Journal of Chromatography, 427 (1988) 219–227
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4138

DETERMINATION OF D,L-*threo*-3,4-DIHYDROXYPHENYLSERINE AND OF THE D- AND L-ENANTIOMERS IN HUMAN PLASMA AND URINE

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(First received December 1st, 1987; revised manuscript received January 25th, 1988)

SUMMARY

DL-*threo*-3,4-Dihydroxyphenylserine (DOPS) is increasingly being investigated for treatment of disorders involving defects of the sympathetic nervous system, such as Parkinson's disease, Shy-Drager syndrome and congenital dopamine-β-hydroxylase deficiency. Whilst L-DOPS is converted by aromatic L-amino acid decarboxylase into natural norepinephrine in vitro, D-DOPS inhibits this process. There are no data on the interaction between D- and L-DOPS in vivo because a reliable method for the measurement of the D- and L-enantiomers in plasma and urine is lacking. We describe here such a method based on reversed-phase chromatography after derivatization with *o*-phthaldialdehyde and N-acetyl-L-cysteine. Good separation was achieved with this procedure (resolution factor 2.33). Two simple and sensitive methods are also presented for total D,L-DOPS estimation, based on reversed-phase chromatography with electrochemical detection after either deproteinization (DP) or liquid–liquid extraction (LE) as sample preparation steps. The two methods gave identical results (regression line DOPS (DP) = 1.026 DOPS (LE) + 33.28; $r = 0.997$; $n = 52$). Excellent agreement was found between the sum of the D- and L-DOPS concentrations and the measured total D,L-DOPS concentration (regression line DOPS (D + L) = 0.955 DOPS (total, LE) + 116.65; $r = 0.992$; $n = 100$).

INTRODUCTION

DL-*threo*-3,4-Dihydroxyphenylserine (DOPS), a non-physiological precursor amino acid of norepinephrine, is increasingly being investigated as a potentially useful drug for the treatment of various disorders associated with defects of the sympathetic nervous system. It has been reported to alleviate symptoms in patients with familial amyloid polyneuropathy [1,2] or the Shy-Drager syndrome [3] and in patients with parkinsonism experiencing freezing symptoms [4]. Recently, it was also reported to be successfully used in the novel orthostatic syndrome of congenital dopamine-β-hydroxylase deficiency [5,6].

0378-4347/88/$03.50

Of the four possible DOPS stereoisomers (D- and L-*threo* and D- and L-*erythro*) only L-*threo* can be converted into natural (−)-norepinephrine by aromatic L-amino acid decarboxylase [7,8]. As yet, only DL-*threo*-DOPS is commercially available. In vitro, the D-*threo* isomer has been reported to be a competitive inhibitor of the decarboxylation of L-*threo* when present in less than equimolar amounts, and a non-competitive inhibitor when present in more than equimolar amounts [9]. Administration of L-*threo*-DOPS might therefore be expected to be more effective than the administration of equivalent amounts of the racemic mixture. Although Suzuki et al. [1] found essentially the same plasma levels of norepinephrine after oral administration of L-*threo*-DOPS as after twice as much DL-*threo*-DOPS in normal subjects, the effectiveness in patients may be quite different.

We have developed a method for determining D- and L-*threo*-DOPS concentrations in plasma and urine in order to be able to evaluate the pharmacokinetic interactions of the enantiomers. The method is based on high-performance liquid chromatographic (HPLC) separation of the diastereoisomers formed by reaction of D,L-*threo*-DOPS with *o*-phthaldialdehyde and N-acetyl-L-cysteine [10–13]. For comparison, we also developed a non-stereospecific method for *threo*-DOPS determination by HPLC after either deproteinization with perchloric acid or isolation of DOPS from plasma or urine by a simple and fast liquid–liquid extraction procedure [14,15]. Both these procedures are simpler to perform than the DOPS assay described before [16], and can be used in clinical monitoring where no measurements of enantiomers are required.

