Journal of Chromatography, 308 (1984) 251- 259
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2090

SENSITIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY USING ELECTROCHEMICAL DETECTION FOR A NOVEL PRODRUG ESTER OF METHYLDOPA, PIVALOYLOXYETHYL 3-(3,4-DIHYDROXYPHENYL)-2-METHYLALANINATE, IN PLASMA AND URINE

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(First received November 8th, 1983; revised manuscript received January 27th, 1984)

SUMMARY

The pivaloyloxyethyl ester of methyldopa is an antihypertensive prodrug possessing improved bioavailability properties over methyldopa. A sensitive cation-exchange, high-performance liquid chromatographic assay using electrochemical detection has been developed for the ester in plasma and urine in order to determine the extent of its hydrolysis after oral administration. The chromatographic conditions involve two Altex Partisil 10 SCX columns (25 cm × 4.6 mm) in series; a mobile phase consisting of methanol, potassium phosphate buffer, pH 3.0, and EDTA disodium dihydrate; and an electrochemical detector set at 0.5 V. The pivaloyloxyethyl ester in plasma or urine is extracted into ethyl acetate, back-extracted into 0.1 *M* sulfuric acid, and analyzed directly by high-performance liquid chromatography. For urine, the ethyl acetate extract is washed with a buffer (pH 8.0) prior to the back-extraction step. The assay gives a linear response over the concentration range of 10—160 ng/ml in plasma and 20—400 ng/ml in urine.

INTRODUCTION

The pivaloyloxyethyl (POE) ester of methyldopa [Fig. 1, I, (*S*)- or (*R*)-pivaloyloxyethyl (*S*)-3-(3,4-dihydroxyphenyl)-2-methylalaninate hydrochloride dihydrate] is a new antihypertensive prodrug of methyldopa (Fig. 1, II). The ester possesses better oral absorption and antihypertensive properties than methyldopa [1—3]. In animals treated orally with a radioactive dose of the ester, the metabolites that have been isolated from urine are pivalic acid (Fig. 1, III) and methyldopa [3].

The POE ester of methyldopa has been developed as a prodrug that would be better absorbed than methyldopa and would readily hydrolyze to methyl-

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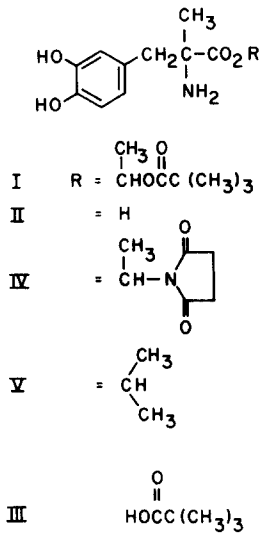

Fig. 1. Chemical structures of the POE ester of methyldopa (I), methyldopa (II), pivalic acid (III), internal standard for the plasma assay (IV) and internal standard for the urine assay (V).

dopa [1]. A sensitive and selective assay for the POE ester was needed to quantify the presence of the intact ester in plasma and urine following oral administration.

Methods available for the analysis of methyldopa include a non-specific fluorescent assay [4], a mass spectrometric assay [5], gas—liquid chromatography [6], and high-performance liquid chromatography (HPLC) using ultraviolet [7,8] and electrochemical detection (ED) [9—13]. The latter technique is the most sensitive for methyldopa and has been developed for the POE ester.

A cation-exchange, HPLC assay using ED is described for the determination of the POE ester of methyldopa. The plasma and urine assays are selective for the ester and are sensitive to 10 ng/ml and 20 ng/ml, respectively. Animal studies and preliminary human studies with prodrug have revealed trace levels in the urines and negligible levels in plasma.

EXPERIMENTAL

Reagents

HPLC solvents were obtained from Burdick & Jackson Labs.; water was purified using a Milli-Q system (Millipore). All chemicals were used as they were received.

Pivaloyloxyethyl 3-(3,4-dihydroxyphenyl)-2-methylalaninate·HCl·$2H_2O$ (*S,S*-diastereomer and an *R,S*- and *S,S*-diastereomeric combination, 50:50), (*S*)-succinimidoethyl (*S*)-(3,4-dihydroxyphenyl)-2-methylalaninate·HCl·$2H_2O$ (Fig. 1, IV, internal standard for the plasma assay), isopropyl (*S*)-2-amino-2-(3,4-dihydroxybenzyl)proprionate (Fig. 1, V, internal standard for the urine assay) were synthesized at Merck Sharp & Dohme Labs. [1].

