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GAS CHROMATOGRAPHIC DETERMINATION OF PHENTOLAMINE (REGITINE®) IN HUMAN PLASMA AND URINE

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SUMMARY

A method for the determination of unconjugated phentolamine at concentrations down to 5 ng/ml in human plasma, and of free and total (free plus conjugated) phentolamine down to 25 ng/ml in urine is described. After addition of 2-[N-(*p*-chlorophenyl)-N-(*m*-hydroxyphenyl)-aminomethyl]-2-imidazoline as internal standard, both compounds are extracted into benzene—ethyl acetate (1:1, v/v) at pH 10, transferred into an acidic aqueous solution and back-extracted at pH 10 into benzene—ethyl acetate. They are then derivatized with N-heptafluorobutyrylimidazole. The derivatives are determined by gas chromatography using a ^{63}Ni electron-capture detector. In urine, total (free plus conjugated) phentolamine is determined after enzymatic hydrolysis. The technique was applied for the study of the plasma concentrations and urinary elimination after oral administration to man.

INTRODUCTION

Phentolamine (Regitine®, Ciba-Geigy) (Fig. 1, I), 2-[N-(*p*-tolyl)-N-(*m*-hydroxyphenyl)-aminomethyl]-2-imidazoline is a sympathicolytic agent. Its determination in blood and urine was described by De Bros and Wolshin [1]. They used a high-performance liquid chromatographic (HPLC) method on a reversed-phase column with an ion-pairing reagent. Pfister and Imhof [2] estimated the plasma concentration of phentolamine on the basis of its inhibitory effect on adrenaline-induced platelet aggregation.

This paper describes a gas chromatographic determination of free and conjugated phentolamine in human plasma and urine, which has been in use in our laboratory since 1974.

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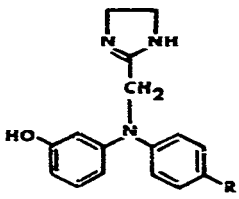

R = -CH_3 (I)
= -Cl (II)
= -COOH (III)

Fig. 1. Chemical structures of phentolamine (I), internal standard (II) and main phentolamine metabolite (III).

EXPERIMENTAL

Chemicals and reagents

Phentolamine and the internal standard (Fig. 1, II) were supplied by Ciba-Geigy (Basle, Switzerland). The solvents and reagents used are all of analytical grade: sulphuric acid (Titrisol, No. 9984, Merck, Darmstadt, G.F.R.), *n*-heptane (Merck 4366), N-heptafluorobutyrylimidazole, HFBI (Pierce, Rockford, IL, U.S.A.), benzene (No. 1043, Mallinckrodt, St. Louis, MO, U.S.A.) and ethyl acetate (Mallinckrodt 3427).

The extraction solvent is benzene—ethyl acetate (1:1, v/v). The acetate buffer, pH 5.5 is prepared with 4.8 ml of 0.2 *M* acetic acid solution and 35.2 ml of 0.2 *M* sodium acetate solution.

The pH 10 buffer (Titrisol, Merck 9890, boric acid—potassium chloride—sodium hydroxide) is prepared by diluting the contents of eight vials with water to a volume of 1000 ml.

The enzyme solution (β-glucuronidase—arylsulphatase) [Calbiochem (Los Angeles, CA, U.S.A.) B grade, 6.66 I.U./ml β-glucuronidase, 3.41 I.U./ml arylsulphatase] is diluted to one-tenth with pH 5.5 buffer.

The two methanolic solutions of internal standard contain 200 ng/ml and 500 ng/ml, respectively.

Equipment

The glassware is washed for 30 min in an ultrasonic bath, first with water and then with methanol.

A Hewlett-Packard Model 5713A gas chromatograph equipped with a Hewlett-Packard Model 18713A electron-capture detector is used. The peak areas are given by a Hewlett-Packard Model 3380A electronic integrator.

