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GAS CHROMATOGRAPHIC MEASUREMENT OF LEVELS OF FENFLURAMINE AND NORFENFLURAMINE IN HUMAN PLASMA, RED CELLS AND URINE FOLLOWING THERAPEUTIC DOSES

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SUMMARY

A specific and sensitive procedure for the determination of fenfluramine and norfenfluramine at therapeutic levels in human plasma, red cells and urine has been developed. The method involves extraction of the drugs from alkalinised samples into diethyl ether or dichloroethane, with subsequent separation and detection on a gas–liquid chromatograph fitted with a flame ionisation detector. N,N-Diethylaniline is used as an internal standard for quantitation by the relative peak area technique. Levels encountered after oral ingestion of a single therapeutic dose are reported.

INTRODUCTION

Fenfluramine (N-ethyl-α-methyl-3-trifluoromethyl-phenethylamine) is widely prescribed in the hydrochloride form as "Ponderax" for the treatment of obesity. A gas–liquid chromatographic (GLC) procedure has been described for the determination of the parent drug and its de-ethylated metabolite, norfenfluramine, in human urine after therapeutic dosage[1]. This method lacks the sensitivity required for the accurate estimation of these compounds in plasma at therapeutic levels[2,3] and has the further disadvantage that the use of amphetamine as an internal standard may lead to erroneous results in patients who may have ingested this compound as well. BRUCE AND MAYNARD[4] have reported a GLC method with blood involving the formation of a heptafluorobutyryl derivative of fenfluramine and the use of an electron-capture detector. Although sensitive to picogram quantities, this procedure is time consuming and is limited to a small linear range of detection, so making it unsuitable for the analysis of a large number of samples with a wide range of levels. The method described in the present work is comparatively quick and simple, one sample taking an hour, and has been successfully applied to the measurement of fenfluramine and norfenfluramine in blood and urine after therapeutic administration, as well as to cases of overdosage.

EXPERIMENTAL

Reagents

Diethyl ether was purified by re-distillation over sodium wire. 1 *N* and 2 *N* sodium hydroxide and 1 *N* sulphuric acid were washed with re-distilled diethyl ether before use. The internal standard was N,N-diethylaniline. All reagents were supplied by Hopkin & Williams Co. Ltd., Chadwell Heath, Essex, Great Britain.

Gas chromatography

A Pye 104 chromatograph equipped with dual-flame ionisation detectors was used. The signal was recorded on a Honeywell —0.1 to 1.0 mV recorder. The column support was 80–100 mesh acid-washed, dimethyldichlorosilane-treated Chromosorb G, coated with 10% potassium hydroxide from methanol and 10% Apiezon L from methylene chloride as stationary phase. This was packed into silanised glass columns (1.5 m long × 4 mm I.D.) and conditioned at 200° in a stream of nitrogen for 24 h before use. The instrument settings were as follows: temperature, injection port 205° and column 155°; gas flow rates, hydrogen 30 ml/min, nitrogen 30 ml/min and oxygen 350 ml/min.

The high degree of electrical "background noise" encountered at low attenuations (10^{-11} A) mitigated against accurate determinations. By using oxygen in place of air in the detector, an improvement of up to 80% in the detector sensitivity enabled higher attenuations to be used. A high flow rate of oxygen was found to be essential in order to prevent burning out of the detector[5].