High-performance liquid chromatography

The liquid chromatographic system consists of a solvent delivery system (Model M6000A) and an autosampler (WISP 710B) from Waters Assoc., a Spectra Physics computing integrator (Model SP4100) and an amperometric controller (LC-2A)/KEL F thin-layer detector cell (TL-8A) from Bioanalytical Systems. The mobile phase flows through the electrochemical detector cell (0.5 V) at 2 ml/min. Two cation-exchange columns from Altex (Partisil 10 SCX, 10-μm particles) are employed in series for the plasma and urine assays. The detector cell and the solvent waste container are enclosed within a copper mesh, Faraday cage (grounded to a water pipe).

The mobile phase for the plasma assay consists of 1.74 g of monobasic potassium phosphate, 850 ml of water, 150 ml of methanol, 50 mg of ethylenediaminetetraacetic acid, disodium salt, dihydrate (EDTA). The pH of the solution is adjusted to 3.0 with phosphoric acid (85%). The mobile phase for the urine assay is prepared similarly except 100 ml of methanol is mixed with 900 ml water. Both mobile phases are filtered (cellulosic membrane filter, 0.45 -μm) and kept under a helium atmosphere.

Extraction

A 1-ml sample of plasma is prepared for HPLC analysis by adding to it 100 μl of sodium fluoride (20 mg/ml), 200 μl of $Na_2S_2O_5$ (0.25 *M*) and 100 μl of (*S*)-succinimidoethyl (*S*)-(3,4-dihydroxyphenyl)-2-methylalanine hydrochloride dihydrate (1 μg/ml) in a 13-ml tube. The sample is then extracted with 5 ml of ethyl acetate (vortex for 1 min, centrifuge for 5 min at 653 *g*). The ethyl acetate layer is transferred to a 13-ml tapered centrifuge tube and back-extracted into 0.5 ml of 0.1 *M* sulfuric acid. The sulfuric acid layer is analyzed by HPLC.

A 2-ml urine sample is prepared by mixing with it isopropyl 2-amino-2-(3,4-dihydroxybenzyl)propionate (100 μl, 2 μg/ml), 100 μl of $Na_2S_2O_5$ and 0.5 ml of borate buffer, pH 9.75. The sample is then extracted with 6 ml of ethyl acetate (vortex for 1 min, centrifuge for 5 min at 653 *g*). The ethyl acetate layer is transferred to a new tube and is washed with 1 ml of phosphate buffer, pH 8.0—$Na_2S_2O_5$ solution (13.4 g Na_2HPO_4, 0.731 g NaH_2PO_4 and 100 ml of 10.25 *M* $Na_2S_2O_5$ in 1 l of water). The ethyl acetate is again transferred to a tapered centrifuge tube and extracted with 0.5 ml of dilute sulfuric acid. The aqueous layer is analyzed by HPLC.

Standard calibration curves

A 10 μg/ml stock solution of the POE ester is serially diluted to concentrations of 100, 200, 400, 800, 1200, 1600, 2000, 2200 and 2500 ng/ml. Standards are prepared by transferring 100 μl of each solution to 1 ml of plasma or 2 ml of urine. The standards are processed as described above.

Standard curves are drawn with peak height ratios plotted against concentrations. Sample concentrations are quantified from these curves which are generated with each set of samples (daily).

Plasma stability studies

The plasma stability of the POE ester was investigated with $Na_2S_2O_4$ and

with sodium fluoride. Aliquots from duplicate test standards were removed every 15 min, treated with internal standard and extracted. The test standards during the studies were mixed at room temperature. The percent of ester remaining overtime was determined from peak height ratios.

Animal and human studies

Animal and human studies were conducted with the *S,S*-diastereomer and/or the diastereomeric mixture to determine the systemic levels of the intact ester and/or bioavailability of methyldopa from the ester. In beagle dogs and rhesus monkeys, the drug was administered orally, 500 mg free base equivalents. Plasma samples were collected in time intervals up to 24 h in heparinized tubes containing 6 mg of sodium fluoride. The blood samples were centrifuged and the plasma frozen until analysis. Urine was collected over dry ice over 0—24 h and 24—48 h after dose. Samples were kept frozen until analysis.

