Journal of Chromatography, 381 (1986) 83—93
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3204

ANALYSIS OF EPININE AND ITS METABOLITES IN MAN AFTER ORAL ADMINISTRATION OF ITS PRO-DRUG IBOPAMINE USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

RALPH GIFFORD, WARREN C. RANDOLPH, FRANCES C. HEINEMAN and JOHN A. ZIEMNIAK*

Department of Drug Metabolism, Smith Kline and French Laboratories, 709 Swedeland Road, Swedeland, PA 19479 (U.S.A.)

(First received December 18th, 1985; revised manuscript received April 17th, 1986)

SUMMARY

Ibopamine (N-methyldopamine O,O'-diisobutyrol ester, hydrochloride) is an ester prodrug of epinine. Epinine is a cardiovascular agent used in congestive heart failure because of its dopaminergic and adrenoreceptor agonist properties Quantitative analytical methods, using high-performance liquid chromatography coupled with electochemical detection, were developed for the determination of epinine and its known metabolites in biological media Epinine was extracted from human plasma and urine via an alumina adsorption procedure; this procedure was also used to estimate epinine conjugates after prior enzymatic hydrolysis. Penicillamine was added to the incubation mixture to inhibit isoquinoline production. Urinary dihydroxyphenylacetic acid levels were obtained using the same alumina adsorption procedure, while a separate analytical procedure utilizing a direct high-performance liquid chromatographic analysis of samples was developed for homovanillic acid and its conjugates. Coefficients of variation for all the assays were below 8%. These methods were used to study the pharmacokinetics and metabolic fate of epinine after oral administration of ibopamine to healthy volunteers.

INTRODUCTION

Ibopamine (SK&F 100168-A), the di-isobutyrol ester of epinine (Fig. 1), is undergoing extensive clinical trials in Europe and the U.S.A. Ibopamine readily undergoes enzymatic hydrolysis by blood and hepatic esterases to yield the presumed pharmacologically active moiety, epinine (N-methyldopamine), a catecholamine whose pharmacologic profile is similar to that of dopamine.

0378-4347/86/$03.50 © 1986 Elsevier Science Publishers B.V.

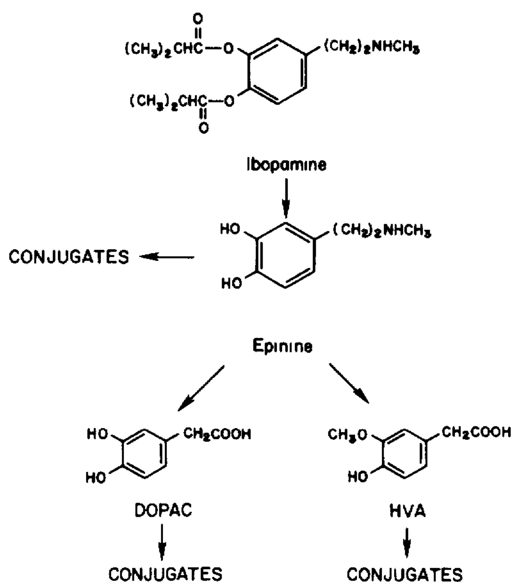

Fig. 1. Identified metabolic profile of ibopamine.

After oral administration, ibopamine produces positive inotropic and diuretic effects [1, 2]. It has been shown to be effective in increasing cardiac output and in decreasing vascular and pulmonary resistance in congestive heart failure, while eliciting no changes in heart rate or mean arterial pressure.

Various animal and human studies have shown that a significant amount of presystemic and systemic metabolism occurs for epinine after ibopamine administration [3]. Epinine can be eliminated by conjugation with inorganic sulfate or glucuronic acid. Epinine can also be metabolized by monoamine oxidase and catechol-O-methyl transferase enzyme systems to yield homovanillic acid (HVA) and dihydroxyphenylacetic acid (DOPAC), and their respective sulfate and glucuronide conjugates.

Disposition and metabolic studies for epinine in man require sensitive analytical methodologies for the quantitation of epinine itself and its various metabolites in biological fluids. This report details the analytical procedures developed to measure these compounds in biological fluids and their application in a preliminary clinical study in man.