The column is operated at 220°C, the injector temperature is 250°C and the detector is set at 300°C with argon—methane (90:10) at a flow-rate of 60 ml/min. The glass column (1 m × 3 mm I.D.) is washed [3] and packed with 3% OV-17 on Chromosorb W HP (80—100 mesh) (Applied Science Labs., State College, PA, U.S.A.). The conditioning procedure was described previously [3].

Enzymatic hydrolysis in urine

Aliquots (1 ml) of urine (diluted with water if necessary), 1 ml water and

500 μl of β-glucuronidase—arylsulphatase mixture, diluted with pH 5.5 acetate buffer (1:10, v/v), are heated for 15 h at 38°C. The extraction is then performed as described below.

Extraction

One ml of the internal standard solution is measured into a stoppered glass tube and dried under a nitrogen stream in a water bath at 37°C. Then 1 ml of the sample (plasma or urine), 2 ml of pH 10 buffer and 5 ml of benzene—ethyl acetate are added. The tube is shaken mechanically (Infors shaker) for 20 min at 180 rpm and centrifuged at 4800 *g* for 10 min. An aliquot of the organic phase is transferred into another tube, and 3 ml of 0.05 *M* sulphuric acid are added. The tube is shaken mechanically for 20 min at 180 rpm and centrifuged. The organic phase is discarded, 3 ml pH 10 buffer and 5 ml benzene—ethyl acetate are added to the acidic phase. The tube is shaken mechanically for 20 min at 180 rpm and centrifuged. An aliquot of the organic phase is transferred into another tube and dried under a nitrogen stream in a water bath at 37°C.

Derivatization and chromatography

To the dry residue are added 100 μl heptane and 10 μl of N-heptafluorobutyrylimidazole. The medium is thoroughly mixed (Vortex mixer) for 30 sec and allowed to stand for 10 min at room temperature. Then, 3 ml of water and 2 ml of heptane are added and the tube is shaken for 30 sec and centrifuged. The aqueous phase is frozen by immersing the tube in a methanol bath containing dry ice. An aliquot of heptane is transferred into another tube and a 3-μl portion is injected into the chromatograph.

The phentolamine content is calculated from the peak area ratio by reference to a calibration curve prepared from a series of methanolic phentolamine solutions between 5 and 100 ng/ml (with 200 ng internal standard), and between 25 and 2000 ng/ml (with 500 ng internal standard). These ranges are used to determine unconjugated phentolamine in plasma, and free and total (free plus conjugated) phentolamine in urine, respectively.

Human study

Three healthy male subjects, who had been advised to take no drugs during the week preceding the experiment and none besides phentolamine throughout the duration of the study, received 20 mg of phentolamine as one tablet of Regitine. Blood samples were collected before and 0.25, 0.50, 0.75, 1 and 2 h after the administration of the drug and centrifuged. Plasma was removed and stored at −20°C until analysis.

Urine was collected at the following intervals: 0—1, 1—2, 2—3, 3—4, 4—5, 5—6, 6—7, 7—8, 8—9, 9—10 and 10—24 h. The volume was measured and an aliquot was stored at −20°C.

RESULTS AND DISCUSSION

Extraction

The extraction yields of [^{14}C]-phentolamine from aqueous solutions at various pH values (Fig. 2) indicate that the drug can be best recovered at pH 10 with benzene—ethyl acetate.