Portal circulation levels of the prodrug were investigated in rhesus and cynomolgus monkeys after oral administration of the ester, 803 mg of the ester's free base. Portal blood samples were collected via a subcutaneously implanted catheter, in heparinized tubes containing 8 mg of sodium fluoride. Blood was collected in time intervals up to 8 h. Urine samples were collected as discussed above.

In human studies, male volunteers received 1000 mg of ester, per os. Blood and urine samples were collected in time intervals up to seven days. The samples were handled and stored as described above.

RESULTS AND DISCUSSION

The HPLC—ED assay is sensitive and specific for the POE ester of methyldopa in human and animal biological fluids. Pivaloyloxyethyl ester of methyldopa selectively elutes on two cation-exchange columns from methyldopa and its major metabolites: α-methyldopamine, α-methylnorepinephrine, 3-O-methyl-2-methyldopa, 3-O-methyl-α-methyldopamine and 3-methoxy-4-hydroxyphenylacetone. There is, however, no chromatographic distinction between the *R,S*- and the *S,S*-diastereomers of the POE ester. Organic extraction of the ester from plasma and urine by ethyl acetate and back-extraction into dilute sulfuric acid increases the selectivity of the method by isolating it from endogenous materials and conjugates that might interfere chromatographically (Figs. 2 and 3).

Investigations into isolation techniques for the POE ester from biological fluids for HPLC analysis were mainly centered on the urine matrix. There were no chromatographic interferences for the POE ester from plasma background using either deproteination (e.g. by acetonitrile precipitation or by ultrafiltration) or extraction methods (e.g. tributyl phosphate (TBP)—hexane extraction, aluminum oxide extraction) [9—13].

Reported isolation methods [9—13] for catecholamines and amino acids involving TBP—hexane and/or alumina extraction were tested on urine with minor success: TBP—hexane extraction—acid back-extraction resulted in interfering peaks; further isolation with aluminum oxide gave a clear chromatogram

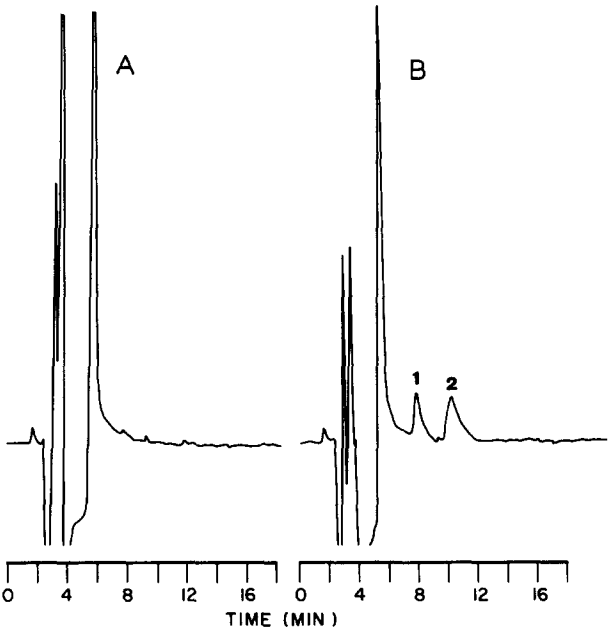

Fig. 2. Chromatograms of (A) a human plasma blank; (B) spiked plasma standard containing internal standard (1) and the POE ester (2) at 20 ng/ml. Electrochemical detector set at 50 nA full scale, offset 2—4.

