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Note

Improved gas—liquid chromatographic method for measuring fenfluramine and norfenfluramine in heparinised plasma

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Fenfluramine, a phenylethylamine derivative, is widely prescribed as an anorectic agent in a range of formulations, including the hydrochloride Ponderax. Previous gas—liquid chromatographic (GLC) methods for measuring fenfluramine and its active N-demethylated metabolite, norfenfluramine [1] have been reported in plasma and urine; however, these methods have either involved derivatisations and electron-capture detection [2], lacked sensitivity or proved difficult to reproduce in our laboratory [3].

Therapeutic monitoring of plasma levels is not only a check of patient compliance, but may also avert reported dose-related side effects [2, 4, 5]. The assay described is currently being applied in a long-term study involving the measurement of steady-state plasma levels of fenfluramine and norfenfluramine in post jaw-wiring obese patients. Blood samples are drawn in an outpatient clinic setting and it is hoped that data obtained will provide information on a therapeutic range for fenfluramine and its metabolite.

MATERIALS AND METHODS

Reagents

All reagents were analytical grade and aqueous solutions prepared using glass-distilled water. The extracting solvent diethyl ether (May and Baker, Melbourne, Australia) was redistilled in glass and *n*-butyl acetate (Mallinckrodt, Australia) was first washed three times with 20% (v/v) pure water (to remove residual acetic acid) and distilled twice in glass. The aqueous solutions, 0.5 mol/l sulphuric acid and 5 mol/l sodium hydroxide, were washed three times with 10% (v/v) diethyl ether before use. The internal standard was N,N-di-

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ethylaniline (BDH). Fenfluramine hydrochloride and norfenfluramine base were kindly donated by Servier (Melbourne, Australia).

Standards

A standard solution containing (DL)fenfluramine (10.8 μmol/l) and norfenfluramine (12.3 μmol/l) was prepared in 0.5 mol/l sulphuric acid. A standard curve was constructed using a concentration range of 0.1 to 2.0 μmol/l. The internal standard was diluted into 0.5 mol/l sulphuric acid to give a final concentration of 13.4 μmol/l.

Preparation of glassware

All procedures relating to the assay were carried out in glassware which had firstly been rinsed in a pyrogen-free detergent solution, rinsed with distilled water and dried. Secondly, the glassware was rinsed with methanol and, before drying, rinsed with ethyl acetate. This latter procedure was found essential to avoid interfering peaks in the final GLC trace.

Chromatography

The separation was performed on a Perkin-Elmer Sigma 3 gas chromatograph equipped with a nitrogen detector. The column (2 m × 2 mm I.D.) was packed with Chromosorb W-AW (80—100 mesh) coated with 10% potassium hydroxide and 10% Carbowax 20 M. The gas flow-rates for nitrogen, air and hydrogen were 60, 110 and 10 ml/min, respectively. The detector and the injection port temperatures were adjusted to 200° C and the column oven temperature to 130° C. The column was conditioned for 3 h at 150° C prior to use.

Assay technique

Aliquots (2 ml) of heparinised plasma were added to 0.4 ml of 0.5 mol/l sulphuric acid in glass-stoppered extraction tubes. In the case of the standard solutions the plasma was drawn from patients who were not receiving fenfluramine. This plasma had been previously shown to contain no compounds which interfered with the peaks of interest on the final GLC trace. The acid in these standard solutions contained known concentrations of fenfluramine and norfenfluramine ranging from 0.1 to 2.0 μmol/l. Aliquots (100 μl) of the internal standard solution were added to each tube and then basified with 1.0 ml of 5 mol/l sodium hydroxide. After briefly vortexing each tube, 2.0 ml of diethyl ether were added, the tubes capped, shaken for 10 min and centrifuged at 1000 *g* for 10 min. The phases were separated by pipetting the organic layer into a second glass-stoppered centrifuge tube. Back extraction was carried out by adding 1.0 ml of 0.5 mol/l sulphuric acid to this organic phase and shaking for 10 min. Tubes were again centrifuged for 10 min at 1000 *g*. The organic phase was aspirated to waste and the aqueous phase basified with 0.5 ml of 5 mol/l sodium hydroxide before adding 0.2 ml of *n*-butyl acetate. The tubes were vortexed for 30 sec and centrifuged for 3 min at 1000 *g*. The aqueous layer was carefully pipetted to waste and 5-μl aliquots of the organic layer subjected to GLC.

