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Note

High-performance liquid chromatographic assay of the metabolites of thymoxamine

P. DUCHENE, J. LE DILY and M. BROMET-PETIT

Biosciences, B.P. 6025, 45100 Orléans 2 (France)

and

J. MOSSER* and C. FENIOU

Laboratoires Sarget, B.P. 100, 33701 Mérignac Cedex (France)

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Thymoxamine, 4-(2-dimethylaminoethoxy)-5-isopropyl-2-methyl phenylacetate, is an α-adrenergic blocking agent which acts by competing with noradrenaline at receptor sites [1]. Thymoxamine is rapidly and completely hydrolysed in the body to deacetylthymoxamine (M_1), which is demethylated to give N-monomethyldeacetylthymoxamine (M_2). These two metabolites are further metabolized to their glucuronide and sulpho derivatives (Fig. 1).

In human pharmacokinetic investigations of thymoxamine, a sensitive and simple method for the determination of its metabolites in biological fluids is needed. Several existing methods based on thin-layer chromatography [2] or fluorescence [3] are not appropriate for the study of human pharmacokinetic parameters. Geahchan and Chambon [4] determined M_1 and M_2 in plasma by using a reversed-phase high-performance liquid chromatographic (HPLC) system with fluorescence detection after extraction, but none of the conjugated metabolites were determined by this method and the sensitivity did not seem to be sufficient for human studies. As the pharmacological activity is due to both conjugated and unconjugated metabolites [5], with conjugated metabolites representing more than 98% of the total plasma metabolites, for pharmacokinetic studies it is necessary to develop a sensitive and specific method to measure the plasma concentration of all these metabolites.

Hengy et al. [6] described a gas–liquid chromatographic method determining M_1 and M_2 and their sulpho conjugates. The technique for necessitates the use

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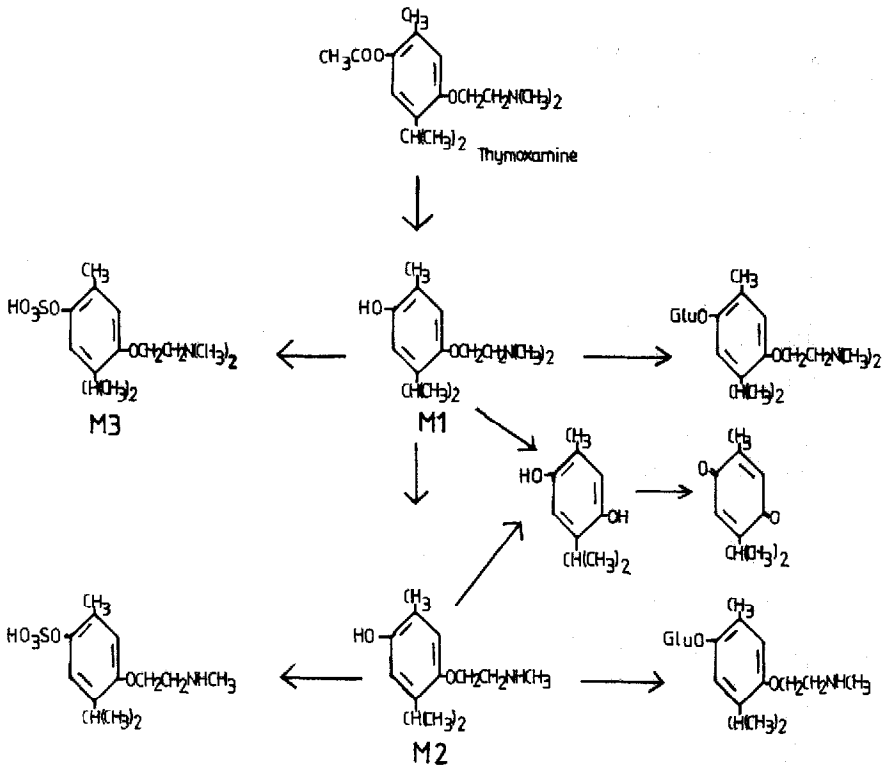

Fig. 1. Proposed scheme for the metabolism of thymoxamine in rat and humans.

of 2 ml of plasma and requires the formation of a silylated derivative coupled with a thermoionic detector.

We have developed an HPLC method using 1 ml of plasma for the rapid, accurate and sensitive determination of free and conjugated M_1 and M_2.