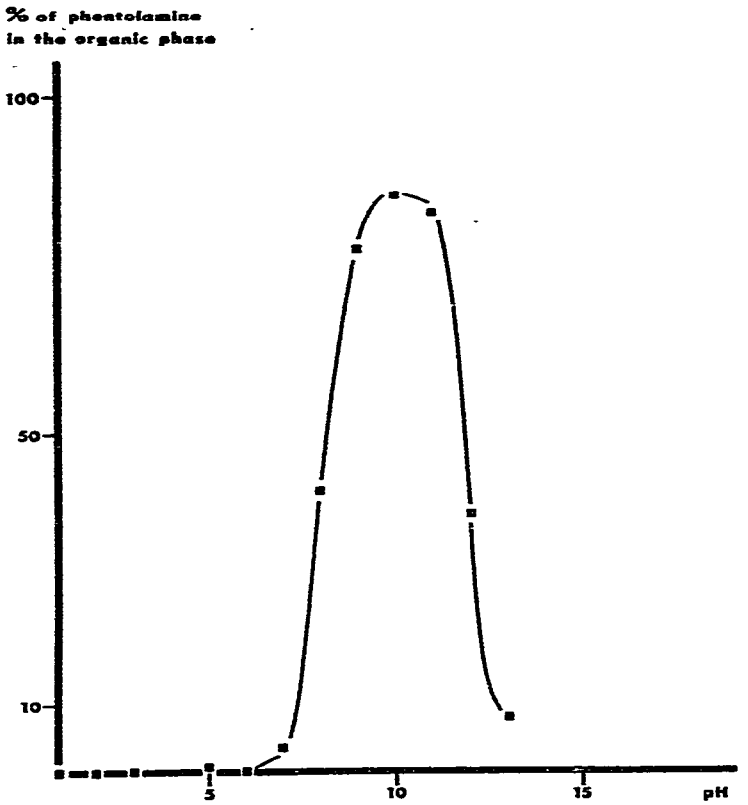

Fig. 2. Partition of phentolamine between the aqueous solution and the extraction solvent benzene—ethyl acetate (1:1, v/v) at different pH values.

The dissociation constants are: pK_a I = 9.5, pK_a II = 11.5 (in 75% methanol by titration).

Derivatization procedure

The derivative obtained with phentolamine and HFBI was heptafluorobutyryl-phentolamine, the phenolic hydroxyl being acylated. Phenolamines are very often derivatized with HFBI [4].

Precision and recovery

Tables I and II give the results obtained when the described procedure was applied to spiked human plasma and urine samples.

These tables demonstrate the good reproducibility of the assay down to concentrations of 5 ng phentolamine per ml plasma and 25 ng phentolamine per ml urine.

Plasma and urine interference

Fig. 3 shows the chromatograms of an extract of human plasma and of the same extract spiked with 100 ng of phentolamine and 200 ng of internal standard. No interference from the normal plasma components was recorded. Urine contains more detectable substances, but phentolamine and the internal stan-

TABLE I

PRECISION AND RECOVERY IN THE DETERMINATION OF UNCONJUGATED PHENTOLAMINE IN SPIKED HUMAN PLASMA

Amount added (ng/ml)	Mean amount found (ng/ml) (n = 6)	Precision (C.V. %)	Recovery (%)
5	5.7	7.5	114.7
10	9.9	6.2	99.5
25	24.6	5.8	98.4
50	50.2	3.6	100.5
100	100.5	2.6	100.5
		Mean	102.7 ± 6.7

TABLE II

PRECISION AND RECOVERY IN THE DETERMINATION OF FREE PHENTOLAMINE IN SPIKED HUMAN URINE

Amount added (ng/ml)	Mean amount found (ng/ml) (n = 6)	Precision (C.V. %)	Recovery (%)
25	23	2.9	92.0
50	50	2.6	100.0
100	99	5.8	98.8
250	264	5.7	105.4
500	522	3.2	104.4
1000	1010	1.7	101.0
2000	1880	4.4	94.0
		Mean	97.4 ± 5.0

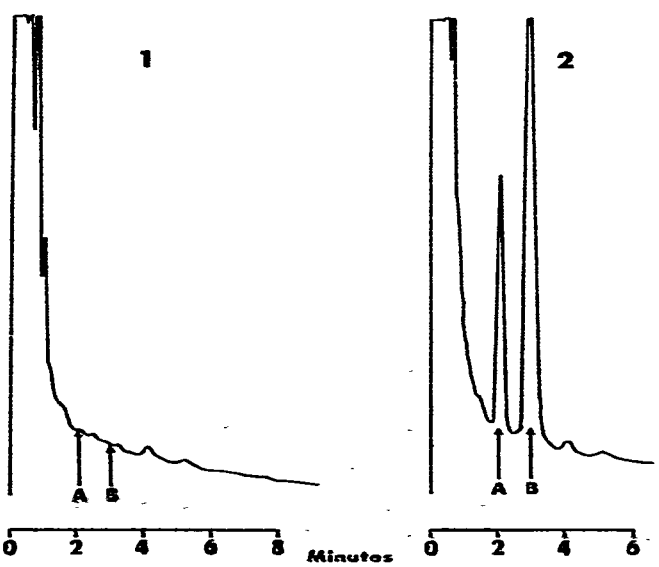

Fig. 3. Chromatograms of (1) human plasma blank (1 ml plasma); (2) same plasma spiked with 100 ng/ml of phentolamine (A), and 200 ng/ml of internal standard (B).