EXPERIMENTAL

Materials

DL-*threo*-3,4-Dihydroxyphenylserine (DL-DOPS) and sodium dodecylsulphate were obtained from Sigma (St. Louis, MO, U.S.A.), *o*-phthaldialdehyde, ethylenediaminetetraacetic acid (EDTA), tetrahydrofuran, boric acid, methanol and 1-octanol from Merck (Darmstadt, F.R.G.), N-acetyl-L-cysteine and tetraoctylammonium bromide from Fluka (Buchs, Switzerland), diphenylborate–ethanolamine complex from Janssen (Beerse, Belgium) and *n*-heptane from J.T. Baker (Deventer, The Netherlands).

Samples of the pure D- and L-enantiomers of both *threo*- and *erythro*-DOPS were kindly donated by Dr. L. Dupuis, Neuropsychiatry Department, Hoffman La Roche (Basle, Switzerland). Boc-L-cysteine was a kind gift of Dr. R.H. Buck, Pharmaceutical Department, Sandoz (Basle, Switzerland).

Blood and urine for DOPS determination were obtained from two patients with congenital dopamine-β-hydroxylase deficiency, who were treated with DL-*threo*-DOPS. Blood was collected in chilled heparinized polystyrene tubes containing 12 mg of glutathione and centrifuged within 15 min at 4°C (15 min, 3000 *g*). Plasma was stored at −70°C. Urine was collected in 1-l plastic bottles containing 250 mg of EDTA and 250 mg of sodium metabisulphite and stored at −20°C.

Apparatus

The instrumentation for the isocratic chromatography consisted of a Kratos SF-400 pump, a Rheodyne 7125 injection valve equipped with a 100-μl loop, a Spark SpH99 column oven, an amperometric electrochemical detector as described by Van Valkenburg et al. [17], and a Merck–Hitachi D-2000 integrator. For gradient chromatography, a Kratos system consisting of two SF-400 pumps, an SF-410 gradient mixer and an SF-450 gradient controller was used, as well as two Rheodyne injection valves equipped with a 10-ml and a 20-μl loop, respectively, a Kontron SFM 23/B spectrofluorimeter (xenon lamp source) and a Merck–Hitachi D-2000 integrator.

All separations were performed on 3-μm Cp^{TM} MicroSpher C_{18} (100 mm$\times$4.6 mm I.D.) columns (Chrompack, Middelburg, The Netherlands).

D- and L-threo-DOPS (D/L method)

The derivatization reagent was prepared freshly every day by dissolving 30 mg of *o*-phthaldialdehyde in 1 ml of methanol and adding 22 ml of 0.4 *M* sodium borate buffer (pH 10.0) and 30 mg of N-acetyl-L-cysteine.

DOPS was extracted from plasma (100 μl to 1 ml) or 100 μl of (diluted) urine by the liquid–liquid extraction procedure described before [15]. Back-extraction of DOPS from the organic phase was carried out with 200 μl of 0.4 *M* acetic acid. To 100 μl of the aqueous layer were added 400 μl of derivatization reagent under vigorous stirring. After exactly 10 min, 20 μl of the solution were injected into the HPLC system.

Mobile phase A was 0.05 *M* sodium phosphate (pH 6.5), and mobile phase B was prepared by adding 20 ml of tetrahydrofuran and 10 ml of methanol to 1 l of mobile phase A. Separation was achieved by a rapid linear gradient of 0 to 100% B in 0 to 1 min, followed by isocratic elution with B, at a flow-rate of 1 ml/min. The fluorescent derivatives were monitored with excitation at 344 nm and emission at 443 nm.

For rapid removal of slow-eluting compounds of unknown identity, 6 ml of methanol–water (60:40) were injected, after elution of the DOPS derivatives, through a second injection valve that was installed in front of the first injection valve and equipped with a 10-ml loop.

A pooled plasma sample and a series of standard solutions of (racemic) *threo*-DOPS were included in each assay. Quantitation was done by comparing peak areas with those of standards of the same enantiomer.

D,L-threo-DOPS

Sample preparation by liquid–liquid extraction (LE method). DOPS was extracted from plasma or urine by the same liquid–liquid extraction procedure as described above. Routinely, 50 μl of plasma or 100 μl of 100-fold diluted urine were extracted, and back-extraction was performed by the addition of 450 μl of 0.4 *M* acetic acid.