Extraction procedure

Plasma. Plasma (10 ml) was made alkaline by the addition of 1.0 ml of 1 *N* sodium hydroxide and extracted by gentle shaking with 10 ml diethyl ether and 1 ml of internal standard solution (0.05 mg% N,N-diethylaniline in diethyl ether) in a mechanical shaker for 5 min. After centrifugation at 4,000 r.p.m., the ether phase was removed from the aqueous phase and stored in a glass-stoppered centrifuge tube at —5° to freeze out dissolved aqueous phase together with plasma constituents which would interfere with subsequent detection. (Drying with anhydrous sodium sulphate resulted in losses of up to 25% fenfluramine.) The organic phase was decanted into a centrifuge tube containing 0.5 ml of 1 *N* sulphuric acid, and the mixture agitated for 5 min on a Fisons "Whirlimixer". The phases were separated by centrifugation at 4,000 r.p.m. for 5 min and the ether layer discarded. The residual aqueous phase was made alkaline by adding 0.5 ml of 2 *N* sodium hydroxide and extracted with 1 ml of diethyl ether by shaking for 1 min on the "Whirlimixer". After further centrifugation at 4,000 r.p.m. for 5 min, the organic layer was transferred to a 15-ml tapered centrifuge tube and carefully concentrated to approx. 50 μl with a slow stream of nitrogen. Evaporation to dryness was avoided, since this resulted in apparent increase of up to 40% in the amount of fenfluramine due to preferential evaporation of the internal standard. The final extracts were stored at —5° in glass-stoppered tubes; this temperature precipitates any aqueous contaminant and prevents evaporation of the organic phase. For analysis the extract was removed from the cold and 3–5 μl were injected immediately onto the gas chromatograph.

Red cells. For the initial extraction of the red cells diethyl ether was found to

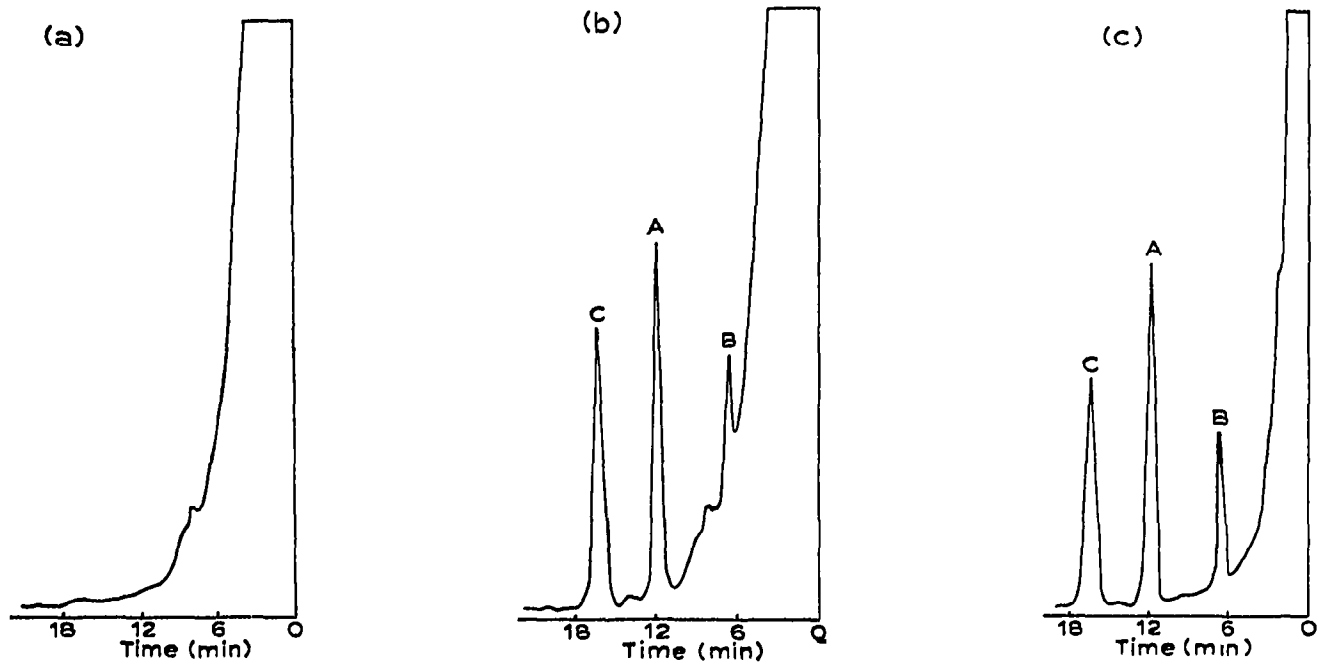

Fig. 1. (a) Gas chromatogram of an ether extract of normal plasma without fenfluramine or norfenfluramine. (b) Gas chromatogram of an ether extract of plasma containing 50 ng/ml of fenfluramine (A), 12 ng/ml of norfenfluramine (B) and N,N-diethylaniline (C) as internal standard. (c) Gas chromatogram of an ether extract of urine containing 4.2 μg/ml fenfluramine (A), 1.3 μg/ml of norfenfluramine (B) and N,N-diethylaniline (C) as internal standard.

remove substances which produced interfering peaks on the chromatograph. By substituting the less polar solvent 1,2-dichloroethane this difficulty was eliminated.

