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# Short Communication

# Determination of dexfenfluramine and nordexfenfluramine in urine by high-performance liquid chromatography using ultraviolet detection

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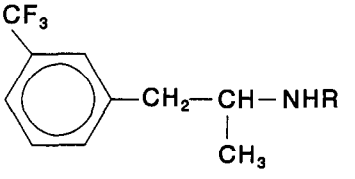
ABSTRACT

A method to determine the concentration of dexfenfluramine and its active metabolite nordexfenfluramine in human urine from healthy volunteers is described utilising a high-performance liquid chromatographic procedure with liquid–liquid extraction and ultraviolet detection. Analytes are measured after extraction of alkalinised urine with diethyl ether and subsequent back extraction with 0.5 *M* $H_2SO_4$ and with chromatography performed on a reversed-phase $C_{18}$ column, using a mobile phase of acetonitrile–50 m*M* $K_2HPO_4$ (25:75, v/v) (flow-rate 1.3 ml/min) and ultraviolet detection at 210 nm. The sensitivity of the technique (10 ng/ml) is appropriate to measure both parent drug and metabolite in urine in humans for up to 5 days after a single 30-mg dose. The method is selective, reproducible (within- and between-day coefficient of variation ranged from 4.2 to 15%) and accurate (bias less than 8%) and thus suitable for dexfenfluramine pharmacokinetic investigations.

## INTRODUCTION

Dexfenfluramine, the *dextro*-rotatory or (+)-enantiomer of the racemic drug fenfluramine, is widely used as a stereochemically pure anorectic drug [1]. In man it is eliminated principally by metabolism, but 9% of the dose appears in the urine as unchanged drug [2]. Up to 6% of the dose is also recovered in urine as an active N-de-ethylated metabolite, nordexfenfluramine [2]. The chemical structures of dexfenfluramine and nordexfenfluramine are given in Fig. 1. In order to investigate further the inter-individual variation in dexfenfluramine disposition [1] we have developed a selective, sensitive and repro-




R = —H Nordexfenfluramine

R = —$CH_2$—$CH_3$ Dexfenfluramine

Fig. 1. Chemical structures of dexfenfluramine and nordexfenfluramine.

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ducible technique to measure both dexfenfluramine and nordexfenfluramine in urine. Unlike previously published methods employing gas chromatography (GC) [3–8], the method presented uses high-performance liquid chromatography (HPLC) with ultraviolet detection. The HPLC method, unlike some GC methods, does not include a derivatisation step and may be useful in laboratories where GC equipment is not available.

## EXPERIMENTAL

### *Standards and reagents*

*S*-(+)-fenfluramine [(*d*-1-(3-trifluoromethyl) phenyl-2-ethylaminopropane) hydrochloride] and *S*-(+)-norfenfluramine [(*d*-1-(3-trifluoromethyl)phenyl-2-aminopropane) hydrochloride] were generously supplied by Servier Laboratories Pty (Melbourne, Australia). Both compounds were greater than 99% stereochemically pure. Diethyl ether (AnalaR, BDH, Kilsyth, Vic., Australia) was initially washed with 2 *M* NaOH, then with 2 *M* HCl and water and subsequently distilled prior to use. Acetonitrile (Unichrom-190) was obtained from Ajax Chemicals (Auburn, N.S.W., Australia). All other reagents were of analytical grade. Double distilled water was used throughout. Glassware was treated with trimethylchlorosilane prior to use [8].

### *Instrumentation and chromatographic conditions*

An octadecasilyl column ($C_{18}$ μBondapak® column: 10 μm, 300 mm × 3.9 mm I.D.; Waters Millipore, Lane Cove, N.S.W., Australia) and a mobile phase consisting of acetonitrile–50 m*M* $KH_2PO_4$ (25:75, v/v) pumped at a flow-rate of 1.3 ml using an M-6000A solvent delivery module (Waters Millipore) were used. A back pressure of 9.6 MPa resulted. Samples were introduced onto the column using an automatic injector (WISP 710B automated sample injector, Waters Millipore) and detected using ultraviolet absorbance at 210 nm (481 Lambda Max, Waters Millipore). Detector output was recorded using an integrator (SP4600 DataJet Integrator, Spectra-Physics, San Jose, CA, USA; attenuation 16). All chromatography was performed at ambient temperature.