The performance of the assay was monitored by assaying a plasma stock containing 0.4 μmol/l fenfluramine and 0.5 μmol/l norfenfluramine. Hence,

coefficients of variation were estimated by comparing eight determinations made in a single assay run, and five determinations made on a weekly run basis.

The limits of detection were determined by preparing further dilutions into plasma of the stock solution of fenfluramine and norfenfluramine, and assaying these solutions as described above. A peak height-to-noise ratio of 2:1 was accepted as the minimum detectable level.

The recoveries of fenfluramine and norfenfluramine through the assay were determined by comparing the peak heights (mean of five injections) of a stock solution of the two compounds (0.4 and 0.5 μmol/l, respectively) prepared in *n*-butyl acetate, with the corresponding peak heights of six samples, containing the same concentrations of the two compounds, prepared in plasma and carried through the assay procedure described above.

RESULTS

The retention times of fenfluramine, norfenfluramine and internal standard (N,N-diethylaniline), were 4.1, 4.8 and 6.0 min, respectively (Fig. 1). The peak height of norfenfluramine was consistently about 60% of that of the parent

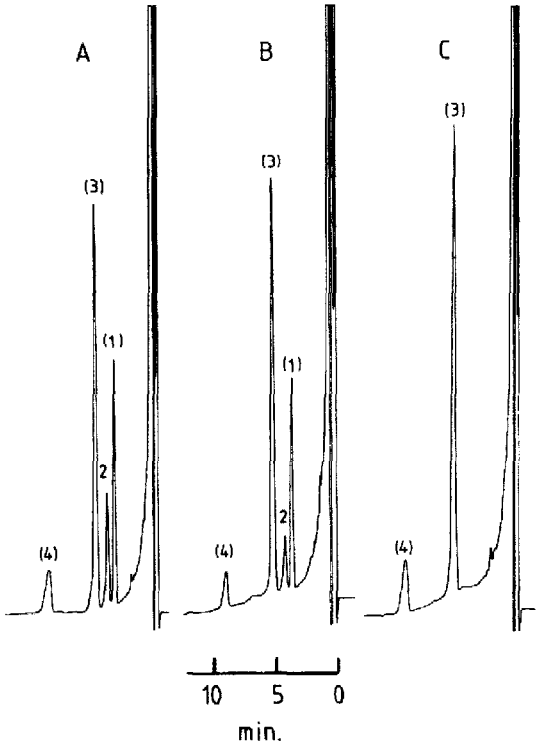

Fig. 1. Sample GLC traces showing fenfluramine (1); norfenfluramine (2); N,N-diethylaniline, internal standard (3); and an unidentified plasma peak (4), with retention times of 4.1, 4.8, 6.0 and 9.3 min, respectively. (A) Blank plasma to which fenfluramine (0.4 μmol/l) and norfenfluramine (0.5 μmol/l) had been added; (B) typical sample from a patient receiving fenfluramine; and (C) sample from an obese patient not receiving fenfluramine. The internal standard (3) is shown in each case.

