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DETERMINATION OF THE MAJOR METABOLITE OF PHENTOLAMINE IN HUMAN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A reversed-phase, high-performance liquid chromatographic method using UV detection is described for the assay of the major metabolite of phentolamine in plasma and urine before or after enzymatic hydrolysis. Plasma is deproteinized with methanol. The sensitivity limit is 200 ng/ml using 150-µl samples. Urine is either diluted with water or purified after enzymatic hydrolysis. Concentrations down to 2—3 µg/ml could be quantified with acceptable precision. This method was applied to plasma and urine samples from subjects given phentolamine.

INTRODUCTION

Phentolamine (Regitine®, Ciba-Geigy) or 2-[N-(*p*-tolyl)-N-(*m*-hydroxyphenyl)-aminomethyl]-2-imidazoline (Fig. 1) is a sympathicolytic agent. Very low plasma levels are reached after oral administration and less than 2% of the dose is recovered unchanged in urine over 24 h [1]. The metabolites in the urine accounted for about 60% of the administered dose [2]. The main one was identified as compound I, 2-[N-(*p*-carboxyphenyl)-N-(*m*-hydroxyphenyl)-aminomethyl]-2-imidazoline (Fig. 1). This compound is amphoteric and therefore difficult to extract from biological materials using conventional solvent extraction. A high-performance liquid chromatographic (HPLC) technique using an aqueous mobile phase and bonded silica gel packing material was developed for its assay in urine and plasma. This technique permitted the possible conjugation of I to be investigated.

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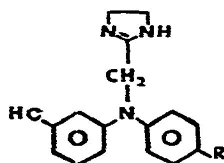


R = CH_3 : PHENTOLAMINE

R = COOH : I

Fig. 1. Phentolamine and its major metabolite.

EXPERIMENTAL

Chemicals

Phentolamine and its metabolite were supplied by Ciba-Geigy (Basle, Switzerland).

β-Glucuronidase—arylsulphatase solution (Helix pomatia) was purchased from Calbiochem (Los Angeles, CA, U.S.A.). One ml of the enzyme solution is diluted with 10 ml of pH 5.5 buffer (sodium acetate—acetic acid).

Apparatus

Chromatography is performed on a Hewlett-Packard Model 1082A instrument, equipped with a fixed-wavelength detector (254 nm) and a loop injector (Rheodyne, Model 7120, Berkeley, CA, U.S.A.).

Column

Stainless-steel columns are used.

For urine, the column (25 cm × 4.7 mm I.D.) is filled with LiChrosorb RP-18 or RP-8, 10 μm (Merck, Darmstadt, G.F.R.), using the balanced-density-slurry packing technique.

For plasma, the column (10 cm × 4.7 mm I.D.) is filled with LiChrosorb RP-8, 5 μm. A precolumn (10 cm × 4.7 mm I.D.) filled with Permaphase ODS (Du-Pont, Wilmington, DE, U.S.A.) is used to protect the analytical column.

Chromatography

The mobile phase and the column are at room temperature.

Urine. The degassed mobile phase $2.6 \cdot 10^{-3}$ *M* orthophosphoric acid—acetonitrile (60:40, v/v) is used at a flow-rate of 3 ml/min.

Plasma. The degassed mobile phase pH 4 buffer (hydrochloric acid—sodium citrate)—acetonitrile (85:15, v/v) is used at a flow-rate of 2 ml/min.

Sample preparation

Precipitation of proteins from plasma. Plasma, 150 μl, 50 μl of distilled water, and 700 μl of methanol, are mixed into a 10-ml conical glass tube. The tube is cooled and then centrifuged for 10 min at 2000 *g*. A 400-μl aliquot of the supernatant is transferred into a 5-ml glass ampoule and evaporated to dryness under nitrogen at room temperature. Distilled water (300 μl) is added to

the residue (method 1). The ampoule is shaken for a few seconds on a Vortex mixer and 50 μl are injected into the column. For concentrations of metabolite lower than 0.50 μg/ml, 600 μl of supernatant are evaporated and 200 μl of distilled water are added to the residue (method 2) and 50 μl are injected.

Urine dilution without enzymatic hydrolysis. Urine, 100 μl, and 900 μl of distilled water are mixed into a 10-ml glass tube; 40 μl are injected.