EXPERIMENTAL

Chemicals

Epinine (deoxyepinephrine hydrochloride), dihydroxyphenylacetic acid, and sulfatase Type H-5 were obtained from Sigma (St. Louis, MO, U.S.A.). α-Ethyldopamine was supplied by Merck. Sodium octyl sulfate and alumina were obtained from Bioanalytical Systems (West Lafayette, IN, U.S.A.) and monochloroacetic acid was obtained from Mallinckrodt (Paris, KY, U.S.A.). Dihydroxybenzylamine (DHBA) and penicillamine were obtained from Aldrich (Milwaukee, WI, U.S.A.). Homovanillic acid was obtained from ICN Nutritional Biochemicals (Irvine, CA, U.S.A.). 1-Phenyl-7,8-dihydroxy-2,3,4,5-tetrahydro-

1H-3-benzazepine, hydrochloride salt (SK&F 38393; I) was obtained from Smith Kline and French Labs. (Philadelphia, PA, U.S.A.). Acetonitrile was HPLC grade from J.T. Baker (Phillipsburg, NJ, U.S.A.). All other chemicals were analytical grade and obtained from commercial suppliers.

Reagents

Enzyme preparation. A C_{18} Sep-Pak cartridge (Waters) was conditioned with 5 ml of acetonitrile followed by 15 ml of water. An aqueous solution of sulfatase Type H-5, prepared by dissolving an amount equivalent to 15 000 U of sulfatase activity in 2 ml of water, was transferred to the pre-conditioned cartridge. Deionized water (3 ml) was then passed through the cartridge until a total of 5 ml of effluent was collected. The enzyme solution was stored at 4°C for up to 30 days without loss of activity.

Acetate buffer, pH 4.7: sodium acetate trihydrate (27.2 g) and glacial acetic acid (11.6 ml) were dissolved in 1000 ml of deionized water.

Tris buffer, pH 8.6: tris[2-amino-2-(hydroxymethyl)-1,3-propanediol] (60.5 g) was dissolved in 250 ml of deionized water. The pH was adjusted to 8.6 with phosphoric acid.

Monochloroacetic buffer, pH 3.0: monochloroacetic acid (28.3 g), sodium hydroxide (9.35 g), disodium EDTA (1.0 g) and sodium octyl sulfate (50 mg) were dissolved in 2000 ml of deionized water. The pH was adjusted to 3.0 with monochloroacetic acid.

Citrate—acetate buffer, pH 5.0: sodium acetate (22.0 g), citric acid (21.0 g), sodium hydroxide (9.8 g), disodium EDTA (0.67 g) and glacial acetic acid (75 ml) were dissolved in 2000 ml of deionized water. The pH was adjusted to 5.0 with 5 *M* sodium hydroxide.

Standard solutions. All stock solutions of epinine, DOPAC, HVA, DHBA, I and α-ethyldopamine were prepared in 0.2 *M* acetic acid. Appropriate plasma standards were prepared daily by the addition of various aliquots of the working standard solution to "preserved plasma" [0.2 ml of 1% disodium EDTA (w/v), 0.1 ml of 5% ascorbic acid (w/v) per 1.0 ml of plasma]. Stock and working standard solutions were stored at 4°C. For the preparation of urine standards, control urine was pretreated with alumina in order to remove endogenous DOPAC and HVA. Blank urine was adjusted to pH 8.6 with sodium hydroxide and alumina was added (≈ 200 mg per 100 ml). The sample was mixed for 15 min. After centrifugation and decanting of the aqueous layer, the procedure was repeated. The urine pH was then adjusted to 4.7 with hydrochloric acid.

Extraction procedure

Epinine in plasma. A 2-ml volume of plasma was transferred to a 75 × 12 mm polystyrene tube and 10 μl of the internal standard (10 μg/ml DHBA) were added, followed by 40 mg of alumina and a 2.0-ml aliquot of pH 8.6 Tris buffer. The tube was immediately capped and vortexed. This step was performed on all samples individually, since the stability of epinine in pH 8.6 buffer is unknown. The samples were then placed on a wrist-action shaker for 15 min, after which the alumina particles were allowed to settle. Plasma was removed by aspiration and discarded. The alumina was washed twice with 1-ml

portions of deionized water, vortexed for 2 min and the aqueous portion discarded. Epinine was eluted from the alumina with 0.5 ml of 0.2 *M* acetic acid after 15—20 min of vortexing. A 50-μl aliquot of the acetic acid extract was analysed via high-performance liquid chromatography with electrochemical detection (HPLC—ED).