### *Sample preparation*

Urine samples (1ml) in polypropylene tubes (Disposable Products, Technology Park, S.A., Australia) to which 200 μl of 5 *M* NaOH was added were extracted with 3 ml of diethyl ether by vortex-mixing for 2 min. After centrifugation at 2200 *g* for 5 min the tube was placed in acetone–dry ice to freeze the aqueous layer. The organic layer was then quantitatively transferred to a second glass tube containing 120 μl of 0.5 *M* $H_2SO_4$. The tubes were vortex-mixed and centrifuged as previously described and the organic layer discarded using a pasteur pipette. Any remaining diethyl ether was evaporated under a stream of nitrogen and 100 μl of the aqueous phase was injected onto the HPLC column.

### *Standardisation, accuracy and reproducibility*

Standard curves were prepared by adding increasing amounts (25, 100, 250, 500, 1000 and 2000 ng) of dexfenfluramine hydrochloride and nordexfenfluramine hydrochloride to drug-free urine and extracting the samples as described above. Linear regression, without weighting or constraint through the origin, of the concentration added and the peak areas of dexfenfluramine or nordexfenfluramine was performed and the concentration of the analytes in unknown samples was obtained from the regression lines. The accuracy of the technique was determined by measuring the concentration of dexfenfluramine and nordexfenfluramine in twelve samples of drug-free urine to which varying amounts of dexfenfluramine and nordexfenfluramine were added and comparing the concentration of the parent drug and metabolite measured with that added. The within-day reproducibility of the method was assessed by measuring the concentration of dexfenfluramine and nordexfenfluramine in six (500 ng/ml) or eight (1000 ng/ml) identical samples extracted and analysed on a single day. To ensure that results obtained on different days could be directly compared, the between-day reproducibility was determined by analysing qual-

ity control samples specifically prepared. These control samples were prepared by adding dexfenfluramine and nordexfenfluramine to drug-free urine, and after thorough mixing, storing the samples in 1.1-ml aliquots at −20°C. Two quality control samples of different concentrations were thawed and assayed on each day that samples were analysed. The bias of the assay was calculated as the percentage difference of the concentration of the quality control samples assayed relative to that added.

*Human studies*

Urine samples were obtained at intervals of 0–2, 2–3, 3–4, 4–5, 5–7, 7–9, 9–11, 11–13, 13–24, 24–36, 36–48, 48–60, 60–72, 72–84 and 84–96 h post-dose from healthy volunteers administered single 30-mg doses of dexfenfluramine hydrochloride. After the urine volume and pH were measured, aliquots of each urine sample were stored at −20°C pending analysis. These studies were approved by the Medical Research Ethics Committee of Royal North Shore Hospital.

## RESULTS AND DISCUSSION

The present method was developed to measure the concentrations of dexfenfluramine and nordexfenfluramine in human urine by high-performance liquid chromatography. Fenfluramine concentrations have previously been measured using gas chromatography [3–8], and thus this new technique will be of interest to laboratories equipped for HPLC but not GC.

A rapid and straightforward technique was developed using a standard $C_{18}$ reversed-phase HPLC column, an isocratic mobile phase and ultraviolet detection. The column initially investigated, the 10-$\mu$m $C_{18}$, was found to separate dexfenfluramine and nordexfenfluramine well when simple mobile-phase mixtures of acetonitrile and phosphoric acid (0.025%) were investigated. The peak shape of both analytes was improved when a phosphate solution (50 m*M*) rather than phosphoric acid was used. The acetonitrile content of the mobile phase was selected in order to optimise the chromatogaphic run time (Fig. 2).

On the basis of ultraviolet absorption spectra of dexfenfluramine obtained in the mobile phase selected, maximum sensitivity was anticipated at low wavelengths. Monitoring at 210 nm was chosen as an appropriate compromise between sensitivity and selectivity. A signal-to-noise ratio of 7:1 was observed for 10 ng of dexfenfluramine on column. Preliminary investigations were also undertaken to establish whether the native fluorescence of dexfenfluramine could be utilised for quantitation. At the optimal conditions identified (excitation 260 nm; emission 305 nm) the signal-to-noise ratio at 10 ng dexfenfluramine on column was 4:1 (RF-535 Fluoresence HPLC Monitor, Shimadzu Corporation, Kyoto, Japan). Ultraviolet absorption was thus selected as the more sensitive detection technique. The sensitivity achieved is appropriate to measure dexfenfluramine and nordexfenfluramine in urine samples from healthy volunteers after single-dose dexfenfluramine administration. However, as peak plasma concentrations of dexfenfluramine and nordexfenfluramine in healthy volunteers after a 30-mg dose have been reported to be 23 and 9 ng/ml, respectively [8], the present method is not of adequate sensitivity for human plasma analyses. The gas chromatographic technique utilising nitrogen specific detection described by Richards *et al.* [8] does have appropriate sensitivity for this matrix.

