.2)		$M-132^+$ 195 (100.0)	$M-260^+$ 167 (13.9)
Phacetoperane	$M^{+\cdot}$ 427 (—)	$M-149^+$ 278 (100.0)		$M-132^+$ 195 (95.2)	$M-260^+$ 167 (12.5)
Dioxadrol	$M^{+\cdot}$ 503 (0.4)	$M-125^+$ 278 (85.5)	$M-298^+$ 225 (47.7)	$M-308^+$ 195 (100.0)	$M-336^+$ 167 (35.5)
Fencamfamine	$M^{+\cdot}$ 409 (—)	$M-214^+$ 195 (63.8)	$M-239^{+\cdot}$ 170 (86.1)	$M-342^+$ 167 (19.6)	$M-367^+$ 142 (100.0)
Fencamfamine metabolite	$M^{+\cdot}$ 381 (—)	$M-186^+$ 195 (65.2)	$M-211^{+\circ}$ 170 (100.0)	$M-214^+$ 167 (13.2)	$M-239^+$ 142 (82.5)
Phenmetrazine	$M^{+\cdot}$ 371 (0.8)	$M-106^+$ 265 (29.6)	$M-125^+$ 246 (29.6)	$M-133^+$ 238 (10.3)	$M-176^+$ 195 (100)

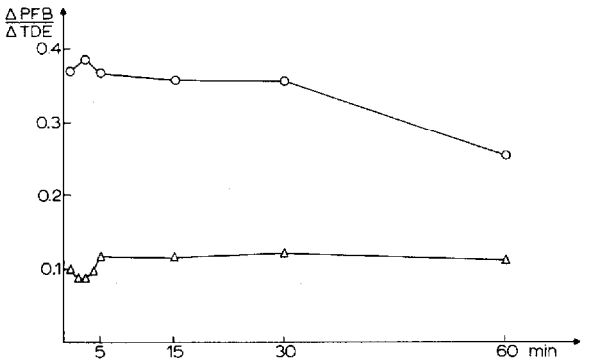

Fig. 5. Effect of reaction time on the pentafluorobenzoylation of amphetamine (△) and phenmetrazine (○).

Although the pentafluorobenzoyl group seems to be the one that confers the greatest sensitivity for electron-capture detection of amines [11], one can see from Table II that the response for derivatives of primary amines is much higher than for secondary amines.

$M-195^+$	$M-120^+$	$M-211^{+\circ}$	$M-212^+$	$M-222^+$	$M-236^+$
132 (9.5)	117 (28.3)	116 (100)	115 (28.9)	105 (27.3)	91 (3.9)
		$M-211^{+\circ}$			$M-252^+$
		132 (7.2)			91 (11.0)
		$M-211^{+\bullet}$			$M-252^+$
		166 (11.9)			125 (9.1)
		$M-211^{+\bullet}$			$M-252^+$
		146 (1.3)			105 (10.7)
		$M-211^{+\bullet}$			$M-238^+$
		118 (32.8)			91 (12.5)
		$M-225^{+\bullet}$			$M-266^+$
		132 (10.8)			91 (16.8)
	$M-224^+$	$M-225^{+\bullet}$	$M-226^+$		$M-252^+$
	119 (1.0)	118 (9.0)	117 (9.6)		91 (16.3)
	$M-238^+$	$M-239^{+\bullet}$	$M-240^+$		$M-266^+$
	119 (1.6)	118 (9.6)	117 (11.0)		91 (19.0)
		$M-239^{+\bullet}$			$M-266^+$
		186 (5.4)			159 (4.9)
		$M-225^{+\bullet}$			—
		110 (2.8)			
$M-398^+$	$M-426^+$				
105 (46.7)	77 (23.0)				
$M-392^+$	$M-394^+$	$M-318^+$			
117 (17.1)	115 (15.8)	91 (30.8)			
$M-264^+$	$M-266^+$	$M-290^+$			
117 (13.3)	115 (11.8)	91 (25.6)			
$M-204^+$	$M-254^+$	$M-266^+$			
167 (18.2)	117 (12.3)	105 (8.5)	70 (19.7)	56 (19.6)	

Taking into account that the doses used in doping practices are still higher than for therapeutic purposes, extractive benzoylation of sympathomimetic amines, especially those with a primary amino group, is a sensitive and rapid method for confirmation of suspected or positive cases.

Further studies with respect to selectivity (fused-silica capillary gas chromatography) and sensitivity (negative chemical ionization mass spectrometry) are currently being investigated.