Epinine and DOPAC in urine. Urine was diluted 1:20 with deionized water and 10 μl of the internal standard (20 μg/ml α-ethyldopamine) were added to 250 μl of the diluted urine. A 20-mg quantity of alumina and 0.5 ml of the Tris buffer were added and the procedure previously outlined for epinine in plasma was followed.

HVA in urine. Urine samples were diluted 1:10 with deionized water. A 100-μl aliquot was then added to 900 μl of the internal standard (200 ng/ml I), and a 10—20-μl aliquot was analyzed via HPLC.

Conjugated metabolites

Hydrolysis procedure. Semi-purified Type H-V sulfatase was used for the hydrolysis of the various sulfate and glucuronic acid conjugates of epinine, DOPAC and HVA. A 0.65-ml aliquot of plasma or urine was transferred to a 17 × 10 mm polypropylene tube followed by 0.5 ml of acetate buffer (pH 4.7), 50 μl of aqueous penicillamine (40 mg/ml) and 0.25 ml of the partially purified enzyme solution. For the determination of urinary glucuronides, 0.5 ml of urine was combined with 150 μl of 1 *M* monobasic potassium phosphate (an inhibitor of sulfatase activity) prior to hydrolysis.

Samples were incubated at 37°C for 16 h. Hydrolyzed urine specimens were diluted 1:10 with deionized water prior to extraction of the liberated aglycone.

Conjugates of epinine/DOPAC. Sample aliquots (0.25 ml) of hydrolyzed plasma or diluted hydrolyzed urine were transferred to a 1.5-ml polypropylene Eppendorf tube. A 10-μl aliquot of the appropriate internal standard (plasma: DHBA 10 μg/ml, urine: α-ethyldopamine, 20 μg/ml) was added, followed by 20 mg of alumina and 0.5 ml of Tris pH 8.6 buffer. The procedure outlined for epinine in plasma was then followed.

Conjugates of HVA. A 200-μl aliquot of 100% (w/v) trichloroacetic acid solution was added to 1 ml of dilute hydrolyzed urine. The samples were vortexed for 1 min and centrifuged at 1000 *g* for 15 min. A 100-μl aliquot of the supernatant was combined with 900 μl of the internal standard (200 ng/ml I) for HPLC analysis.

Chromatography

Chromatography was performed using a Model 110A isocratic pump (Beckman, Palo Alto, CA, U.S.A.) coupled with a WISP 710B autosampler (Waters Assoc., Milford, MA, U.S.A.) suitable for variable-volume injections. The electrochemical detector (BAS, New Lafayette, IN, U.S.A.) consisted of an LC-4A or LC-4B controller and a TL-5 thin-layer glassy carbon electrode. It was operated in the oxidative mode at a potential of 0.65 V vs. an Ag/AgCl reference electrode. All separations were performed on a 5-μm C_{18} Ultrasphere-IP (25 cm × 4.6 mm I.D.) column (Beckman) using one of the following mobile phases at a flow-rate of 1 ml/min. For plasma epinine: monochloroacetate buffer—acetonitrile (97:3). For urine epinine/DOPAC: citrate—acetate

buffer—acetonitrile (82:18). For urine HVA: citrate—acetate buffer—acetonitrile (89:12).

Assay validation

Validation studies were conducted for plasma epinine, total plasma epinine conjugates and for epinine, DOPAC and HVA conjugates in urine. Since authentic reference standards were unavailable for the various conjugates, all validations were conducted using the unconjugated compounds.

The accuracy and precision of the epinine assay was determined by analysis of five replicate samples containing known concentrations of analyte on each day of a three-day study period. Reproducibility of the analysis of total plasma epinine conjugates was determined by analysis of replicate plasma samples obtained from a beagle dog after oral administration of ibopamine (5 mg/kg). Reproducibility studies of the urinary epinine, DOPAC and HVA assays were conducted on replicate samples of authentic clinical specimens analyzed over a three-day study period.

