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Note

Measurement of medifoxamine metabolites in urine by high-performance liquid chromatography

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Medifoxamine, a new monoamine reuptake inhibiting antidepressant drug [1,2], is structurally unrelated to other tricyclic antidepressants (Fig. 1). Animal studies have shown that medifoxamine is rapidly and almost totally absorbed and undergoes extensive biotransformation giving a large number of metabolites. N-Oxide, N-desmethyl and acidic metabolites (Fig. 1) were the major metabolites excreted in urine of different animal species [3]. Analytical studies in serum and urine of volunteers and of patients using a specific high-performance liquid chromatographic (HPLC) method [4] have shown very little of the parent drug, suggesting that it undergoes extensive hepatic extraction and metabolism [5].

Because of species and inter-individual differences in rates and routes of metabolism, it was necessary to investigate the biotransformation of medifoxamine in man. A method using HPLC and solvent extraction has been developed to measure four metabolites of meditoxamine in biological fluids and to study the metabolites of medifoxamine following intravenous administration.

EXPERIMENTAL

Standard samples

The medifoxamine metabolites were obtained from Anphar-Rolland Labs. (Paris, France). Standards were prepared from stock solutions of 100 mg/l in distilled water which were added to drug-free urine to given final concentra-

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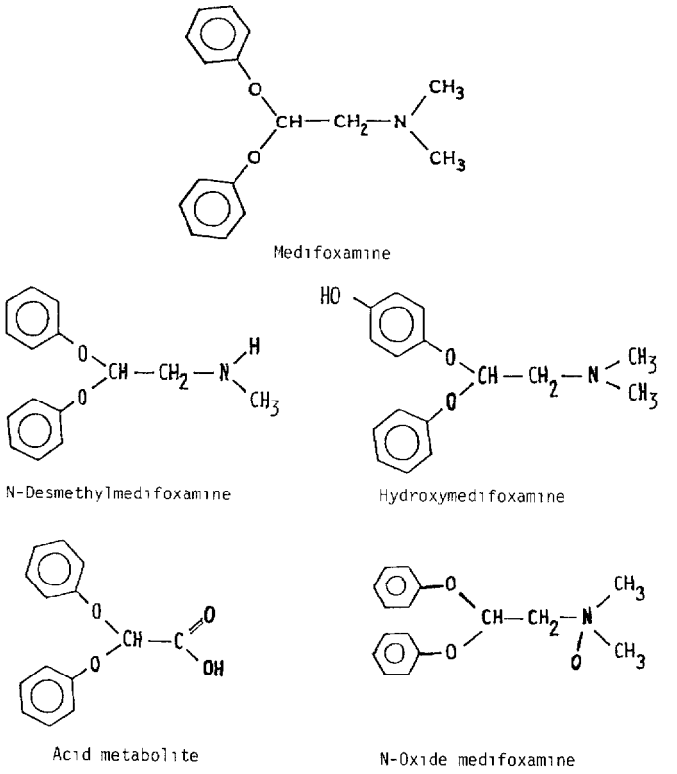

Fig. 1. Structures of medifoxamine and its metabolites.

tions of 0, 125, 250, 500, 1000, 2000, 5000 and 10 000 μg/l for acidic medifoxamine, N-oxide medifoxamine and hydroxymedifoxamine and 0, 25, 50, 100, 200, 400, 1000 and 2000 μg/l for N-desmethylmedifoxamine.

Sample extraction

To establish the most appropriate extraction conditions, the following solvents were studied under different acidic and alkaline conditions: ethyl acetate, diethyl ether, *n*-heptane, *n*-hexane, cyclohexane, butanol, *n*-butyl acetate and acetonitrile. The following method gave the best results. To a screw-capped glass tube containing 1 ml of sample were added 1 ml of borate–sodium hydroxide buffer (pH 10), 50 μl of aqueous internal standard solution (1 μg/l 5,6 benzoquinoline; Aldrich, Gillingham, U.K.) and 6 ml of ethyl acetate–diethyl ether (1:1) (HPLC grade, Rathburn, Walkerburn, U.K.). After 10 min of gentle shaking the tubes were centrifuged at 350 *g* (2000 rpm) for 5 min (Centaur 2MSE, Fisons Scientific Supplies, Loughborough, U.K.), and the organic layer

was decanted into a conical glass tube containing an antibump granule. The organic layer was evaporated to dryness using a hot plate at 70°C. The residue was redissolved in 120 μl of the HPLC mobile phase.