Sample preparation by deproteinization (DP method). To a polypropylene tube were added 100 μl of plasma or 100-fold diluted urine and 100 μl of 0.01 *M* hydro-

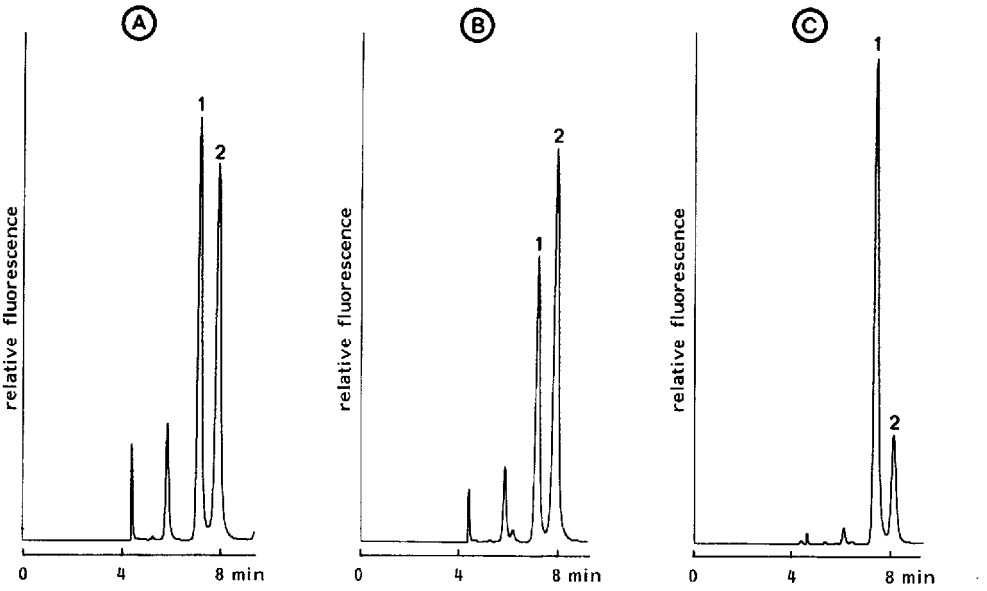

Fig. 1. Separation of D- and L-*threo*-DOPS after derivatization with *o*-phthaldialdehyde and N-acetyl-L-cysteine. (A) Standard mixture (2500 ng/ml each); (B) plasma sample (D- and L-*threo*-DOPS, 1860 and 2980 ng/ml, respectively, D/L ratio 0.62); (C) urine sample (D- and L-threo-DOPS, 7500 and 1800 ng/ml, respectively, D/L ratio 4.17). For conditions see Experimental. Peaks: 1 = D-*threo*-DOPS; 2 = L-*threo*-DOPS.

chloric acid (or DOPS standard). Under vigorous stirring 100 μl of 1.2 *M* perchloric acid were added, and the tube was centrifuged (15 min, 4 °C, 8000 *g*). Then 50 μl were carefully transferred to a new tube and mixed with 250 μl of 0.01 *M* hydrochloric acid.

Chromatography. A 100-μl volume of the solution resulting from the LE or DP sample preparation was injected into the HPLC system. The isocratic separation was achieved at 35 °C with a flow-rate of 1 ml/min. The mobile phase was 0.025 *M* disodium hydrogenphosphate, containing 0.347 m*M* sodium dodecylsulphate, 0.269 m*M* EDTA and 10% methanol; the pH was adjusted to 2.1 with phosphoric acid. The electrochemical detector was operated at +800 mV vs. an Ag/AgCl reference electrode at a sensitivity setting of 250 nA/V. Calculated peak areas were compared with those of standard amounts of DOPS for determination of plasma DOPS concentrations.

Standards (125, 25 and 2.5 ng of DOPS) and two plasma pools, both with and without added DOPS (25 ng) were included in each assay.