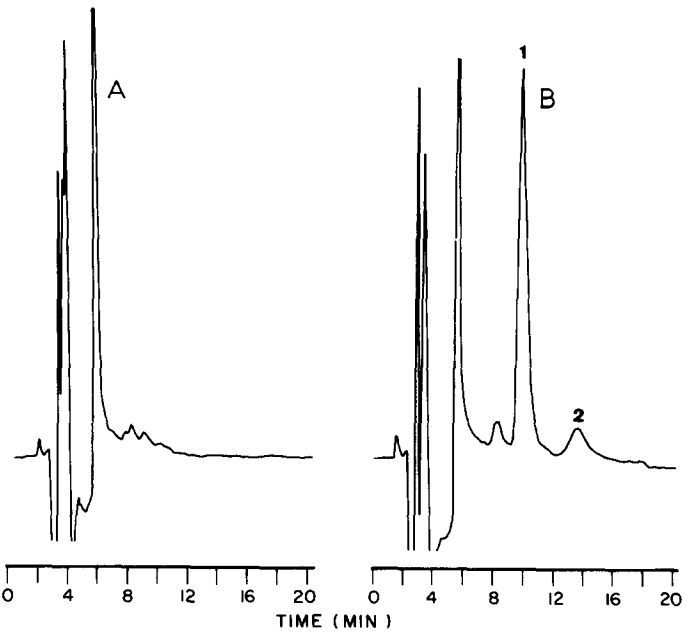

Fig. 3. Chromatograms of (A) a human urine blank; (B) a spiked urine containing the internal standard (1) and the POE ester (2) at 40 ng/ml. Electrochemical detector set at 50 nA full scale, offset 2—4.

for the POE ester, but recovery was less than 10%. Urine clean-up using $ZnSO_4 \cdot 7H_2O$—$Ba(OH)_2$ [14] was not any better than the TBP—hexane extraction—back-extraction technique.

POE ester extraction from urine with ethyl acetate [3] followed by back-extraction into dilute sulfuric acid was chromatographically successful with 70—80% recovery. An additional washing step in the isolation sequence was necessary to further clear the urine chromatogram for a suitable internal standard, isopropyl ester of methyldopa.

The POE ester's structure characteristically suggests chemical sensitivity to oxidation of its catechol and hydrolysis of its ester. The enzymatic hydrolysis of this ester in human plasma has been reported [3]. The enzymatic hydrolysis of esters in serum and their protection with sodium fluoride is known [15,16]. Therefore, the stability of the POE ester was tested at ambient temperature in human plasma, in plasma with $Na_2S_2O_5$ and in plasma with sodium fluoride. The results (Table I) indicate protection for the ester with sodium fluoride rather than with the reducing agent $Na_2S_2O_5$. Thus, sodium fluoride was added to the samples during collection to prevent enzymatic hydrolysis. The reducing agent was added later to the samples to prevent oxidation during preparation for analysis. Similar enzymatic losses for the POE ester of methyldopa in urine do not occur. The chemical stability of the ester in dilute hydrosulfuric acid was also tested, revealing no losses with time.

Intra-day coefficients of variation of replicates for the human plasma and the human urine assays are presented in Table II. These values were measured at concentrations over the linear range. The recovery yield for the POE ester extracted from plasma and back-extracted into dilute sulfuric acid is 79.7 ± 8.3%; for urine, 74.7 ± 5.9%. The plasma assay for the POE ester gives a linear plot ($r^2 = 0.9982$) of peak height ratios versus concentration described by the equation $y = 0.0069 + 0.0043x$. The concentration range is 10—160 ng/ml. The urine assay gives a linear plot ($r^2 = 0.9999$) described by the equation $y = 0.0030 + 0.0019x$ over the concentration range of 20—400 ng/ml. The assay is sensitive for the ester to 10 ng/ml in plasma and to 20 ng/ml in urine. Lower concentration levels of the ester (approximately 1 ng/ml) can be obtained by adjusting

TABLE I

STABILITY OF POE ESTER (*S,S*-DIASTEREOMER) IN HUMAN PLASMA AND IN PLASMA CONTAINING $Na_2S_2O_5$ OR NaF AT AMBIENT TEMPERATURE

Time (min)	Percent remaining		
	Plasma*	Plasma with $Na_2S_2O_5$**	Plasma with NaF***
15	96.9	83.0	100
30	64.6	56.5	103
45	63.1	46.1	87.6
60	63.1	38.1	87.0

*POE ester in plasma at 5.8 μg/ml.
**POE ester (5.8 μg/ml) in plasma with 3 mg/ml $Na_2S_2O_5$.
***POE ester (5.8 μg/ml) in plasma with 2 mg/ml NaF.