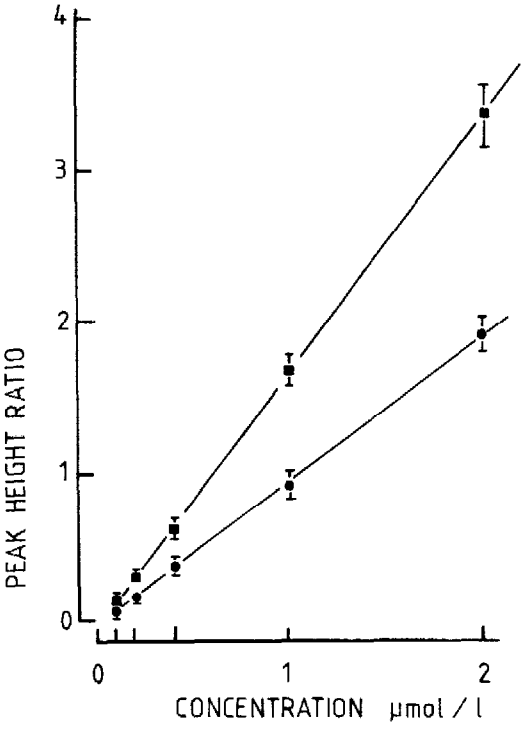

Fig. 2. Standard curves for fenfluramine (▪) and norfenfluramine (•) showing the peak height ratios [i.e., sample peak (mm) : internal standard (mm)] over a concentration range of 0.1 to 2.0 µmol/l. Values are means (± S.E.M.) of determinations made in four different assay runs. The correlation coefficients (r^2) for fenfluramine and norfenfluramine were 1.0000 and 0.9993, respectively.

compound, fenfluramine. The ratios of the peak heights of fenfluramine and of norfenfluramine to the internal standard were linear over the concentration range of 0.005 µmol/l (the detection limit of the assay) to at least 2.0 µmol/l. However, as shown in Fig. 2, the concentration range routinely employed for estimating patient sample levels was 0.1 to 2.0 µmol/l. The method was standardised by assaying in each assay run a plasma sample which had been previously spiked with fenfluramine (0.4 µmol/l) and norfenfluramine (0.5 µmol/l), divided into 2.5-ml aliquots and stored at −20°C. These estimations indicated coefficients of variation of 2.0% and 5.9% between assays (n = 5), and 2.0% and 4.5% within a single assay (n = 8) for fenfluramine and norfenfluramine, respectively. Further, the recoveries through the assay at the same concentrations were 65% and 82% for fenfluramine and norfenfluramine, respectively.

Amphetamine, the structurally related compound from which fenfluramine was derived, and methylamphetamine, when assayed by this method were found to interfere with norfenfluramine and the internal standard in the final GLC trace. The retention times for amphetamine and methylamphetamine were 5.0 and 5.5 min, respectively.

DISCUSSION

The method presented here for the assay of fenfluramine and norfenfluramine in plasma represents an improvement over previously published methods as it employs nitrogen detection with GLC. The methodology is less complex than some of the previous methods as no derivatisation is required. Hence, the assay run time, the resolution of peaks and the assay sensitivity have been improved over other methods.

The performance of this sensitive assay has proved to be dependent on the purity of the reagents used and the cleanliness of the glassware employed as described above. The time spent initially in decontaminating reagents and glassware has proved valuable in terms of the continuing satisfactory performance of the assay.

The method is currently being applied to plasma samples from obese patients in an outpatient clinic setting. The aim of this on-going study is to attempt to establish a dose–response relationship and a possible therapeutic range for fenfluramine and norfenfluramine. Hence, although the method is equally applicable to measurement of these two compounds in urine, such levels are of little value in terms of the above aim since it has been shown [3, 6] that, like many drugs, urinary excretion of fenfluramine and norfenfluramine is very dependent on the volume and pH of the urine. Such levels could, however, be utilized to monitor pharmacodynamics or compliance.

Of the 50 patient samples assayed so far, none have shown any interfering compounds on the final GLC trace, as evidenced by the presence of other peaks or shoulders on expected peaks.

The inclusion of amphetamine was based on its structural and pharmacological (in terms of its anorectic properties) relationship to fenfluramine. Methyl amphetamine [2] and amphetamine [6] have also been used as internal standards in previous methods. Hence, in the unlikely event that their fenfluramine assay were applied to a sample containing amphetamine and/or methylamphetamine, a false result would be obtained. The present method would at least recognise, although not resolve, the presence of the amphetamine compounds. Further, with the appropriate choice of standards, the assay could be equally applied to the measurement of amphetamine and methylamphetamine in patient samples.

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