Journal of Chromatography, 163 (1979) 135—142
Biomedical Applications

CHROMBIO. 332

URINARY PHENYLETHYLAMINE EXCRETION: GAS CHROMATOGRAPHIC ASSAY WITH ELECTRON-CAPTURE DETECTION OF THE PENTAFLUOROBENZOYL DERIVATIVE

KARL BLAU and IRENE M. CLAXTON

Department of Chemical Pathology, Bernhard Baron Memorial Research Laboratories and Institute of Obstetrics and Gynaecology, Queen Charlotte's Maternity Hospital, London W6 0XG (Great Britain)

GÜL ISMAHAN

University of Paris, Unité de Neuropharmacologie, Laboratoire de Physiologie Générale, F-91405 Orsay (France)

and

M. SANDLER

Department of Chemical Pathology, Bernhard Baron Memorial Research Laboratories and Institute of Obstetrics and Gynaecology, Queen Charlotte's Maternity Hospital, London W6 0XG (Great Britain)

(First received November 20th, 1978; revised manuscript received February 23rd, 1979)

SUMMARY

Phenylethylamine was extracted into *n*-hexane from alkaline urine saturated with sodium chloride, and back-extracted into dilute acid. The acid extract was freeze-dried and the residue converted to a pentafluorobenzoyl derivative for analysis by gas chromatography on a column of OV-225 with electron-capture detection. Quantification was achieved by adding an internal standard of tolylethylamine to each sample prior to extraction. Output values in normal subjects and in some patients with phenylketonuria and hyperphenylalaninaemia were in agreement with those in some other recent reports.

INTRODUCTION

Jepson et al. [1] were the first to measure the urinary excretion of phenylethylamine in normal and phenylketonuric subjects, and since that time interest in the endogenous role and metabolism of this amine has gradually increased [2—4]. Disagreement over the range of normal urinary output [1, 5—7] has made it difficult to evaluate claims of changes in pathological condi-

tions. This situation largely reflects differences in methodological approach and points to variations in specificity. We therefore felt it necessary to try to develop a reliable and sensitive gas chromatographic procedure of high specificity, and we describe below such a method based on electron-capture detection of a pentafluorobenzoyl derivative [8].

MATERIALS AND METHODS

Reagents

Solvents were redistilled before use. Phenylethylamine hydrochloride was obtained from Koch-Light Labs. (Colnbrook, Great Britain). *p*-Methylphenylethylamine (tolylethylamine, Aldrich, Gillingham, Great Britain) was converted to its hydrochloride with 1 *M* HCl, evaporated to dryness and recrystallized twice from ethanol. Pentafluorobenzoyl chloride was purchased from Pierce and Warriner (Chester, Great Britain) and stored at —25°.

Preparation of standards

Stock standard solutions of phenylethylamine and tolylethylamine hydrochlorides (1 mg free amine per ml) were made up in 0.01 *M* HCl and stored frozen. Working standards were diluted to a final free amine concentration of 1 μg/ml.

Extraction of amines from urine

Urine (1 ml) in a 15 × 110 mm test-tube, to which tolylethylamine (10 ng) had been added, was mixed on a vortex mixer and adjusted to pH 12.5 with 2.5 *M* NaOH (0.2 ml). After adding solid NaCl (0.3 g), the mixture was extracted with *n*-hexane (2 ml) by vortex mixing for 30 sec and centrifuged at 1500 *g* for 10 min. The upper organic layer was then carefully transferred to a 15-ml conical centrifuge tube with a Pasteur pipette. The hexane layer was back-extracted into 1 *M* HCl (0.4 ml) by vortex mixing for 60 sec, and the mixture was centrifuged at 1500 *g* for 5 min. The lower aqueous layer was transferred to a 2-ml screw-cap glass vial with a Pasteur pipette. Vials in a batch of analyses were put into a rack which was stood on edge in a deep-freeze, so that the extracts were frozen in a nearly horizontal position to increase their surface area. When the extracts were frozen, the whole rack, with the tubes still horizontal, was subjected to freeze-drying with anhydrous "Linde" molecular sieve 3Å as desiccant and KOH pellets to absorb acid. This procedure took about 2 h and resulted in barely visible residues. Salt crystals in the vial indicated that aqueous solution had inadvertently been transferred, and such samples were rejected, because alkali in the aqueous layer liberates the free amines, including the internal standard, which are then lost during the drying stage. The amines were isolated in 75—85% yield.