RESULTS

D- and L-threo-DOPS (D/L method)

Under the conditions employed, the D-*threo*-DOPS derivative eluted before the L-*threo*-DOPS derivative at respective retention times of 7.14 and 7.90 min (Fig. 1). The resolution factor was calculated to be 2.33. The specific fluorescence with

TABLE I

REPRODUCIBILITY OF THE D/L METHOD FOR THE DETERMINATION OF THE D- AND L-*threo*-DOPS ENANTIOMERS

	Intra-assay, pool 1 ($n=9$)		Inter-assay, pool 2 ($n=15$)	
	Mean (ng/ml)	C.V. (%)	Mean (ng/ml)	C.V. (%)
D-*threo*-DOPS	3379	2.3	4392	5.2
L-*threo*-DOPS	3358	2.6	4409	5.4
D/L Ratio	1.006	0.9	0.996	0.7

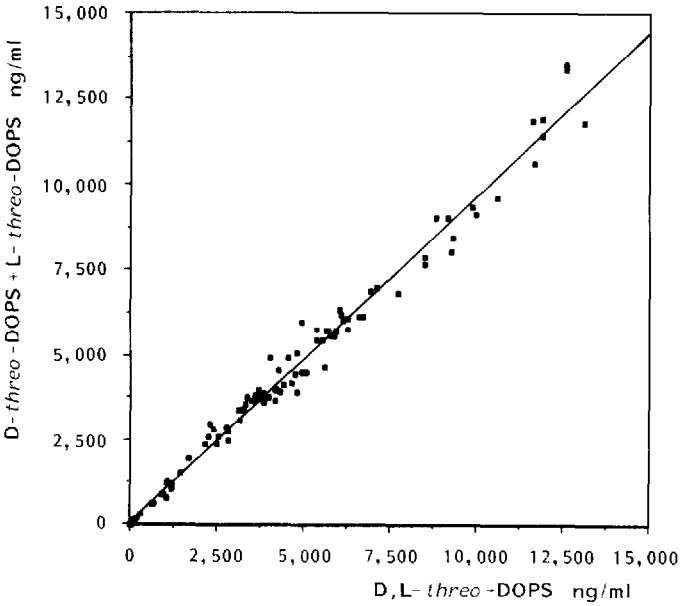

Fig. 2. Correlation between the sum of D- and L-*threo*-DOPS concentrations (D/L method) and total D,L-*threo*-DOPS concentrations (LE method). Regression line: $y=0.955x+116.65$; correlation coefficient, 0.992; $n=100$.

D-*threo*-DOPS is 93.9% of that with L-*threo*-DOPS ($n=9$, coefficient of variation 0.5%). No interfering peaks were detected. The *erythro*-DOPS derivatives eluted much later, at 17.19 min (D) and 19.13 min (L), with a resolution factor of 2.57.

The measured fluorescence intensity decreased slowly as a function of the derivatization reaction time (ca. 20% from 1 to 30 min); 10 min was found to give good and reproducible results, as noted before [13].

Reproducibility of the procedure was established with plasma pools of DL-*threo*-DOPS (Table I). Mean recovery of a standard in 31 consecutive assays was 96.2% for both L- and D-DOPS, with standard deviations of 3.8 and 4.3%, respectively.

As a check on the validity of the D/L method, the sum of the D- and L-*threo*-DOPS concentrations as measured by the method was compared with the total concentration as determined by the LE method. As can be seen in Fig. 2, excellent agreement was obtained.

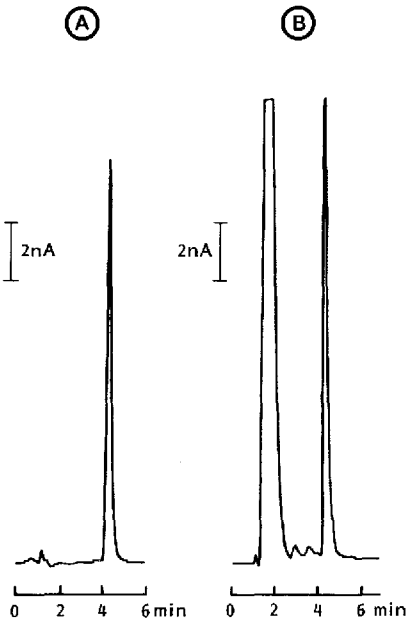

Fig. 3. Chromatograms of D,L-*threo*-DOPS determinations in plasma. (A) LE method (plasma D,L-*threo*-DOPS, 2350 ng/ml); (B) DP method (plasma D,L-*threo*-DOPS, 5075 ng/ml). For conditions see Experimental.