Chromatography

Several solvents were studied to find the most appropriate mobile phase, namely methanol, ethyl acetate, acetonitrile and water, using different buffers and pH values. The most appropriate mobile phase was found to be a mixture of 560 ml of methanol and 400 ml of phosphate buffer pH 8 (2.375 g Na_2HPO_4 + 0.135 g KH_2PO_4 dissolved in 400 ml distiled water) at a flow-rate 2 ml/min using a Shimadzu LC-6A liquid chromatography pump (Dyson Instruments, Tyne and Wear, U.K.). The analytical column used was a 150 mm × 5 mm I.D. stainless-steel column at room temperature containing 5-μm Hypersil silica 5 ODS (Hichrom, Reading, U.K.). The effluent was monitored using a Spectromonitor III Model 1204A UV detector (Laboratory Data Control, Milton Roy U.K., Staffordshire, U.K.) spectrometer at 266 nm connected to a Trio integrator recorder. Under these conditions the retention times were approximately 1.6 min for the acidic derivative, 3.7 min for the N-oxide derivative, 4.6 min for the hydroxy derivative, 5.7 min for the internal standard, 9.4 min for the N-desmethyl derivative and 15.6 min for medifoxamine.

Enzymatic hydrolysis

β-D-Glucuronide glucuronidase hydrolase from *Helix pomatia* was used (Sigma, Poole, U.K.). The crude solution was diluted with 0.2% sodium chloride immediately before use to produce a 1000 U/ml glucuronidase enzyme solution (1 U will liberate 1 μg of phenolphthalein from phenolphthalein glucuronide per hour at pH 5 at 37°C). Sodium acetate buffer pH 5 (0.1 *M*) was prepared by dissolving 681 mg of sodium acetate trihydrate in 40 ml of deionized water, equilibrating at 37°C and adjusting to pH 5 with 1 *M* hydrochloric acid. This was then diluted to a final volume of 50 ml with deionized water. To a glass tube containing 1 ml of urine sample were added 1 ml of sodium acetate buffer and 100 μl of β-glucuronidase enzyme solution. After a gentle shaking the tubes were incubated for 24 h at 37°C using a water bath (Kerry Ultrasonic, Hitchin, U.K.).

Data analysis

Medifoxamine metabolite concentrations were calculated from peak-height ratios of each metabolite and internal standard versus concentrations of the calibration standards using the linear regression software program Lincal.

Reproducibility of the method

Precision was quantified by calculating the coefficient of variation using ten aliquots of the same sample.

RESULTS

Using this procedure, medifoxamine metabolites and internal standard gave a chromatogram with well shaped peaks and short retention times. There was no interference from other UV-absorbing endogenous substances. A chromatogram of medifoxamine metabolites and internal standard in urine are shown in Fig. 2. The amount of metabolites in the lipid organic phase was determined to establish the most appropriate solvent medium for extraction purposes. The efficiency of the extraction method was 89% for acidic medifoxamine, 76% for N-oxide medifoxamine, 94% for hydroxymedifoxamine and 97% for N-desmethylmedifoxamine ($n=10$) at concentrations of 250 μg/l.

Linear regression of peak-height ratios against medifoxamine concentrations typically gave correlation coefficients of more than 0.99 for the range tested. The detection limits were 10 μg/l for N-oxide medifoxamine, 10 μg/l for hydroxymedifoxamine, 5 μg/l for acidic medifoxamine and 30 μg/l for N-desmethylmedifoxamine. The results of the coefficient of variation of the analytical method are summarised in Table I.