Quantitation of the resulting peaks was based on peak-height ratio (analyte/internal standard) analysis versus an appropriate set of standards prepared daily. Peak-height data were obtained via a computer-automated laboratory system (Computer Inquiry Systems, Waldwich, NJ, U.S.A.). The equation for the linear regression line (fitted by least squares) of concentration versus peak-height ratio was obtained. Analyte concentrations for samples were calculated from the following equation:

$$\text{concentration of analyte} = (c/d - a)/b$$

where b = slope of regression line, a = intercept of regression line, c = peak height of analyte and d = peak height of internal standard.

Clinical study

Six fasted healthy male volunteers each received a single 200-mg oral dose of ibopamine (equivalent to 109 mg of epinine). Blood samples were collected at various time periods in heparinized vacutainers, immediately chilled on ice and centrifuged at 4°C. A measured aliquot of plasma was then transferred to a 100 × 17 mm polypropylene tube to which 0.2 ml of 1% disodium EDTA and 0.1 ml of 5% ascorbic acid per ml of plasma were immediately added. Samples were stored at −20°C until analysis. Stability studies showed epinine to be stable under these storage conditions for three months.

Urine was collected prior to (−24 to 0 h) and during (0—8, 8—24 h) the study period. A 10-ml aliquot of 6 *M* hydrochloric acid was added to the containers prior to each collection period.

Estimates of baseline urinary excretion of DOPAC and HVA were obtained during the control periods and normalized for creatinine excretion during the same period. This ratio was used to predict endogenous urinary levels during the ibopamine treatment period according to the following equation:

$$\text{study day excretion of DOPAC (mg)} = \frac{\text{control DOPAC (mg)}}{\text{control creatinine (mg)}} \times \text{creatinine excretion during study day (mg)}$$

These predicted values were used to estimate the urinary excretion of DOPAC and HVA for endogenous levels during the actual study period.

RESULTS AND DISCUSSION

Several analytical HPLC—ED procedures have been developed for the quantitation of epinine, DOPAC and HVA and other catechol-containing compounds [4--9]. Many utilize an alumina extraction step, known to be highly specific for catechols [10]. The analytical procedures described in this report used modifications of these methods and combined them for routine quantitative use to assess the pharmacokinetics and metabolism of epinine after ibopamine administration.

Early animal experimentation revealed that epinine and its metabolites can undergo conjugation with sulfate and/or glucuronic acid. An enzymatic hydrolysis procedure coupled with HPLC—ED was developed for the quantitation of these conjugated metabolites. A number of commercially available sources of glucuronidase and sulfatase mixtures were evaluated. Most of these preparations were found to contain many additional substances, which either eluted with the peaks of interest or resulted in an excessive number of extraneous peaks. Sulfatase Type H-V from Sigma is a mixture of sulfatase and glucuronidase from Helix pomatia, partially purified via gel chromatography. It was found to contain no chromatographic interferences and, of all the enzyme preparations analyzed, resulted in the fewest additional peaks. In addition, the Sep-Pak clean-up procedure that we employed was believed to further reduce the number of extraneous peaks.

During the development of the enzymatic hydrolysis procedures, it became apparent that epinine itself was unstable under the incubation conditions used. Approximately 25% of the epinine added to the incubation mixture was degraded; however, the decomposition was linear with respect to concentration and time. It was previously shown that in the presence of ascorbic acid and elevated temperatures, epinephrine can react with formaldehyde formed through the oxidation of ascorbic acid to yield tetrahydroisoquinolines [11]. Another group of investigators found that isoquinoline formation could be

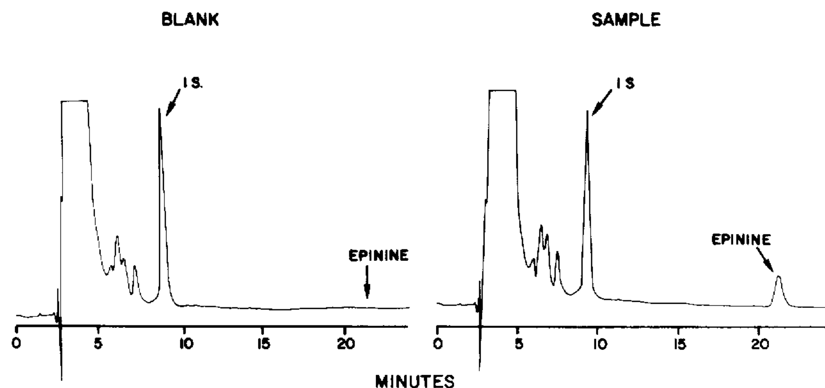

Fig 2. Chromatograms obtained from human plasma for the determination of plasma epinine (20 ng/ml) after oral administration of 200 mg of ibopamine.