Calibration

Duplicate standards containing increasing ratios of phenylethylamine:tolylethylamine were prepared by measuring, say, 10, 20, 30, 40, 50 μl working standard solution into 2-ml screw-cap vials with 50-μl Hamilton syringes and adding 10 μl tolylethylamine to each. To each vial were also added 10 μl 1 *M*

HCl to prevent amine loss. Solvent was removed rapidly in vacuo and the residues derivatized as described below.

Derivative formation

To the residue in each vial, test or standard, was added freshly prepared 1% pentafluorobenzoyl (PFB) chloride in dry diethyl ether (40 μl). The vial was covered with a glass microscope cover-slip and left at 37° for 5 min. Excess reagent was evaporated off in vacuo for about 15 min, after which the procedure was stopped to avoid losses. All-glass derivatization vessels were used because it was found that screw-caps lined with polytetrafluoroethylene caused losses of up to 75% and often gave rise to a large interfering peak of pentafluorobenzoic acid which emerged just before that of the phenylethylamine derivative (Fig. 1). The dried residue of derivatized amine in each vial was dissolved in ethyl acetate (40 μl) for analysis. Where it was desirable to use a reference standard to check the volume injected, the pesticide *p,p*-DDD (1,1-dichloro-2, 2-bis(*p*-chlorophenyl)ethane, Field Instruments, Twickenham, Great Britain) was added to the ethyl acetate used to dissolve the samples, at a concentration of 2.5 μg/l, but this is optional. The use of an injection standard does, however, identify samples with low yields and analyses where the correct volume was not injected for some reason.

Gas chromatography

Analyses were done on a Hewlett-Packard 5713A instrument with an 18713A ^{63}Ni electron-capture detector and a 5709A pulse-modulated electron-capture control module. A coiled pyrex column (180 cm × 0.3 cm I.D.) packed with 3% silicone OV-225 on 80—100 mesh Chromosorb W HP (Applied Science Labs.) was used isothermally at 190°. Injection port temperature was 250° and detector 300°. The carrier gas was 5% methane in argon at 55 ml/min. Before columns were put into service they were conditioned with several injections of the strongest working standard solution.

RESULTS

The isolation procedure was chosen to discriminate against phenolic amines by extracting phenylethylamine at pH 12.5. Various solvents (diethyl ether, ethyl acetate, benzene, chloroform, methylene chloride and hexane) were investigated but hexane proved to be the most suitable because of clean separation, specific extraction and optimal amine recovery. The PFB derivative [8] was chosen as one of the most sensitive for the determination of primary and secondary amines by electron-capture detection [9—11]. PFB chloride reacted rapidly and completely with both phenylethylamine and internal standard, and the resulting derivatives gave clean sharp peaks, well-resolved from other peaks in the urinary profile and from one another. A typical profile from a normal urine extract is shown in Fig. 2. The profile includes unknowns and peaks identified as the PFB derivatives of other amines; our analytical scheme can, in fact, be used to quantify a range of these compounds both endogenous and exogenous and these findings have been reported elsewhere [18, 19]. Table I shows relative retention times of certain amine PFB derivatives and of some reference compounds.