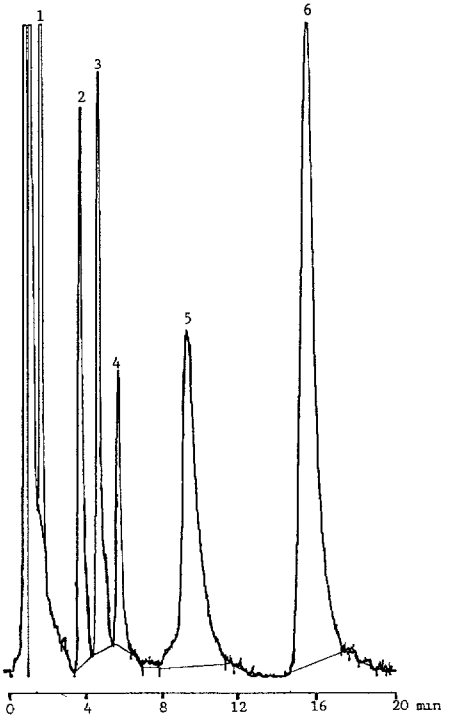

Fig. 2. Standard chromatogram of (1) acid medifoxamine (1.5 mg/l), (2) N-oxide medifoxamine (4 mg/l), (3) hydroxymedifoxamine (4 mg/l), (4) internal standard (0.1 mg/l), (5) N-desmethylmedifoxamine (2.5 mg/l) and (6) medifoxamine (5 mg/l) extracted from urine.

TABLE I

REPRODUCIBILITY OF THE METHOD FOR THE THREE METABOLITES

The values in parentheses are coefficients of variation (%).

Spiked concentration (μg/l)	Mean concentration found (μg/l)		
	N-Desmethylmedifoxamine	Hydroxymedifoxamine	N-Oxide medifoxamine
50	58 (22.4)	54 (8.6)	48 (8.4)
125	121 (13.7)	128 (5.3)	123 (19.1)
250	247 (12.1)	253 (4.1)	255 (6.0)
1000	1022 (9.3)	1008 (1.8)	997 (2.8)

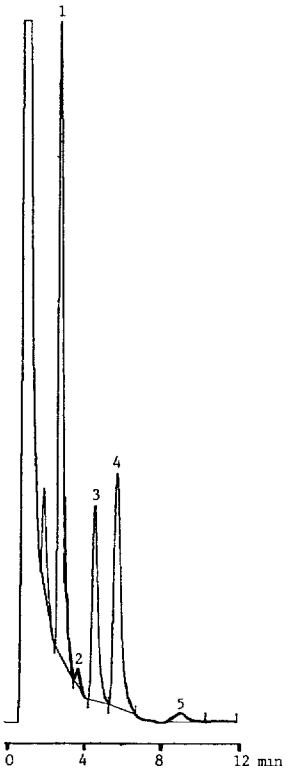

Fig. 3. Chromatogram of medifoxamine metabolites after extraction from urine of subject who received 50 mg intravenous medifoxamine. Peaks: 1 = unknown metabolite; 2 = N-oxide medifoxamine (729 μg/l); 3 = hydroxymedifoxamine (480 μg/l); 4 = internal standard; 5 = N-desmethylmedifoxamine (312 μg/l).

Following an intravenous tolerance study of medifoxamine (5–100 mg), we took the opportunity to collect the urine from 0 to 3 h after administration, for estimation of medifoxamine metabolites using this method.

The amount of N-desmethylmedifoxamine and N-oxide medifoxamine excreted in urine was each less than 2% of the dose administered. Hydroxymedifoxamine excreted was less than 8% of the dose. The majority of both the hydroxy and N-desmethyl metabolites of medifoxamine were excreted in the conjugated form (>90%) but the N-oxide was excreted unchanged. The amounts of these metabolites were directly proportional to the dose given. No acidic medifoxamine metabolite was detected in the urine.

Under these condition, another unknown metabolite was detected in the urine samples with retention time 2.5 min (Fig. 3). The peak height of this metabolite was higher than any other metabolite peak, and when we calculated the concentration using the response factor of the N-oxide, and amount of drug excreted (as conjugate) was up to 37% of the doses administered and the amount excreted was directly proportional to the dose.

CONCLUSION

The method described allows the measurement of four metabolites of medifoxamine in urine with a run time of less than 10 min (excluding medifoxamine). The method is selective, rapid, simple and utilizes widely available equipments and reagents. After 3 h the percentage of drug excreted as known metabolites was less than 11% of the given dose. We are trying to identify the structure of an unknown metabolite.

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