Urine dilution with enzymatic hydrolysis. Urine, 1 ml, 1 ml of water and 500 μl of enzyme solution are mixed into a 10-ml glass tube and heated for 15 h at 38°C. Hydrolysate, 500 μl, and 1500 μl of methanol are mixed into a 10-ml conical glass tube. The tube is cooled and centrifuged at 2000 *g* for 10 min. A 1-ml aliquot of supernatant is transferred into a 2-ml glass ampoule and evaporated to dryness under nitrogen at room temperature. The dry residue is dissolved in 1 ml of pH 10.7 buffer (sodium carbonate—hydrogen carbonate) and extracted with 1 ml of chloroform. A 500-μl aliquot of the aqueous phase and 500 μl of $36 \cdot 10^{-3}$ *M* phosphoric acid are mixed into a 2-ml glass ampoule. The aqueous phase is washed with 1 ml of dichloroethane; then 40 μl of the upper aqueous phase are injected.

Calibration curves

Calibration samples are prepared by adding aliquots of different aqueous solutions of the metabolite to plasma and urine. A calibration curve is established every day.

RESULTS AND DISCUSSION

Recovery and precision

Spiked plasma and urine samples were prepared and analysed several times. The results summarized in Tables I and II show that the described method permits the accurate and precise determination of the metabolite of phentolamine at concentrations down to 0.2 μg/ml in plasma and 2—3 μg/ml in urine.

TABLE I

PRECISION AND RECOVERY IN SPIKED PLASMA SAMPLES

Concentration added (μg/ml)	Within-day reproducibility			Day-to-day reproducibility		
	Concentration found (μg/ml) (mean of *n* replicates)	Coefficient of variation (%)	Mean recovery (%)	Concentration found (μg/ml) (mean of *n* replicates)	Coefficient of variation (%)	Mean recovery (%)
0.20	0.20 (9)	8.8	101			
0.54	0.60 (8)	7.35	112	0.55 (8)	9.2	101
1.1	1.0 (10)	4.8	91	1.1 (10)	6.6	98

TABLE II

PRECISION AND RECOVERY IN SPIKED URINE SAMPLES

	Concentration added (μg/ml)	Within-day reproducibility			Day-to-day reproducibility		
		Concentration found (μg/ml) (mean of n replicates)	C.V. (%)	Mean recovery (%)	Concentration found (μg/ml) (mean of n replicates)	C.V. (%)	Mean recovery (%)
Without hydrolysis	2.2	2.15 (8)	6.25	96			
	4.7	5.0 (9)	6.4	106	4.8 (12)	4.6	101
	18.9	18.9 (9)	2.7	100	19.0 (12)	2.0	100
With hydrolysis*	3.0	3.2 (10)	9.5	105			
	9.1	9.2 (9)	6.0	101	9.2 (8)	7.9	100
	12.2	12.2 (9)	5.3	100	12.0 (7)	5.4	99

*Urine spiked with the unconjugated compound.

Specificity

Plasma. The metabolite of phentolamine is well separated from the plasma components (Fig. 2). The parent drug, phentolamine, which is eluted as a large peak within 50 min, did not interfere since the concentrations reached after administration are in the nanogram range [1].

Urine. The metabolite of phentolamine is well separated from the urine components with or without hydrolysis. No interference was observed using urines from different volunteers. The parent drug is eluted within 20 min and does not interfere.

