J. Pharm. Pharmacol., 1964, **16**, *Suppl.*, 27*T*–31*T* Received May 1, 1964

A specific method for the determination of amphetamine in urine

A. H. BECKETT AND M. ROWLAND

A specific and sensitive method for the quantitative determination of amphetamine in urine, by gas chromatography, is described.

A NUMBER of methods have been used for the determination of amphetamine in urine, but all are non-specific. Richter (1938) used a picric acid dye-complexing method and this was modified by Jacobsen & Gad (1940), and by Harris, Searle & Ivy (1947). The methyl orange method of Brodie & Udenfriend (1945) was modified by Keller & Ellenbogen (1952) and found to be more sensitive than previous methods. Subsequent modifications of this have been used by Axelrod (1954), Utena, Ezoe & Kato (1955), Connell (1956) and Chapman, Shenoy & Campbell (1959).

Amphetamine has also been determined by coupling the molecule with diazotised *p*-nitroaniline (Beyer & Skinner, 1940) and measuring spectrophotometrically the red colour produced under alkaline conditions. Combined with protein precipitation and steam distillation this has been used by McNally, Bergman & Polli (1947).

Alles & Wiesgarver (1961) claim that the method of McNally & others (1947) gave inconsistent results, but by suitable standardisation obtained a working procedure. The method was slightly modified by Krivulka (1962).

Primary amines are present in urine and these react in the diazotisation method. Other amines are also present in urine and all these bases will interact to some extent in the complexing method. Since the total amine content in the urine may vary for example with the diet, time of day and smoking, a more selective method for the determination of amphetamine in urine is required for biological studies.

We now describe the development of an analytical method of sufficient specificity and sensitivity to measure the urinary excretion of amphetamine in man after oral doses of as low as 5 mg of amphetamine sulphate.

Experimental

REAGENTS

Chloroform was refluxed (12 hr) with 1% amphetamine base, washed with 1 N HCl, and twice with distilled water. It was dried over $MgSO_4$ (anhyd.) distilled, and the middle 80% fraction collected: 2% v/v absolute ethanol was then added. Absence of interfering substances was checked by evaporating 6 ml, dissolving the residue in amine-acetone solvent (see below) (150 μl) and testing for the absence of a peak (with about the same t_R value as amphetamine) on the gas chromatograph. Checks were also

From Chelsea School of Pharmacy, Chelsea College of Science and Technology, London, S.W.3.

made by placing known amounts of amphetamine hydrochloride in the chloroform solvent and assaying by the procedure given below to see that there was no interaction of amphetamine with the particular batch of chloroform.

Methoxyphenamine hydrochloride. 1% w/v in distilled water was stored at 4°.

Amine-acetone solvent. Triethylamine 5% v/v, distilled water 10% v/v, acetone to 100%.

APPARATUS AND OPERATING CONDITIONS

The equipment used was a Griffin & George V.P.C. Apparatus M.K.IIB fitted with a hydrogen flame ionisation detector and an integrator (Gas Chromatography Limited type IE.165).

Stationary phase. Polyethyleneglycol 6,000 (PEG), 10% w/w and potassium hydroxide 5% w/w on Celite 545 (acid washed 100–120 mesh). The column was prepared by applying the potassium hydroxide in methanol to the Celite, removing the methanol, then applying the polyethyleneglycol in chloroform.

Working conditions. Column length, 4 ft. (copper tubing); column temperature, 150°; mobile phase, $H_2 : N_2$ (4 : 1); flow rate, 2 litres/hr.

PROCEDURE

To urine (50 ml), add 20% sodium hydroxide solution (10 ml). Using all glass distillation apparatus, steam distil until 50 ml distillate is collected in a receiver containing hydrochloric acid solution (dilute, B.P.) (2 ml). To the acid solution add methoxyphenamine hydrochloride (300 μg) and pass steam through the acid solution until 50 ml distillate is collected. Evaporate the residual acid solution to dryness over a steam-bath. Transfer the residue to a 5 ml flask, and wash the evaporating dish with 3 × 1 ml of chloroform, transferring the chloroform to the flask. Reflux for 5 min, cool, and pipette the chloroform into a wide necked short boiling tube, via a No. 2 porosity glass filter. Reflux the residue with another 2 ml of chloroform and transfer the solution as above. Wash the sintered filter with chloroform (0·5 ml) and collect this solution. Evaporate the combined chloroform extracts to dryness by passing hot air over the tubes. Dissolve the residue in chloroform (0·5 ml) and transfer to a burette equipped with a Teflon tap. Flash distil the solution in a B7 container (0·3 ml total capacity) in a bath at about 98°. The boiling tube is then washed with chloroform (0·5 ml) and this solution also concentrated in the B7 container. Dissolve the residue in amine-acetone solvent (150 μl) in the B7 container, close with a slightly silicone-greased stopper, leave for 2 hr and then chromatograph 10 μl of this solution. Obtain the ratio of the amphetamine to methoxyphenamine peak areas.