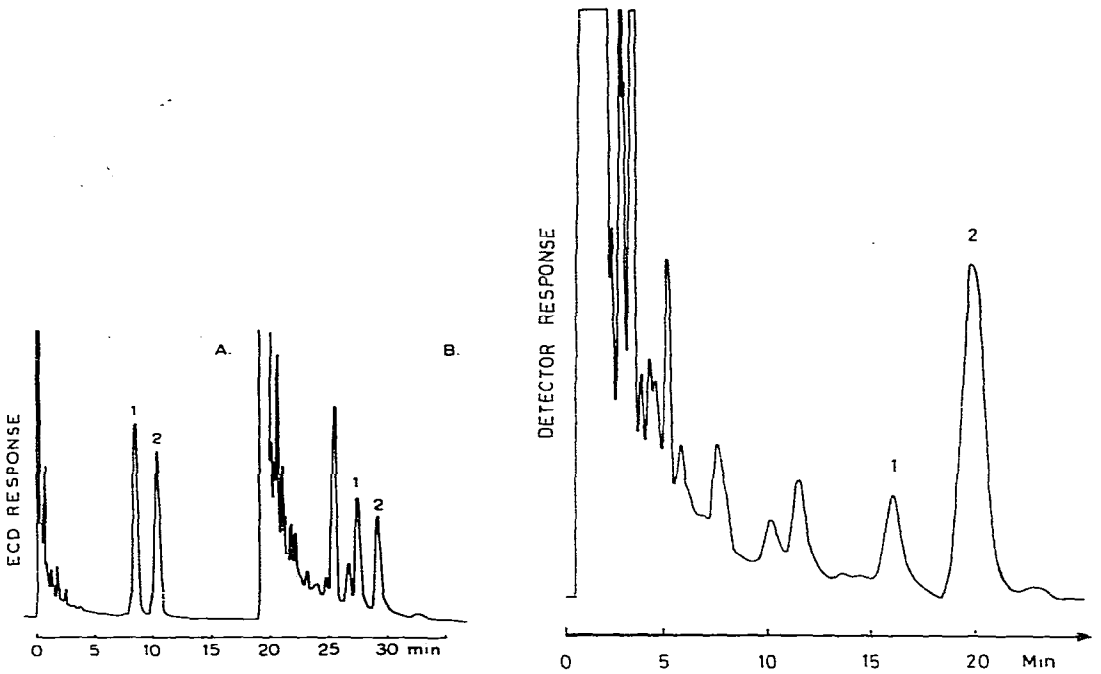

Fig. 1. The effect of polytetrafluoroethylene on derivatization. PFB derivatives of 10 ng each of amine and internal standard were prepared as described in the text. A, Vial was sealed with a glass cover-slip. B, Vial sealed with a PTFE-lined screw-cap. Note reduced peak heights, interfering peaks and slower fall of solvent and reagent peaks in B. Gas chromatography was performed at 210°.

Fig. 2. Gas chromatographic profile of a typical derivatized extract of normal human urine. Peak 1, PFB-phenylethylamine; peak 2, PFB-tolylethylamine. Conditions as in text.

TABLE I

RETENTION TIMES OF PFB-AMINES AND REFERENCE COMPOUNDS, RELATIVE TO PFB-PHENYLETHYLAMINE

Chromatography was carried out on a 180 × 0.3 cm column of 3% OV-225 at 190°. For further details see text.

Compound	Relative retention time
PFB-Phenylethylamine*	1.0
PFB-N-Methylphenylethylamine	0.74
PFB-N-Methylamphetamine	0.75
PFB-Benzylamine	0.79
PFB-Amphetamine	0.82
PFB-*p*-Methylphenylethylamine (tolylethylamine)	1.20
1,1,1-Trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT)	1.62
PFB-Phenylpropylamine	1.65
1,1-Dichloro-2,2-bis(*p*-chlorophenyl)ethane (*p,p*-DDD)	1.68
PFB-*trans*-Phenylcyclopropylamine (tranylcypromine)	1.87

*Retention time under these conditions was 15.6 min.

The structure of the PFB derivative of phenylethylamine was confirmed mass spectrometrically (Fig. 3). Several urinary extracts were investigated by combined gas chromatography—mass spectrometry under conditions similar to those used for the standard procedure, and gave mass spectra of the PFB-phenylethylamine peak indistinguishable from that in Fig. 3. No evidence of any interfering compound with a retention time equal to that of the phenylethylamine derivative was found in any of these extracts, so that the amine could be determined with high specificity. Fig. 4 shows the linearity of peak height ratios of the phenylethylamine derivative to that of the internal standard, tolylethylamine, plotted against phenylethylamine concentration, using a set of standards as described in the section on calibration.