EXPERIMENTAL

Reagents and chemicals

Thymoxamine, M_1, M_2, sulphodeacetylthymoxamine (M_3) and 4-[2-(ethylmethylamino)ethoxy]-2-methyl-5-(1-methylethyl)phenol (EMMP), used as an internal standard, were supplied by Sarget Labs. (Bordeaux, France) and were all of laboratory quality. Acetonitrile (far-UV grade), diethyl ether, cyclohexane and hydrochloric acid (Carlo Erba, Milan, Italy), dichloromethane, sodium hydroxide, orthophosphoric acid, sodium carbonate and sodium hydrogen carbonate (Merck, Darmstadt, F.R.G.) and heptanesulphonic acid (Kodak, Touzart et Matignon, Vitry sur Seine, France) were of analytical-reagent grade. 4-(2-Dimethylamino-[^{14}C]-1-ethoxy)-5-isopropyl-2-methylphenyl acetate hydrochloride ([^{14}C]thymoxamine), 7 mCi/mmol, with a radiochemical purity superior to 97%, was obtained from Centre d'Etude Atomique (Saclay, France).

Chromatography

The HPLC system consisted of a Model 6000A pump (Waters Assoc., Milford, MA, U.S.A.), a Model 710B WISP injector (Waters Assoc.) coupled to a Schoeffel (Model FS 970) variable-wavelength spectrofluorimeter, operated at 198 nm for excitation (without emission filter) (Cunow, Clichy, France), and a Linseis L650 recorder (Linseis, Saint Cloud, France).

The column (25 cm×4 mm I.D.) was packed with Spherisorb RP-18 (5 μm) (Beckman, Paris, France). The working pressure of the pump was 175 bar at a flow-rate of 1 ml/min. The eluent composition was acetonitrile–water–heptanesulphonic acid solution (5.5 g of heptanesulphonic acid in 100 ml of water with 50 μl of orthophosphoric acid) (40:54:6). A 1.36-g amount of potassium dihydrogen phosphate was added to each litre of eluent.

Extraction procedure

A 1-ml volume of plasma, 100 ng of internal standard solution (1 mg/l EMMP in methanol) and 1 ml of 2 *M* hydrochloric acid were placed in a 10-ml glass tube. The contents were mixed for 30 s on a Whirlimixer, then heated at 120°C for 1 h. After cooling the mixture at room temperature (about 1 h), the pH was adjusted to 7–8 with about 1 ml of 2 *M* sodium hydroxide; 1 ml of 0.5 *M* carbonate buffer (pH 10) and 5 ml of dichloromethane were added, and the mixture was shaken for 15 min. After centrifugation at 4°C, the aqueous phase was discarded, 0.5 ml of 1 *M* hydrochloric acid and 2 ml of water were added, and the mixture was shaken for 10 min. The aqueous phase was transferred into another glass tube, then washed with 5 ml of diethyl ether by shaking for 15 min.

The organic layer was removed after centrifugation and the pH of the aqueous phase was increased to 7–8 with about 0.5 ml of 1 *M* sodium hydroxide and 1 ml of 0.5 *M* carbonate buffer (pH 10) was added. The metabolites of thymoxamine were then extracted twice with 4 ml of cyclohexane. The combined cyclohexane layers were evaporated to dryness under a gentle stream of nitrogen at 40°C.

The residue was dissolved in 200 μl of mobile phase, and 10–100 μl were injected into the liquid chromatograph. The parent drug can be measured by using this method, but without the hydrolysis step (2 *M* hydrochloric acid, 120°C, 1 h).

Measurement of radioactivity

The total radioactivity in plasma samples was determined by liquid scintillation counting. Each sample was supplemented with 4 ml of Aqualuma Plus (Radiomatic, La Queue les Yvelines, France), and the radioactivity was measured in a scintillation spectrometer (Intertechnique, Plaisir, France).

RESULTS AND DISCUSSION

Hydrolysis

During the metabolism studies, the enzymic hydrolysis of conjugated metabolites by using juice of *Helix pomatia* did not result in the complete cleavage of the sulphate metabolites. Therefore, we attempted to achieve chemical hydrolysis with hydrochloric acid. To increase the reproducibility of the method, a technique

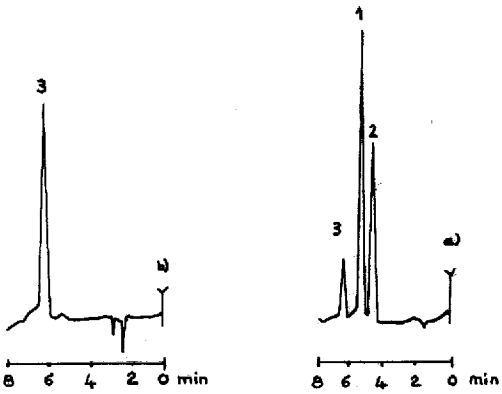

Fig. 2. Chromatograms of hydrolysed and extracted human plasma obtained after oral administration of 60 mg of thymoxamine. (a) HPLC separation of 19 ng of M_1 (1), 15 ng of M_2 (2) and 5 ng of internal standard (I.S.) (3); 10 μl injected from 200 μl of residue (1.5 h after dose). (b) Chromatogram of blank human plasma; 70 μl injected from 200 μl of residue (24 h after dose).