Although the sensitivity of the fluorimeter is such that the derivative of 1 ng of DOPS can be well detected, the dilution by the derivatization procedure and the smaller injection volume cause the D/L method to be less sensitive than the LE method for plasma measurements (see below). The detection limit of the D/L method is ca. 60 ng/ml for each enantiomer when 1 ml of plasma is used for sample preparation. On the other hand, the extra selectivity provided by the derivatization procedure makes possible the use in the extraction procedure of 100 μl of undiluted urine, thereby leading to a detection limit of ca. 600 ng/ml in urine.

D,L-threo-DOPS (LE and DP methods)

Under the chromatographic conditions employed, D,L-*threo*-DOPS gives a single sharp peak with a retention time of 4.1 min. In the DP method a large front peak is always present, which is absent when the more selective LE method is used (Fig. 3). No interferences were ever seen, except when undiluted urine was used. The detector response is linear in the range 0.1–100 ng DOPS injected, whereas the LE method described is useful at least in the range 2.5–625 ng DOPS. Recovery of standards with the LE method was $98.4 \pm 3.6\%$ ($n=25$) in a concentration range of 125–3125 ng/ml.

A high- and low-DOPS plasma pool, both with and without added DOPS, were used to evaluate intra- and inter-assay variabilities and recoveries of the added DOPS. Variabilities are presented in Table II. Recoveries of added DOPS were 97.1 ± 3.5 and $95.6 \pm 8.5\%$, respectively, for 5000 and 500 ng/ml added DOPS in

TABLE II

REPRODUCIBILITY OF THE LE AND DP METHODS FOR D,L-*threo*-DOPS DETERMINATION

Pool No.	LE method						DP method					
	Intra-assay			Inter-assay			Intra-assay			Inter-assay		
	n	Mean (ng/ml)	C.V. (%)	*n*	Mean (ng/ml)	C.V. (%)	*n*	Mean (ng/ml)	C.V. (%)	*n*	Mean (ng/ml)	C.V. (%)
1	6	4834	3.2	8	4878	3.2	6	5292	2.3	8	5092	4.2
1*	6	9695	0.6	8	9733	2.5	6	8108	1.2	8	7833	4.5
2	6	514	2.8	8	517	5.7	6	525	2.1	8	504	4.1
2*	6	999	1.9	8	989	7.7	6	794	3.3	8	754	3.5

*Pool spiked with a known amount of DL-*threo*-DOPS.

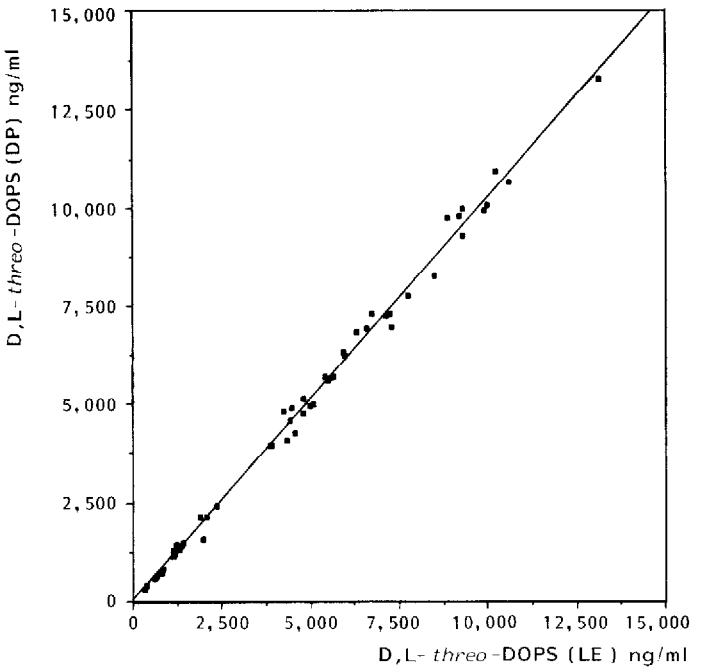

Fig. 4. Correlation between D,L-*threo*-DOPS concentrations measured with the LE and the DP method. Regression line: $y = 1.026x + 33.28$; correlation coefficient, 0.997; $n = 52$.

the LE method, and 110.9 ± 5.2 and 103.1 ± 9.2%, respectively, for 2500 and 250 ng/ml added DOPS in the DP method ($n = 14$ in all cases).