Urine. Urine (5 ml) was made alkaline with 0.5 ml of 1 *N* sodium hydroxide and extracted with 5 ml diethyl ether and 1 ml of the internal standard (1.0 mg% N,N-diethylaniline in diethyl ether) by gentle shaking for 5 min. After centrifugation at 4,000 r.p.m. for 5 min, the organic layer was transferred to a 15-ml tapered cen-

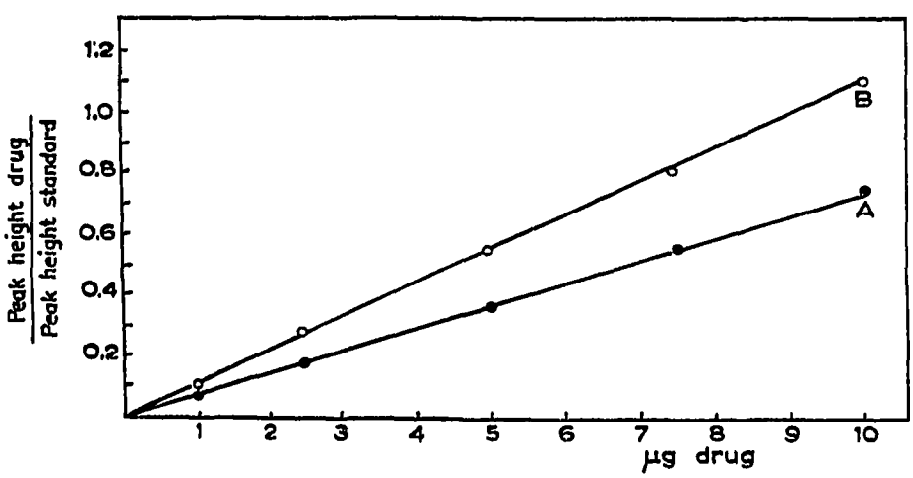

Fig. 2 The standard calibration graph for urine relating the ratio of the peak heights of fenfluramine (A), norfenfluramine (B) and N,N-diethylaniline.

trifuge tube, carefully evaporated to about 50 μl with a stream of nitrogen and 3–5 μl of this extract were injected onto the gas chromatograph.

Measurement

Losses of the compounds due to adsorption on the solid phase of the column were minimised by presaturating the active adsorption sites prior to each set of determinations. This was achieved by repeated injection of an ethereal solution of nicotine (1 μg/ml) until a constant base-line was maintained. The retention times of fenfluramine, norfenfluramine and N,N-diethylaniline were 11.8 min, 6.5 min and 16.2 min, respectively (Fig. 1). Over the range 1–10 μg of fenfluramine and norfenfluramine, the ratios of peak heights of the compounds to that of the internal standard were linear (Fig. 2). At the lower range of 0.02–1.0 μg, a plot of the ratios of peak areas (height × width at half height) gave a more linear calibration curve (Fig. 3). Recoveries of fenfluramine and norfenfluramine from plasma and red cells over the range 0.1–0.5 μg were 85 ± 4% and from urine over the range 2.0–20 μg, 99 ± 0.5%. The limit of detection of both compounds in plasma and red cells was 2 ng/ml.