Calculate the concentration of the amphetamine in the sample by making reference to a calibration curve obtained by plotting the ratio of amphetamine to methoxyphenamine peak areas against the concentration of amphetamine using a fixed concentration of methoxyphenamine (0·2%

w/v solution of methoxyphenamine hydrochloride in the amine-acetone solvent).

Results and discussion

PREPARATION OF THE SAMPLE FOR GAS CHROMATOGRAPHY

Problems were encountered in the attempted gas chromatography of free amphetamine. For example pronounced tailing was experienced with an alkaline-treated PEG column, a PEG glass bead column, or with a silicone elastomer column (Fales & Pisano, 1962) coated on Diatoport S (a silizanised treated celite) or glass beads. This tailing was not due to lack in uniformity in coating, or to improper packing, since other materials, e.g., naphthalene always produced a symmetrical peak. By using the amphetamine-acetone derivative, a more symmetrical peak resulted (Brochmann-Hanssen & Svendsen, 1962).

In the method described, amphetamine is present as the hydrochloride throughout the concentration procedure, to prevent loss of the compound. Before gas chromatography the free base has to be generated, since protonated primary amines do not react with acetone (Bergel & Lewis, 1955). Triethylamine was most convenient for this purpose. It is a stronger base than either amphetamine or methoxyphenamine and emerges from the chromatography column with the solvent peak. In addition, 10% water was required since in anhydrous acetone no amphetamine-acetone complex was formed. This proportion of water allowed the acetone to react with the amphetamine but not with unknown interfering compounds sometimes present in urine. The hydrochloride of methoxyphenamine produced marked tailing in gas chromatography and, therefore, had to be converted to the free base (Nicholls, Makisum & Saroff, 1963).

The conversion of the free base to the acetone derivative occurs slowly, being almost complete in 2 hr. The equilibrium mixture is stable for at least two months. The small proportion of free amphetamine in the equilibrium mixture does not prejudice the assay procedure, since the calibration curve is linear.

In the steam distillation, much ammonia is collected along with the amphetamine in the hydrochloric acid solution used to trap the bases. Evaporation of this solution gave large quantities of ammonium chloride which blocked the gas chromatography column. Amphetamine hydrochloride is much more soluble in chloroform than ammonium chloride and, therefore, chloroform extraction was used to separate the two. The chloroform was specially purified because of the known interaction of impurities in commercial chloroform with many bases (Caws & Foster, 1957). Ethanol was added to purified chloroform to prevent formation of carbonyl chloride because of the latter's reaction with amphetamine and consequent interference with the assay procedure.

INSTRUMENTAL CONSIDERATIONS

The column. Tailing of the amphetamine derivative occurs if the

column is not uniformly coated. The columns described have a long working life (2,000 chromatograms over 5 months).

Choice of internal marker. Benzylamine and methoxyphenamine with suitable t_R values were chosen.* The use of benzylamine had the disadvantage that to get complete conversion to the benzylamine acetone derivative, the water content in the acetone solution could not be allowed to exceed 1%. At this concentration of water, materials in the urine gave complexes with acetone with t_R values in the region of the amphetamine-acetone derivative peak. Since methoxyphenamine does not form an acetone adduct, the water content of the acetone is unimportant to its gas chromatographic peak. A typical gas chromatograph from a urine sample to which the internal marker has been added, is shown in Fig. 1.

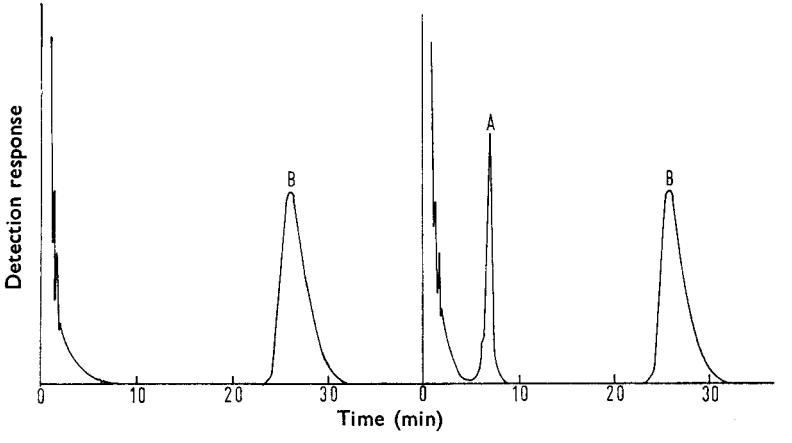

FIG. 1. Chromatograms of urine + marker and urine + marker containing 2 μg/ml amphetamine base. A. Amphetamine-acetone derivative (t_R=6·5 min). B. Methoxyphenamine (t_R 26 min).