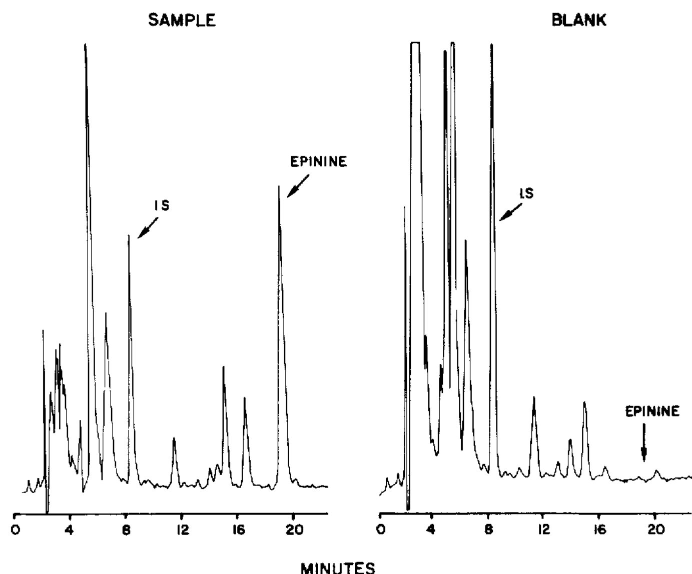

Fig. 3. Chromatograms obtained from human plasma for the determination of plasma epinine conjugates (550 ng/ml) after oral administration of 200 mg of ibopamine.

inhibited by addition of penicillamine to the incubation mixture [12]. By incorporating penicillamine in our enzymatic hydrolysis procedure, we reduced epinine decomposition to less than 5%.

The quantifiable lower limit of detection for epinine in plasma defined as a signal-to-noise ratio of 5, was 0.5 ng/ml. A linear relationship existed for plasma epinine over concentration ranges of 0—100 ng/ml and 0—500 ng/ml for total plasma epinine conjugates. The alumina extraction procedure yielded recoveries of 55 ± 2.3% for the epinine assay and a value of 70 ± 1.5% for the epinine conjugate assay. At present, we have not identified the source of this disparity.

Chromatograms obtained from blank plasma and authentic clinical specimens are shown in Figs. 2 and 3, respectively. Acceptable chromatography was seen for all the assays, with no interfering peaks present. As seen in the epinine conjugate assay on blank plasma (Fig. 3), additional peaks were present after enzymatic hydrolysis, the majority of which came directly from the enzyme material used.

Chromatograms obtained from blank urine and actual clinical samples after ibopamine administration are shown in Figs. 4 and 5. The linear range of the urinary epinine and DOPAC assays was 0—50 μg/ml and that for HVA was 0—125 μg/ml. The within- and between-day variability for the plasma epinine and various urine assays are included in Tables I and II.

The methods described here were used for the analysis of samples from an inital single-dose ibopamine pharmacokinetic study in man. Mean plasma concentrations for epinine and total epinine conjugates are shown in Fig. 6. Epinine plasma concentrations peaked at 0.5 h, indicating the rapid hydrolysis of the prodrug ibopamine to epinine. These data are consistent with earlier studies using a radioenzymatic assay, which demonstrated a four-fold increase

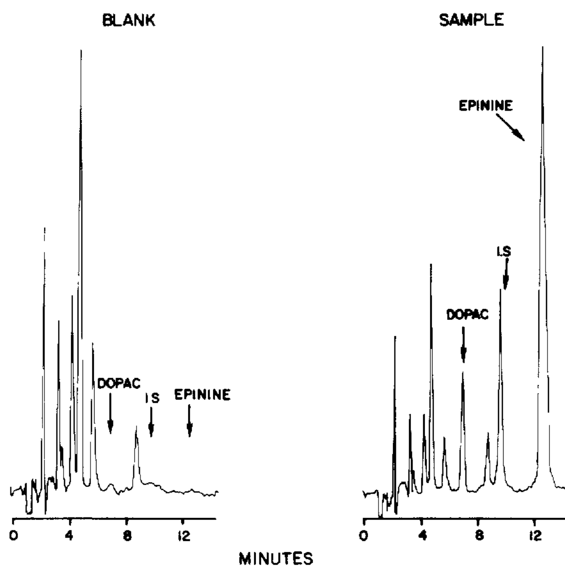

Fig. 4. Chromatograms obtained from human urine for the determination of epinine (4 μg/ml) and DOPAC (7 μg/ml) conjugates in urine after oral administration of 200 mg of ibopamine.