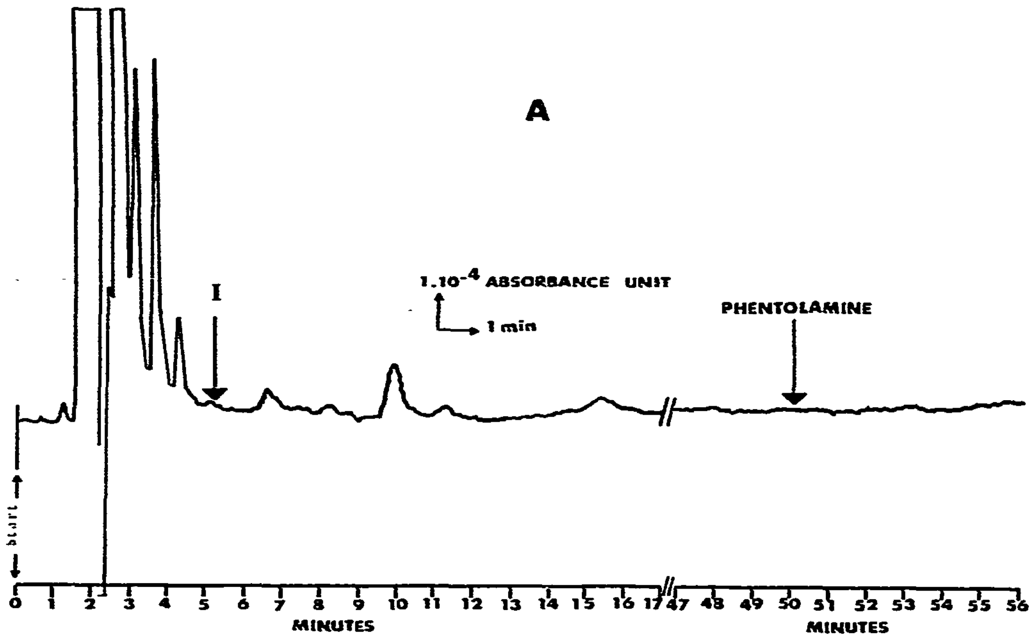
Stability

The metabolite of phentolamine remains stable in frozen urine samples for at least three months.

Application

The present method was used to determine the urinary excretion of the metabolite of phentolamine after the administration of single intravenous and oral doses of phentolamine to volunteers. The corresponding urinary excretion of the parent drug has been also determined [1]. The results summarized in Table III indicate that phentolamine is metabolized to a greater extent after oral than after intravenous administration. About 40% of the given dose was eliminated in the urine in the form of metabolite I within 24 h after oral administration (Table III).

The plasma concentrations and the urinary excretion of the metabolite were also determined in a patient receiving chronic treatment with phentolamine (oral doses of 400 mg daily). The data in Table IV indicate that the metabolite



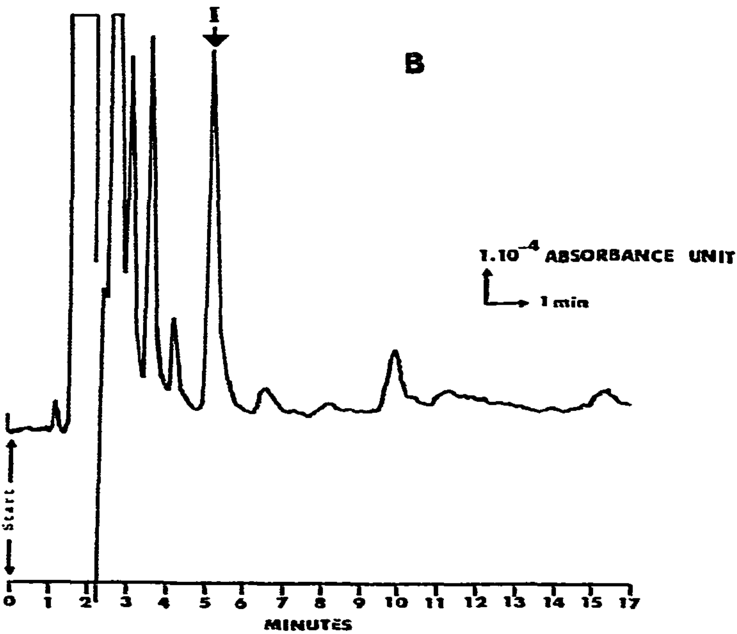


Fig. 2. Chromatograms of (A) blank human plasma and (B) spiked with the metabolite (method 1, see Sample preparation).

TABLE III

URINARY EXCRETION OF PHENTOLAMINE AND ITS MAJOR METABOLITE AFTER SINGLE INTRAVENOUS AND ORAL ADMINISTRATIONS

Mean values from 3 volunteers

	Dose			
	10 mg intravenous		20 mg oral	
	Phentolamine	I	Phentolamine	I
Total urinary excretion* (% of the dose)	15.3	16.8	8.0	41.6
Unconjugated fraction (% of total)	85	100	22.5	96.5

*Free + conjugated compound.

TABLE IV

PLASMA CONCENTRATIONS AND URINARY EXCRETION OF PHENTOLAMINE METABOLITE DURING REPETITIVE ADMINISTRATION

Plasma		Urine		
Time after administration (h)	Concentration (μg/ml)	Time interval	Amount excreted (mg)	Per cent of the dose
0	4.6	6 p.m.—6 a.m.	79.0	17.8
1	5.3	6 a.m.—6 p.m.	96.2	21.7
4	4.6	24 h	175.2	39.5

seems to accumulate in the plasma during such treatment. As after the administration of a single oral dose, about 40% of the dose was eliminated in the urine over 24 h in the form of the unconjugated metabolite (Table IV).

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