dard are well separated from the normal components of the urine extract. Enzymatic hydrolysis did not change the chromatogram.

Hexane could replace benzene when plasma and urine blanks are suitable.

Stability of calibration curve samples of phentolamine in plasma and urine

For routine analysis, a calibration curve in plasma and two calibration curves in urine (one for free and one for total phentolamine) are used. The calibration curve samples can be stored for up to three days in a refrigerator at +4°C, but a significant decrease in the peak area ratio of phentolamine:internal standard is observed after four days.

Specificity

The main metabolite of phentolamine, 2-[N-(*p*-carboxyphenyl)-N-(*m*-hydroxyphenyl)-aminomethyl]-2-imidazoline (Fig. 1, III), is present in plasma and urine. This compound is amphoteric (in fact it is an amino acid) and therefore difficult to extract using conventional solvent extraction. Amino acids can be extracted if ion-pair principles are used. This compound does not interfere when the described technique is applied. Two HPLC methods have been developed using an aqueous mobile phase to determine this metabolite in plasma and urine [5].

Application

The technique was applied in a study of the elimination of phentolamine after oral administration to three healthy subjects. Very low plasma levels were measured at all sampling times. Measurable concentrations of free and conjugated phentolamine were found in the urine. Fig. 4 shows the average (n = 3) curves of free and total (free plus conjugated) phentolamine urinary excretions.

On average, 1.80% of the administered dose was recovered in the 24-h urine as free phentolamine and 7.95% as total phentolamine.

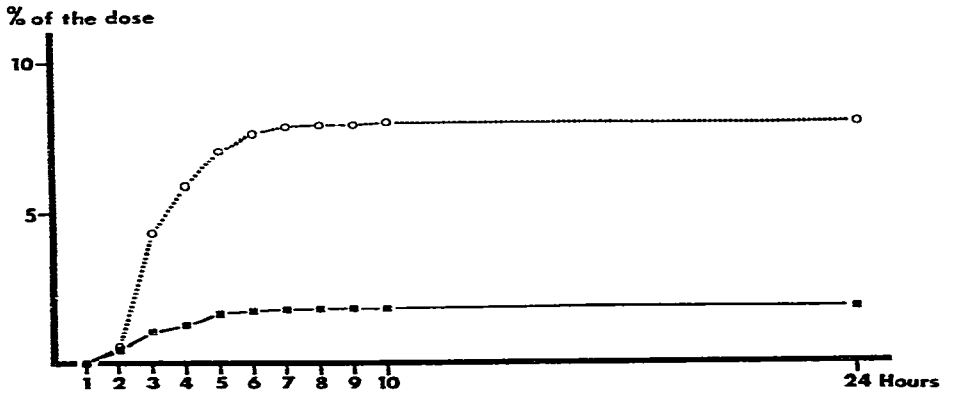

Fig. 4. Average cumulative urinary excretions in three healthy subjects after oral administration of 20 mg of phentolamine. (○), Total phentolamine; (■), free phentolamine.

REFERENCES

1 F. de Bros and E.M. Wolshin, Anal. Chem., 50 (1978) 521.
2 B. Pfister and P. Imhof, Brit. J. Clin. Pharmacol., 5 (1978) 175.
3 A. Sioufi and F. Pommier, J. Chromatogr., 183 (1980) 33.
4 S.D. Seeley and L.E. Powell, Anal. Biochem., 58 (1974) 39.
5 J. Godbillon and G. Carnis, J. Chromatogr., 222 (1981) 461.