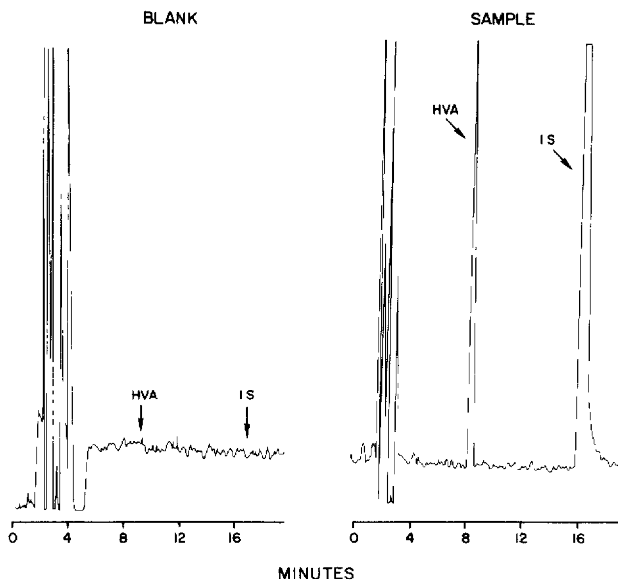

Fig 5. Chromatograms obtained from human urine for the determination of HVA conjugates (2.7 μg/ml) in urine after oral administration of 200 mg of ibopamine.

in peak epinine concentrations and a three-fold increase in the area under the curve when ibopamine is administered compared to epinine itself [3]. In the present study, the area under the curve for epinine (23 ± 10 ng·h/ml) represents less then 0.5% of the total epinine conjugates in plasma (4736 ± 1886 ng·h/ml), indicative of the extensive metabolism it undergoes.

The extensive metabolism noted in plasma is also reflected in the urine data

TABLE I

ACCURACY AND PRECISION OF PLASMA ASSAYS

Analyte	Concentration (ng/ml)	Time (h)	Assay concentration (mean ± S.D., n = 5) (ng/ml)	Coefficient of variation (%)	
				Within-day	Between-day
Epinine	1.0		0.95 ± 0.13	7.1	3.2
	5.0		4.90 ± 0.09	5.5	2.9
	9.0		9.20 ± 0.41	4.3	3.0
Epinine conjugates		1.0	205 ± 5	4.6	4.1
		4.0	218 ± 3	3.0	4.7
		6.0	95 ± 2	3.0	4.3

TABLE II

PRECISION OF URINE ASSAYS

Analyte	Assay concentration (mean ± S.D., n = 5) (μg/ml)	Coefficient of variation (%)	
		Within-day	Between-day
DOPAC conjugates	2.45 ± 0.20	7.1	7.6
	25.15 ± 1.22	4.0	2.5
	40.69 ± 2.23	4.7	4.9
Epinine conjugates	2.03 ± 0.08	4.5	3.5
	13.08 ± 1.28	2.6	4.9
	21.85 ± 0.69	3.0	0 8
HVA conjugates	3.33 ± 0.06	2.8	4.6
	21.34 ± 0.30	2.7	2.9
	35.65 ± 1.05	1.8	3.2
	98.27 ± 2.86	2.4	3.7

TABLE III

URINARY EXCRETION (0—24 h) OF EPININE, DOPAC AND HVA IN HUMAN VOLUNTEERS (n = 8) FOLLOWING ORAL ADMINISTRATION OF IBOPAMINE, A PRODRUG OF EPININE

	Percentage of dose (S.D.)*			
	Unchanged	Glucuronide	Sulfate	Total
Epinine	0.7 (0.85)	2.3 (1.3)	1.8 (2.9)	4.8 (3.4)
DOPAC	18 6 (14.2)	4.7 (3.1)	2.7 (2.9)	25.9 (16.6)
HVA	30 5 (10.5)	2.1 (2.2)	11.3 (7.4)	44.0 (13.0)

*Expressed as epinine equivalents.