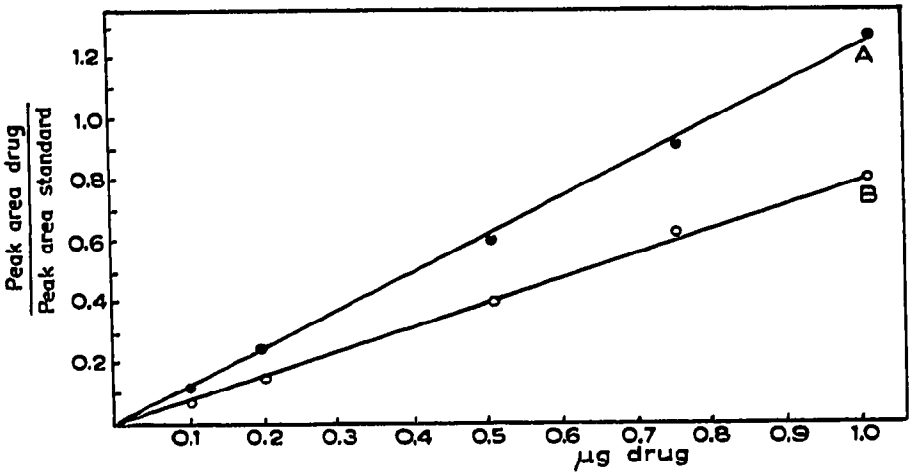

Fig. 3. The standard calibration graph for blood relating the ratio of peak areas of fenfluramine (A), norfenfluramine (B) and N,N-diethylaniline.

Specificity

The chromatographic system gave a separation of fenfluramine and norfenfluramine from the related congeners, amphetamine and methylamphetamine, which had retention times of 9.4 min and 13.0 min, respectively, under the same conditions.

Application

Two male subjects each received oral doses of 60 mg of fenfluramine hydrochloride. Venous blood samples were withdrawn at hourly intervals over a period of 8 h; and three further samples at 24 h, 30 h and 50 h. The red cells and plasma were separated immediately by centrifugation and both were analysed for fenfluramine and norfenfluramine. Urine samples were collected at hourly intervals between blood sampling for the initial 8½ h after dosage, and thereafter at intervals between 2 and 4 h over a period of 48 h.

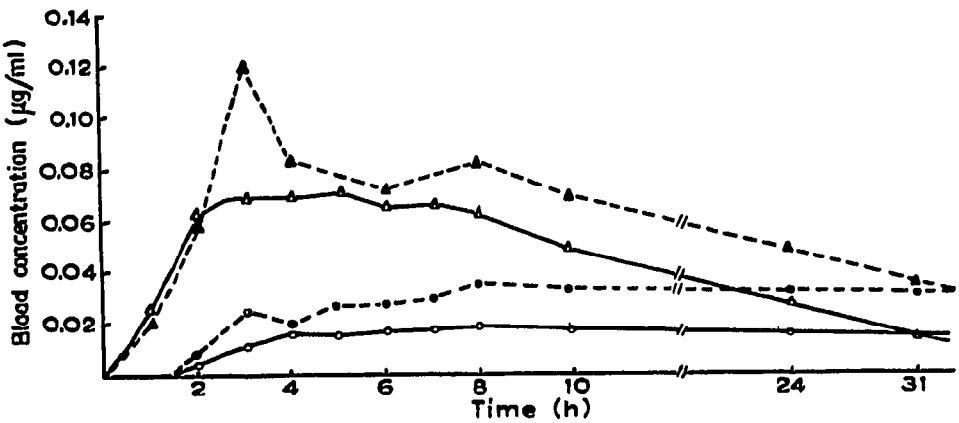

Fig. 4. Plasma and red cell concentration–time curves indicating levels found in subject A following an oral dose of 60 mg of fenfluramine hydrochloride. △—△, plasma fenfluramine; ▲— —▲, red cell fenfluramine; ○—○, plasma norfenfluramine, ●— —●, red cell norfenfluramine.

After immediate measurement of the pH and volume, urine samples were stored at −20° prior to analysis.

RESULTS AND DISCUSSION

Plasma levels of fenfluramine reached a peak of between 0.05–0.07 μg/ml approximately 3 h after oral administration of fenfluramine hydrochloride. For norfenfluramine, peak plasma levels of between 0.015–0.02 μg/ml occurred approximately 4 h after dosage (Fig. 4).