was used that allowed us to obtain complete hydrolysis of M_1 and M_2. The ratio of the hydrolysis used during the assay was determined for M_3, and converted to 100% of M_1. The results for both conjugates can be used for pharmacokinetic studies because the percentage metabolite composition in human and rat plasma and urine after administration of thymoxamine does not show significant changes [5,7]. We determined the percentage of sulpho-conjugated derivatives of M_1 and M_2 in plasma by using the hydrolysis described by Hengy et al. [6] for several subjects various times after administration of thymoxamine, and obtained consistent results, viz., $31.0 \pm 8.5\%$ ($n=17$) for M_1 and $80.9 \pm 16.8\%$ ($n=15$) for M_2.

Extractions

A succession of extractions were carried out using different solvent systems to extract M_1 and M_2 to obtain the highest specificity possible, in order to prevent the risk of interference during the HPLC analysis of a large number of biological samples.

Chromatograms

Fig. 2 shows a chromatogram of human plasma obtained 1.5 h after oral administration of 60 mg of thymoxamine (Carlytene) and a chromatogram obtained 24 h after dosing. The retention volumes are: M_1, 5.6 ml; M_2, 4.8 ml; I.S., 6.7 ml.

Linearity

Standard plasma samples obtained by adding different amounts of M_1 and M_2 to plasma were hydrolysed and M_1 and M_2 were determined by using the technique described here. The correlation between the peak-height ratio (M_1/I.S., M_2/I.S.) and the concentration was determined. M_1 and M_2 showed linearity from 5 to 2000 ng ml^{-1} ($r=0.999$ for M_1 and M_2).

TABLE I

BETWEEN-DAY REPRODUCIBILITY AND RECOVERY OBTAINED FOR THE ASSAY OF FIVE SPIKED HUMAN PLASMA SAMPLES AT TWO LEVELS OF CONCENTRATION

Metabolite	Added (ng ml^{-1})	Found (ng ml^{-1})	Mean ± S.D. (ng ml^{-1})	C.V.* (%)	Recovery (%)
M_1	50	47, 50, 43, 45, 50	47 ± 3.1	6.6	94
	500	487, 486, 492, 489, 469	485 ± 9.0	1.9	97
M_2	50	58, 58, 50, 50, 53	54 ± 4.0	7.4	108
	500	470, 485, 508, 463, 444	474 ± 24.1	5	95

*C.V. = coefficient of variation.

TABLE II

HUMAN PLASMA CONCENTRATIONS OF M_1 PLUS M_2, AFTER ADMINISTRATION OF 60 mg OF [^{14}C]THYMOXAMINE (50 μCi) AND DETERMINED BY TWO DIFFERENT METHODS (RESULTS EXPRESSED AS EQUIVALENT M_1)

B.D. = below the detection limit (20 ng ml^{-1} for the radioactivity method and 5 ng ml^{-1} for the HPLC method.

Time after administration (h)	$M_1 + M_2$ concentration (ng ml^{-1})	
	Radioactivity	HPLC
0	B.D.	B.D.
0.25	494	503
1	1052	1188
1.5	826	742
2	555	419
3	302	370
4	173	291
7	30	46
10	B.D.	19
24	B.D.	B.D.
34	B.D.	B.D.

Reproducibility and recovery

Human plasma was spiked with known amounts of M_1 and M_2 and five samples at each concentration were hydrolysed, then extracted and measured. Table I shows the added and measured concentrations, demonstrating good reproducibility. The recoveries were calculated using a calibration graph constructed from the assay of plasma samples in the same concentration range.

Selectivity

In order to detect any interferences, several plasma samples obtained from subjects who had not taken any drug were analysed. No peaks were visible at the retention volumes of M_1, M_2 or the internal standard.

Limit of detection

For 1 ml of plasma the lowest measurable concentration was 5 ng ml^{-1} for both metabolites.

Storage of samples

The stability of human plasma samples stored in darkness at $-18°C$ has been checked over a period of two years.

Validation of the method

The method was applied to the determination of plasma concentrations of M_1 and M_2 after oral administration of 60 mg of [^{14}C]thymoxamine (50 μCi) to human subjects. The plasma concentrations were determined by measurement of the total radioactivity in the plasma samples and by the method described here, and the results obtained were compared. No parent drug was found and the results in Table II show no significant differences between the results obtained by the two methods ($p=0.05$).

It can be concluded that this HPLC method using only 1 ml of plasma is a single and accurate procedure and can be used to study the pharmacokinetics of thymoxamine in man.

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