The internal standard was introduced before analysis, and carried through the entire procedure. It was therefore subjected to the same manipulations as endogenous phenylethylamine, behaved like phenylethylamine during isolation and derivatization, but was resolved from it by gas chromatography. Peak height ratios were calculated and used to determine phenylethylamine concentrations

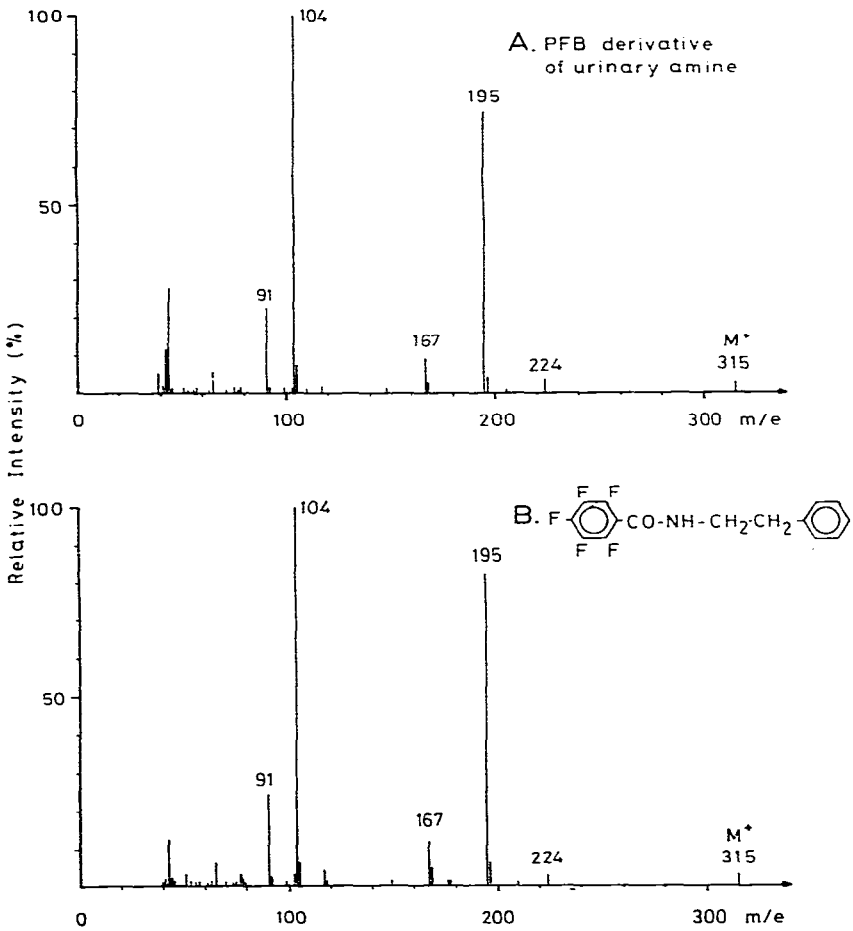

Fig. 3. Electron-impact mass spectra of a PFB derivative of the amine extracted from urine (A) and of authentic PFB-phenylethylamine (B).