Chromatograms of extracts of drug-free urine and from a healthy volunteer after administration of dexfenfluramine are shown in Fig. 2. The peak shapes of both the parent drug and metabolite using the chromatographic conditions described were good and the retention times of nordexfenfluramine and dexfenfluramine were 8.6 min and 13.5 min, respectively. The overall analysis run time was, however, lengthened to 25 min in order to ensure that a late eluting peak observed in the urine of some healthy subjects (Fig. 2C) did not interfere with subsequent analyses. The chromatographic technique described can be readily automated and thus even with the longer analysis time, over 25 unknown samples can be assayed in addition to the appropriate control samples daily. No interfering peaks from endogenous substances were apparent at the retention

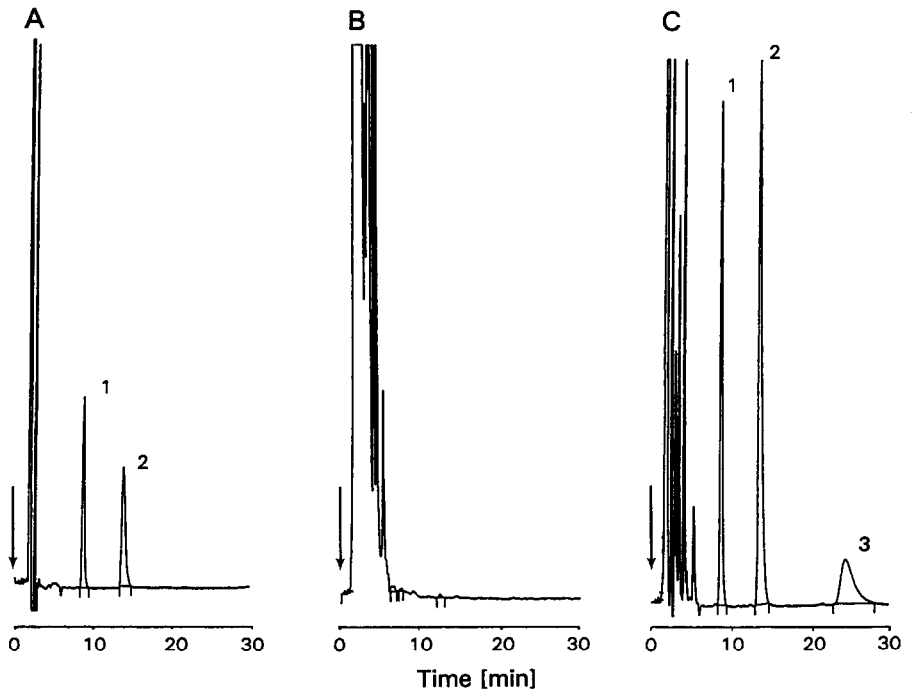


Fig. 2. Representative chromatograms of (A) standard solutions of nordexfenfluramine (1) and dexfenfluramine (2) : 100 ng of each on column, (B) extract of drug-free urine, and (C) extract of urine from a healthy volunteer collected over a 2-h interval from 7 to 9 h after ingestion of 30 mg dexfenfluramine. The concentrations of nordexfenfluramine (1) and dexfenfluramine (2) are 282 ng/ml and 503 ng/ml, respectively. An unidentified, late-eluting peak (3) was observed in some subjects.

time of dexfenfluramine in any urine sample investigated. However, in urine samples from some healthy volunteers an endogenous peak eluted close to the retention time of nordexfenfluramine. Baseline resolution of the peaks from the unidentified endogenous substance and nordexfenfluramine could be readily achieved by decreasing the concentration of acetonitrile in the mobile phase from 25% to 22%.

The technique used to extract dexfenfluramine and nordexfenfluramine from urine was both straightforward and rapid. Diethyl ether has been used previously to quantitatively extract fenfluramine [4,7,8]. The subsequent back extraction into 0.5 *M* sulphuric acid, as used previously as part of the sample extraction prior to GC analysis [4,7], was also quantitative as the overall recovery observed by comparing the peaks areas obtained from extracts with those of standards injected directly on-column was 96% for dexfenfluramine (250 ng/ml, $n = 6$) and 99% for nordexfenfluramine (250 ng/ml, $n = 6$). The lowest concentration routinely prepared for the standard curve was 25 ng/ml of dexfenfluramine hydrochloride and 25 ng/ml nordexfenfluramine hydrochloride. Dexfenfluramine and nordexfenfluramine were observed as peaks with signal-to-noise ratios of 8:1 in extracted urine samples containing 10 ng/ml of each analyte.