RECOVERY, REPRODUCIBILITY AND SPECIFICITY OF THE METHOD

The corrected recovery of amphetamine from urine containing from 1–6 μg amphetamine hydrochloride per ml by the above method, was 100 $\pm$ 5%. The actual amount of amphetamine recovered was about 85%. Amphetamine concentrations in the urine as low as 0·2 μg/ml, may be detected with ease. The amphetamine was stable in the urine, stored at 4°, for at least three days, with no concomitant rise in the urine blanks.

Specificity. The assumption is made that, in man, over 24 hr, 30% of any amount of amphetamine administered may be recovered unchanged (Beyer & Skinner, 1940). For a dose of 10 mg amphetamine sulphate and the normal urine output of 1,500 ml daily the concentration of amphetamine in the urine is 1·5 μg/ml base. Hence if a detailed urinary excretion

* 4-benzylpyridine; *NN*-diethylaniline; benzylmethylamine; 1-phenylpiperidine; aminodimethylmethane; anisidine; *o*-chlorobenzylamine; *p*-chlorobenzylamine; 4-methylbenzylamine; α-ethylbenzylamine; 2,4-dichlorobenzylamine were also investigated.

study is to be conducted the "blank" urine levels must be much lower than 1·5 μg/ml.

Previously published methods gave the following blank values (as μg apparent amphetamine base/ml) based upon the assumed urine output of 1,500 ml daily: with the picric acid assay, Jacobsen & Gad (1940), obtained 0–3; Alles & Wiesgarver (1961) 5–12: with the diazotisation method, Harris, Searle & Ivy (1947 found 1·76 (s.d. 1·11); Alles & Wiesgarver, (1961) gave 1·6 and Krivulka (1962) 1·2–1·7; the methyl orange procedure gave 0·9–2·0 (Utena & others 1955), 0·65 (Connell, 1958), 1·5 (s.d. 0·35) (Chapman & others, 1959).

The chromatographic method described herein gave virtually zero blank values and on no occasion did a value reach 0·1 μg amphetamine/ml of urine. This method is being used for a detailed examination of the urinary excretion pattern of amphetamine in man and other species.

Acknowledgement. One of us (M.R.) thanks the Medical Research Council for a grant held while carrying out this research.

References

Alles, G. A. & Wisegarver, B. B. (1961). *Toxicol. App. Pharmacol.*, **3**, 678–688.
Axelrod, J. (1954). *J. Pharmacol.*, **110**, 315–326.
Bergel, F. & Lewis, G. E. (1955). *Chem & Ind.*, 774–775.
Beyer, K. H. & Skinner, J. T. (1940). *J. Pharmacol.*, **68**, 419–432.
Brochmann-Hanssen, E. & Svendsen, A. B. (1962). *J. pharm. Sci.*, **51**, 938–940.
Brodie, B. B. & Udenfriend, S. (1945). *J. biol. Chem.*, **158**, 705–714.
Caws, A. C. & Foster, G. E. (1957). *J. Pharm. Pharmacol.*, **9**, 824–832.
Chapman, D. G., Shenoy, K. G. & Campbell, J. A. (1959). *Canad. med. Ass. J.*, **81**, 470–477.
Connell, P. H. (1957). *Biochem. J.*, **65**, 7P.
Connell, P. H. (1958). *Amphetamine Psychosis*, p. 92. London: Oxford University Press.
Fales, H. M. & Pisano, J. J. (1962). *Analyt. Biochem.*, **3**, 337–342.
Harris, S. C., Searle, L. M., & Ivy, A. C. (1947). *J. Pharmacol.*, **89**, 92–100.
Jacobsen, E. & Gad, I. (1940). *Arch. exp. Path. Pharmak.*, **196**, 280–289.
Keller, R. E. & Ellenbogen, W. C. (1952). *J. Pharmacol.*, **106**, 77–82.
Krivulka, R. L. (1962). M.Sc. Thesis. University of California.
McNally, W. D., Bergman, W. L. & Polli, J. F. (1947). *J. Lab. clin. Med.*, **32**, 913–916.
Nicholls, C. N., Makisumi, S. & Saroff, H. A. (1963). *J. Chromatog.*, **11**, 327–331.
Richter, D. (1938). *Biochem. J.*, **32**, 1763–1769.
Utena, H., Ezoe, T. & Kato, N. (1955). *Psychiatr. et. Neurol. Jap.*, **57**, June.

The paper was presented by MR. ROWLAND.