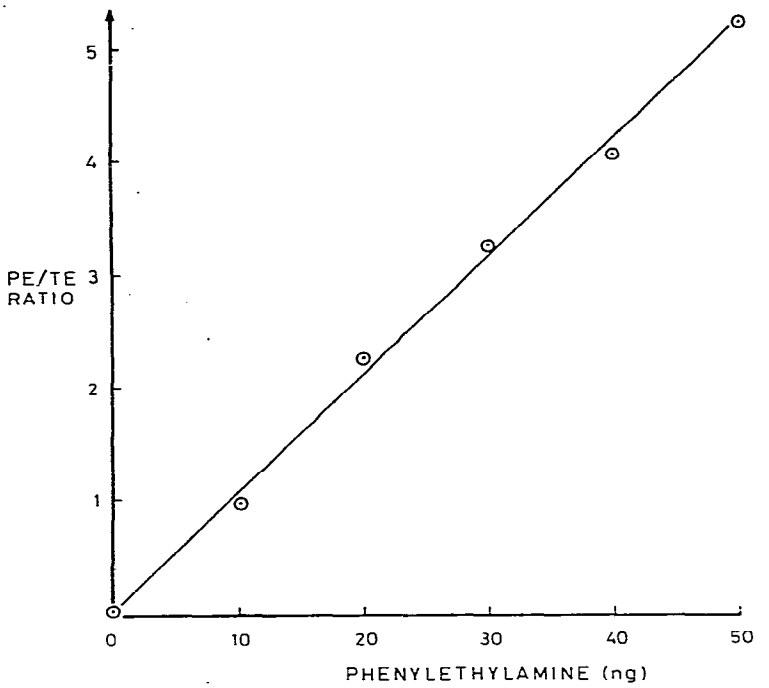

Fig. 4. Typical calibration curve obtained as described in the text. Each point is the average of duplicate determinations. PE, phenylethylamine; TE, tolylethylamine.

in urine samples by reference to the calibration curve (Fig. 4). To check the simplicity and reproducibility of the method, phenylethylamine was determined in ten aliquots of the same urine by a worker who had not previously performed gas chromatographic analyses. The result was 2.31 ± 0.238 μg/l, a standard deviation of just over 10%, and better results have generally been obtained by experienced workers in our department.

Urinary excretion of free phenylethylamine was determined in normal subjects, in a group of adults with hyperphenylalaninaemia not on phenylalanine-depleted diets, and in three infants with confirmed classical phenylketonuria on phenylalanine-reduced diets whose blood phenylalanine concentrations were not well controlled (Table II). These values broadly agree with those given in recent investigations [12—15] in which specific gas chromatographic or mass fragmentographic methods were employed.

DISCUSSION

The present method was developed in response to the need for a sensitive and reliable way of determining phenylethylamine specifically. The analytical conditions were designed accordingly, because earlier reports of high phenylethylamine excretion (for example, refs. 5 and 7) suggested that contributions from interfering substances may have been included. Our procedure proved to be rapid and simple, and has routinely delivered reliable and reproducible results over the last two years. Because of its high sensitivity, only a

TABLE II

URINARY PHENYLETHYLAMINE EXCRETION IN NORMAL SUBJECTS, AND HYPERPHENYLALANINAEMIC AND PHENYLKETONURIC PATIENTS

Patient or subject		Sex	Urinary phenylethylamine		
			μg/l	μg/g creatinine	μg/24 h
Normal subjects					
S.C.		F	1.1	3.7	
C.R.		M	3.8	5.7	
R.J.	1	M	3.0	4.6	
	2		2.3	2.7	
W.W.		M	3.3	1.9	
L.N.		F	2.4	1.9	
C.B.		M	3.9	4.4	
S.B.		F	5.6	2.3	
K.B.	1	M	3.8	8.6	6.5
	2		5.5	6.9	9.0
	3		3.3	4.9	6.2
	4		4.2	4.8	7.2
Hyperphenylalaninaemic patients					
B.J.	1	F	14.0	19.7	21.5
	2		7.6	10.6	12.0
L.A.	1	M	15.2	8.1	15.6
	2		20.0	11.2	20.8
E.A.	1	M	17.0	18.1	33.2
	2		14.2	13.0	30.1
E.B.	1	F	14.8	5.0	
	2		10.0	8.5	
A.F.	1	F	7.0	11.7	
	2		9.6	7.7	
L.M.		M	6.2	5.5	
L.C.		F	8.2	5.6	
Phenylketonuric patients					
V.M.		F	29.9	21.2	
R.S.		M	40.2	33.5	
K.F.		M	51.6	32.2	

small volume of urine is needed, which simplifies experimental manipulations. The method is also applicable to biological fluids other than urine, and with minor modifications has successfully been applied to the determination of phenylethylamine in blood, cerebrospinal fluid and amniotic fluid, and in liver perfusates and tissue homogenates.