During assay development it was noted that the quality of the diethyl ether used was critical for ensuring that clean chromatograms were obtained. Extracting dexfenfluramine with other organic solvents including chloroform, dichloromethane, ethyl acetate and dichloromethane–isopropyl alcohol (90:10, v/v) was investigated when initial chromatograms with unwashed/distilled diethyl ether were not promising. However, the dexfenfluramine extraction recovery using these solvents was lower than that observed with purified diethyl ether. All diethyl ether used, therefore, was washed and distilled prior to use. The

relatively high concentration of $H_2SO_4$ (0.5 *M*) used for the back extraction ensured reproducible recovery over the entire range of dexfenfluramine concentrations studied. The low-pH solution used to inject the urine extracts did not have any adverse effect on the HPLC column as no deterioration in column performance was observed after assaying more than 600 urine samples for dexfenfluramine and nordexfenfluramine.

The method developed has good linearity over the whole range of concentrations measured. A typical standard curve for dexfenfluramine is $y = 1797x - 1664$ ($r^2 = 0.999$, $n = 12$) and for nordexfenfluramine $y = 1566x - 7280$ ($r^2 = 0.999$, $n = 12$). The accuracy of the determination of both dexfenfluramine and nordexfenfluramine was good and a bias of less than 8% was observed for the quality controls prepared (Table I). For samples prepared over the whole range of the standard curve, there was excellent agreement between the concentration added and that measured in urine ($y = 1.0x - 4.1$, $r = 0.999$, $n = 22$). The within-day and between-day reproducibilities for the determination of dexfenfluramine and nordexfenfluramine in urine are given in Table I. The within-day reproducibilities are less than 6%. The between-day reproducibilities at the lower concentration of both dexfenfluramine and nordexfenfluramine are higher, however, still acceptable.

The technique described does not use an internal standard. The reproducibility of the method was, however, not compromised (Table I), partly because back extraction rather than evaporation and reconsitution [3–5], was used to concentrate the initial extract. A number of compounds with appropriate chromatographic characteristics (including benzocaine and doxazosin) were investigated as possible internal standards, however, the reproducilility of the technique was not improved with their use.

Dexfenfluramine was developed as a stereochemically pure drug from the marketed racemate, fenfluramine. Stereospecific analytical techniques have therefore not been required to quantitate the single enantiomer and the method described would not resolve the enantiomers of fenfluramine. As chiral inversion of *dextro-* or *levo-* rotatory fenfluramine has not been observed [9], this method would be suitable for the analysis of *levo*-rotatory fenfluramine and its metabolite *levo*-norfenfluramine if administered in a stereochemically pure form. After administration of the racemate fenfluramine, the mixture of the enantiomers of the parent drug and metabolite would be measured in urine using the method developed. However, as the enantiomers of fenfluramine differ in their activity and disposition [2], meaningful data on the fate of racemic fenfluramine will only be obtained if the individual enantiomers are measured. A stereospecific method to analyse the individual enantiomers of fenfluramine in biological fluids after administration of the racemate has been reported [9].

TABLE I

WITHIN-DAY AND BETWEEN-DAY REPRODUCIBILITIES OF THE MEASUREMENT OF DEXFENFLURAMINE AND NORDEXFENFLURAMINE IN URINE

Two quality control (QC) samples were used to establish the between-run variability.

| Concentration (ng/ml) | *n* | Concentration assayed (mean ± S.D.) (ng/ml) | Bias (%) | C.V. (%) |
|---|---|---|---|---|
| *Dexfenfluramine:* | | | | |
| *Within-day* | | | | |
| 500 | 6 | 482 ± 21 | −3.6 | 4.4 |
| 1000 | 8 | 974 ± 39 | −2.6 | 5.6 |
| *Between-day (QC)* | | | | |
| 75 | 20 | 79 ± 12 | +5.3 | 15.0 |
| 1100 | 20 | 1171 ± 83 | +6.5 | 7.1 |
| *Nordexfenfluramine:* | | | | |
| *Within-day* | | | | |
| 500 | 6 | 507 ± 21 | +1.0 | 4.2 |
| 1000 | 8 | 983 ± 50 | −1.7 | 5.1 |
| *Between-day* | | | | |
| 120 | 20 | 115 ± 14 | −4.2 | 12.6 |
| 1500 | 20 | 1602 ± 106 | +6.8 | 6.6 |

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