BROOKES[2] reported a peak plasma fenfluramine level of 0.16 μg/ml occurring 3–4 h after oral administration of three times the daily therapeutic dose (3 × 60 mg)

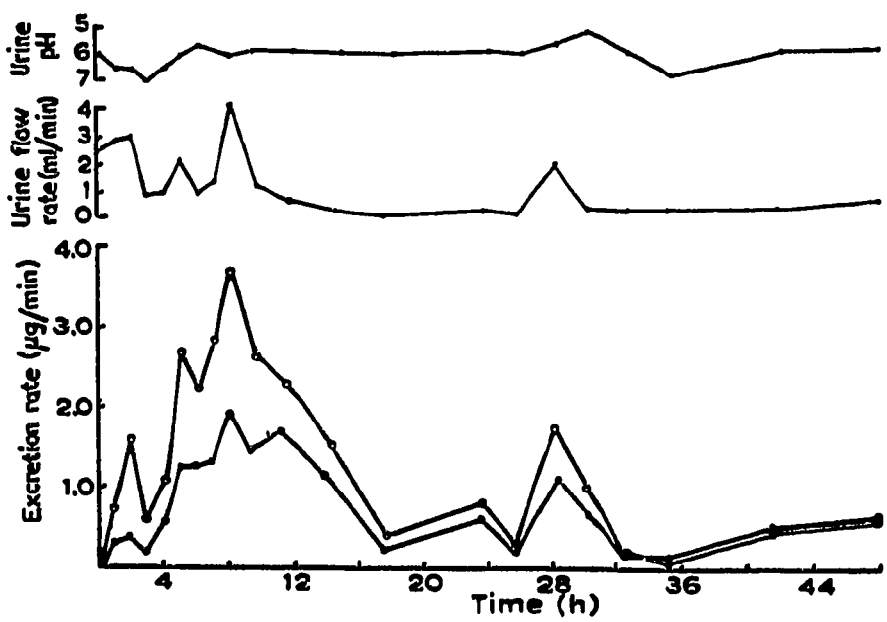

Fig. 5. The corresponding urinary excretion rates of fenfluramine (○—○) and norfenfluramine (●—●) from subject A.

to a male subject. Using the same procedure, BRUCE AND MAYNARD[3] quoted a peak plasma fenfluramine level of only 0.03 μg/ml after oral ingestion of 60 mg of the drug. Their refined technique, however, when applied to a subject who ingested 5 mg of the drug, indicated a peak plasma fenfluramine level of 0.005 μg/ml (ref. 4). It would seem that the initial findings of BROOKES[2] and the latter findings of BRUCE AND MAYNARD[4] have been substantiated by the present work. Plasma levels of fenfluramine and norfenfluramine remained relatively constant for 5 h after peak levels were achieved and then declined at a constant rate. Fenfluramine and norfenfluramine could be detected in plasma and red cells over 50 h after a single therapeutic dose. In human subjects, following the ingestion of 60 mg of fenfluramine hydrochloride, the distribution of fenfluramine and norfenfluramine between plasma and red cells was found to be 40% and 60% for both compounds.

The rate of urinary excretion of these compounds fluctuated considerably over the same period (Fig. 5), this finding being in agreement with previous work of BECKETT AND BROOKES[1], which showed that the rate of excretion of fenfluramine and norfenfluramine was a function of urinary volume and pH.

Work presented elsewhere[6-8] has demonstrated the applicability of this procedure in cases of fenfluramine overdosage.

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REFERENCES

1 A. H. BECKETT AND L. G. BROOKES, *J. Pharm. Pharmacol.*, 19, Suppl. (1967) 42S.
2 L. G. BROOKES, *Ph. D. Thesis*, London University, 1968.
3 R. B. BRUCE AND W. R. MAYNARD, JR., *J. Pharm. Sci.*, 57 (1968) 1173.
4 R. B. BRUCE AND W. R. MAYNARD, JR., *Anal. Chem.*, 41 (1962) 977.
5 D. B. CAMPBELL, *J. Pharm. Pharmacol.*, 21 (1969) 129.
6 D. B. CAMPBELL AND B. W. R. MOORE, *Lancet*, II (1969) 1307.
7 M. R. FLEISHER AND D. B. CAMPBELL, *Lancet*, II (1969) 1306.
8 A. S. RICHARDS, *Lancet*, II (1969) 1367.