The results in Table II are in accordance with previous findings [13, 15] on the general relationship between urinary phenylethylamine output and blood phenylalanine concentration. The phenylketonuric children had the highest phenylethylamine output, while that of the hyperphenylalaninaemic subjects, like their phenylalanine blood levels, was intermediate between the values of the normal subjects and the higher values of phenylketonuric patients.

Comparison of the urinary phenylethylamine excretion relative to creatinine with 24-h phenylethylamine output when both values were available (Table II) indicates that the correlation between them is not very good. This is due partly

to diurnal fluctuation in blood phenylalanine concentrations affecting random urinary phenylethylamine values [16] and partly also to variations in the pH at which the urine was excreted by the kidneys [17]. For reliable metabolic studies one should ideally collect 24-h urine samples and also arrange for metabolic acidification of the urine by ammonium chloride ingestion.

The agreement of the more recent reports with the present results makes it clear that specific chromatographic methods must be used to resolve phenylethylamine from interfering substances in order to achieve reliable quantitative results. The availability of such methods should now enable us to obtain more dependable indications of the significance of phenylethylamine both in normal physiology and in pathological conditions.

ACKNOWLEDGEMENTS

We thank Dr. G.S. King for performing the mass spectrometry and Dr. G.P. Reynolds for valuable discussions.

REFERENCES

1 J.B. Jepson, W. Lovenberg, P. Zaltzman, J.A. Oates, A. Sjoerdsma and S. Udenfriend, Biochem. J., 74 (1960) 5P.
2 M. Sandler, S.B. Carter, B.L. Goodwin and C.R.J. Ruthven, in E. Usdin and M. Sandler (Editors), Trace Amines and the Brain, Marcel Dekker, New York, 1976, p. 233.
3 H.C. Sabelli, R.L. Borison, B.I. Diamond, H.S. Havdala and N. Narasimhachari, Biochem. Pharmacol., 27 (1978) 1707.
4 M. Sandler and G.P. Reynolds, Lancet, i (1976) 70.
5 E. Fischer, H. Spatz, J.M. Saavedra, H. Reggiani, A.H. Miro and B. Heller, Biol. Psychiat., 5 (1972) 139.
6 A.A. Boulton, and L. Milward, J. Chromatogr., 57 (1971) 287.
7 A.D. Mosnaim, E.E. Inwang, J.H. Sugerman and H.C. Sabelli, Clin. Chim. Acta, 46 (1973) 407.
8 G.R. Wilkinson, Anal. Lett., 3 (1970) 289.
9 E. Änggård and A. Hankey, Acta Chem. Scand., 23 (1969) 3110.
10 S.B. Matin and M. Rowland, J. Pharm. Sci., 61 (1972) 1235.
11 A.C. Moffat, E.C. Horning, S.B. Matin and M. Rowland, J. Chromatogr., 66 (1972) 255.
12 J.W. Schweitzer, A.H. Friedhoff and R. Schwartz, Biol. Psychiat., 10 (1975) 277.
13 G.P. Reynolds and D.O. Gray, Clin. Chim. Acta, 70 (1976) 213.
14 H. Andersen and C. Braestrup, Scand. J. Clin. Lab. Invest., 37 (1977) 33.
15 G.P. Reynolds, J.W.T. Seakins and D.O. Gray, Clin. Chim. Acta, 83 (1978) 33.
16 R.D. Feigin, W.R. Beisel and R.W. Wannemacher, Amer. J. Clin. Nutr., 24 (1971) 329.
17 G.P. Reynolds, P.M. Ceasar, C.R.J. Ruthven and M. Sandler, Clin. Chim. Acta, 84 (1978) 225.
18 G.P. Reynolds, J.D. Elsworth, K. Blau, M. Sandler, A.J. Lees and G.M. Stern, Brit. J. Clin. Pharmacol., 6 (1978) 542.
19 M.B.H. Youdin, J.K. Aronson, K. Blau, A.R. Green and D.G. Grahame-Smith, Psychol. Med., in press.