TABLE II

REPRODUCIBILITY (n = 5–6) OF THE URINE AND THE PLASMA ASSAY OF THE *S,S*-DIASTEREOMER

Concentration (ng/ml)	Coefficients of variation (%)
Plasma assay	
10	18.3
20	7.5
40	5.0
80	6.1
160	2.2
Urine assay	
20	8.3
40	8.1
80	8.4
125	8.8
200	10.4
400	6.2

a lower attenuation or by extracting from a larger volume of fluid (in the urine assay, a different internal standard would be required). However, at lower attenuation baseline stability and assay reproducibility would be compromised.

The POE ester of methyldopa was developed with the objective of obtaining a prodrug which would be more efficiently absorbed from the gastrointestinal tract compared to oral methyldopa but which would also be readily hydrolyzed in the body to deliver the active moiety to the general circulation. When small oral doses (200 mg) of the *S,S*-diastereomer, labeled with ^{3}H, were administered to man, about 70% of the label was recovered in urine within 36 h and methyldopa was the major urinary metabolite [3]. In comparison, about 40% of a ^{14}C-labeled oral dose of methyldopa is recovered in urine [17]. With larger oral doses of the prodrug, equivalent to 500 and 1000 mg of methyldopa, the availability of methyldopa to the general circulation of man averaged 64% of the dose compared to 27% after oral methyldopa [2]. In vivo hydrolysis of the prodrug (*S,S*-diastereomer) was initially studied in rats, dogs, and rhesus monkeys after oral administration of the drug labeled with ^{3}H in the methyldopa moiety or ^{14}C in the pivalic acid moiety [3]. The differing temporal pattern of the two labels in the portal circulation and their selective solvent extraction profile from portal plasma was indicative that hydrolysis of the prodrug occurred pre-systematically and was rapid and extensive. In vitro experiments also demonstrated that plasma esterases are capable of hydrolyzing the ester at an appreciable rate in rat, dog, and human plasma [3] (see also Table I).

These data indicate that after oral administration of the POE ester, concentrations of the intact ester in biological fluids would be extremely low relative to the active hydrolysis product, methyldopa. The present HPLC method is highly sensitive and selective for the ester and was developed and applied in several animal and human studies to assess the degree to which the body is

exposed to the prodrug relative to the active therapeutic moiety, methyldopa. In these studies, blood was collected in the presence of sodium fluoride to limit any hydrolysis of the prodrug during collection and processing of the plasma samples. The assay was also used to determine if there is any stereoselectivity in absorption and/or hydrolysis of the ester by comparing results after administration of the *S,S*-diastereomer and an equimolar mixture of the *S,S*- and *R,S*-diastereomers of the drug.

In beagle dogs (n=6) given 500-mg oral doses of the single diastereomer and the mixture of diastereomers, the intact ester was not detected in plasma (<10 ng/ml); only a third of the 0—24 h urine samples contained detectable levels of the ester (<0.0003% of the dose), all below the minimum detection limit. In contrast, the urinary recovery of free methyldopa averaged 34.8% of the dose in either treatment. At the same dose level in rhesus monkeys (n=6), plasma samples contained trace levels of the intact ester varying between 8.4 and 32.8 ng/ml. The drug was present in the urine of three monkeys and accounted for less than 0.034% of the dose. No differences were observed in the bioavailability of methyldopa between the two treatments. The intact drug was detected in the portal circulation of rhesus monkeys (n=3) for 8 h after an oral 803-mg dose of the diastereomeric mixture. The area under the curve in portal plasma averaged 2.09 μg·h/ml compared to 135 μg·h/ml for methyldopa.

In a disposition study in man (n=4), intact ester was not detected in plasma after a 1000-mg oral dose of the *S,S*-diastereomer. The drug was detected in the initial 0—2 h urine collection of one subject and accounted for less than 0.0013% of the dose. Its identity was also verified by gas—liquid chromatographic—mass spectrometric analysis [18]. The single and mixed diastereomers were also compared in man (n=6) at oral doses of 1000 mg. The intact ester was detected in both treatments but did not exceed 7 ng/ml in plasma or 11.8 μg/ml (0.0012% of the dose) in urine. Methyldopa bioavailability in the two treatments was equivalent with about 40% of the dose recovered in urine as free methyldopa.

These results indicate that the prodrug is well absorbed and efficiently hydrolyzed to the active drug prior to reaching the general circulation. No stereoselectivity in absorption and hydrolysis is observed between the *S,S*- and *S,R*-diastereomers of the POE ester of methyldopa.

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