(Table III). Because of detectable endogenous urinary levels of DOPAC and HVA, urinary excretion data needed to be corrected for baseline values. Endogenous urinary excretions of DOPAC and HVA were obtained during the control period and normalized for creatinine excretion. Control values of DOPAC (1.4 ± 0.8 mg) and HVA (5.2 ± 1.2 mg) agree with daily excretion

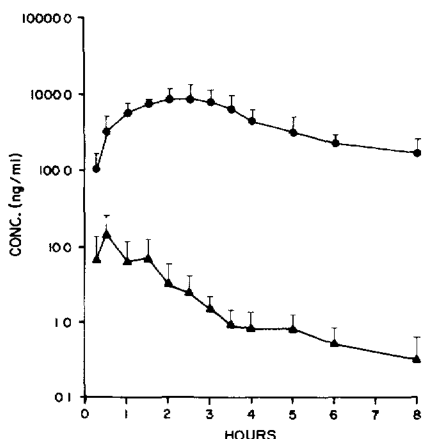

Fig. 6. Mean (± S.D.) plasma concentrations of epinine (▲) and epinine conjugates (●) in healthy male volunteers (n = 6) after oral administration of 200 mg of ibopamine.

rates reported for healthy volunteers [4, 8]. Endogenous urinary levels of epinine were not detectable. After ibopamine administration, endogenous DOPAC and HVA levels were increased seven- to ten-fold higher than baseline values. Radiolabeled ibopamine studies in animals have shown that the additional DOPAC and HVA present were products of ibopamine metabolism. These increases in DOPAC and HVA demonstrate that epinine produced from ibopamine is eliminated in man via existing metabolic pathways for endogenous catecholamines.

As seen in Table III, less than 1% of the orally administered dose of ibopamine (as epinine equivalents) was recovered in the urine as unchanged epinine. A substantial amount of the dose was eliminated as HVA (44%) and DOPAC (26%). A total of 74.7 ± 25.8% of the dose was recovered in the urine as epinine and its various metabolites. The fate of the remaining 25% in man is unknown.

In summary, we have presented several analytical HPLC—ED procedures for the determination of epinine and its various metabolites in human plasma and urine after administration of its prodrug, ibopamine. These procedures are currently being used to study the clinical pharmacology and pharmacokinetics of epinine.

REFERENCES

1 J.H. Ren, M.E. Leithe, P. Huss, D.V. Unverforth and C.V. Leier, Curr. Ther. Res., 34 (1983) 667.
2 G.C. Reffo, M. Turin, A. Gabellini and C. Forattini, Eur. J. Clin. Pharmacol., 26 (1984) 19.
3 M. Boyce, N. Dalton, B. Goodwin, P. Walker, M. Weg and M. Saldler, Br. J. Clin. Pharmacol., 19 (1985) 143 P.
4 L.J. Felice and P.T. Kissinger, Anal. Chem., 48 (1976) 794.
5 G. Sperk, J. Neurochem , 38 (1982) 840.
6 M.H. Joseph, B.V. Kadam and D. Risby, J. Chromatogr., 226 (1981) 361.
7 Y. Dalmaz and L. Peyrin, J. Chromatogr., 145 (1978) 11.

8 L.J. Felice, C.S. Bruntlett and P.T. Kissinger, J. Chromatogr., 143 (1977) 407.
9 I.N. Mefford, M.M. Ward, L. Miles, B. Taylor, M.A. Chesney, D.L. Keegan and J.D. Barchas, Life Sci., 28 (1981) 477.
10 A.H. Anton and D.F. Sayre, J. Pharmacol. Exp. Ther., 138 (1962) 360.
11 A. Shirahata, M. Yashioka and Z. Tamura, Chem. Pharm. Bull., 30 (1982) 1352.
12 T. Kenyhercz and P. Kissinger, J. Pharm. Sci., 67 (1978) 112.
13 J.L. Morrisey and Z.K. Shihabi, Clin. Chem., 25 (1979) 2045.
14 E.R. Smith and H.W. Weil-Malherbe, Clin. Chim. Acta, 35 (1971) 505.