TABLE II

DETECTION LIMITS (ng/ml) OF PFB DERIVATIVES OF SOME DOPING AGENTS STARTING WITH 2 ml OF BIOLOGICAL FLUID

Compound	Urine	Plasma	Compound	Urine	Plasma
Amphetamine	2.5	0.35	Phenmetrazine	50	25
Chlorphentermine	2.5	1	Fenfluramine	125	20
Phentermine	5	2.5	Methylphenidate	250	25
Fencamfamine metabolite	2.5	0.5	Mephentermine	500	200
Tranylcypromine	1	0.125	Ethylamphetamine	750	125

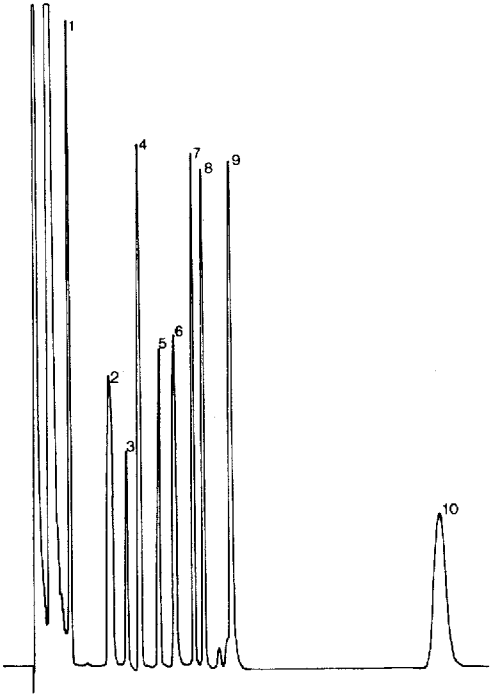

Fig. 6. Gas chromatography of N-pentafluorobenzoyl derivatives of doping agents. For GC conditions see section on gas chromatography. Peaks: 1 = cyclopentamine; 2 = ethylamphetamine, fenfluramine, mephentermine and methylamphetamine; 3 = phentermine; 4 = amphetamine; 5 = phenmetrazine; 6 = chlorphentermine, tranylcypromine, fencamfamine; 7 = phacetoperane; 8 = methylphenidate; 9 = fencamfamine metabolite; 10 = dioxadiol.

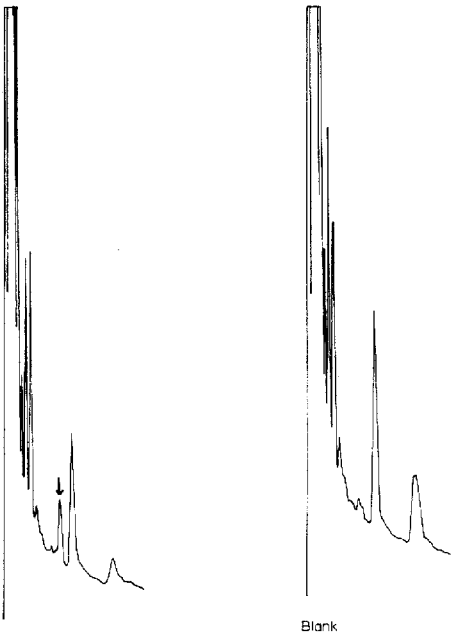

Fig. 7. Detection limit (2.5 ng/l) for the N-pentafluorobenzamide of amphetamine (arrow) in urine (2 ml).

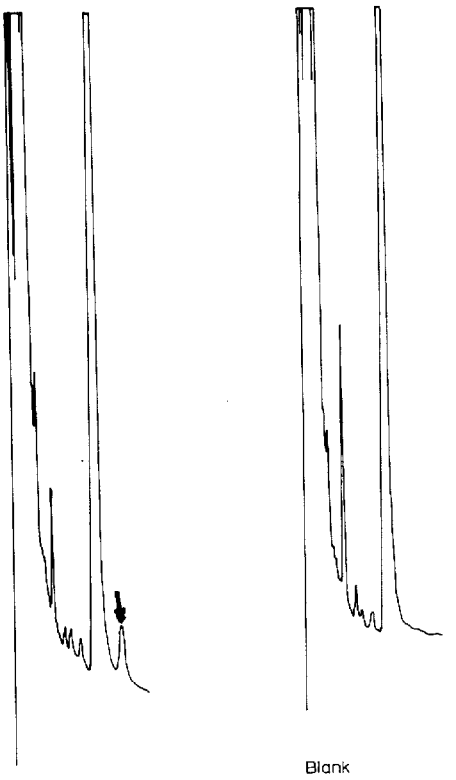

Fig. 8. Detection limit (50 ng/ml) for the N-pentafluorobenzamide of phenmetrazine (arrow) in plasma (2 ml).

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