Detection limits in plasma with the standard assay procedures were 10 ng/ml for the LE method and 20 ng/ml for the DP method at a signal-to-noise ratio of 3. If necessary, the sensitivity of the LE method can be increased 100-fold by using more plasma (up to 1 ml), by performing the back-extraction with 200 µl of 0.4 *M* acetic acid and by resetting the detector sensitivity to 100 nA/V. In urine,

detection limits are much higher (± 1 μg/ml), mainly due to the 100-fold dilution step. For all practical purposes this is adequate, and attempts to increase the sensitivity by using undiluted urine were found to be both unnecessary and unsuccessful because of the appearance of large interfering peaks.

The nature of the deproteinizing process and the good recoveries with the LE method make the use of an internal standard dispensable. The use of a suitable internal standard, such as α-methylnorepinephrine, would greatly lengthen the chromatographic analysis time (to ca. 15 min).

Plasma levels of DOPS determined with the LE and the DP method are in good agreement with one another (Fig. 4).

DISCUSSION

In recent years, several papers [10–13] have described the separation of enantiomeric amino acids by reversed-phase chromatography after derivatization with *o*-phthaldialdehyde and a chiral mercaptan to form highly fluorescent diastereoisomeric isoindole derivatives. This method was found to be applicable for the separation of D- and L-*threo*-DOPS. Derivatization with N-acetyl-L-cysteine as the chiral mercaptan gave better resolution than with Boc-L-cysteine, with the added advantage that the first is commercially available. The poor resolution that we first obtained with methanol as the sole organic modifier in mobile phase B was greatly improved by (partly) substituting tetrahydrofuran for methanol.

The peaks of unknown identity, which seemed to be formed during the derivatization procedure from the ammonium chloride used in the LE extraction procedure, eluted slowly (at ca. 40 min) with mobile phase B. As an increase in organic modifier concentration in mobile phase B is not practical owing to the limited solubility of tetrahydrofuran in water and the decrease in resolution with more methanol, a simple and economical solution was found by installing a second injection valve with a 10-ml loop. By flushing the column with 6 ml of water–methanol after elution of the DOPS derivatives, these unknown compounds eluted within 5 min, and the time between sample injections could thus be reduced to 20 min. Where available, a ternary gradient system can of course be used for the same purpose.

A similar difference in specific fluorescence between diastereoisomeric derivatives as we found for DOPS has been reported for tryptophan, aspartate and phenylalaninol [10,13].

The LE method originally described by Smedes et al. [14] has, with some modifications [15], been successfully used in our laboratory for the nearly quantitative isolation and concentration from biological fluids of many compounds containing the catechol moiety. Although in preliminary experiments recovery of DOPS did not exceed 80–90%, we found that increasing the molarity of the acetic acid employed in the back-extraction step from the usual 0.08 to 0.4 *M* raised the recovery to nearly quantitative levels. Impurities in some batches of the diphenylborate–ethanolamine complex and of 1-octanol can give rise to interfering ghost peaks in the chromatograms, which can be prevented by stirring the diphenyl-

borate-containing buffer with alumina before use (45 g/l, 2 h) and by first washing the octanol with 0.08 *M* acetic acid. When acid-washed octanol is used, the addition of 200 or 450 μl of acetic acid in the back-extraction step causes concentration of DOPS in 250 or 500 μl of acetic acid.

The DP method is somewhat easier and quicker to perform than the LE method, but both are simpler than the method described before [16], which involves selective desorption of DOPS from boric acid gel columns by a 10% sorbitol solution. The LE method, however, is more sensitive, more versatile and more selective, resulting in cleaner chromatograms and better stability in the column and detector. Usually, therefore, we prefer the LE method and use the DP method only when a few samples need to be analysed quickly.

In two patients with congenital dopamine-β-hydroxylase deficiency treated with 500 mg of DL-*threo*-DOPS twice daily, we found DOPS plasma levels of 4000–8000 and 300–600 ng/ml at 2 and 12 h after dosing, and 24-h urinary concentrations ranged from 100 to 250 μg/ml. Ratios between the measured D- and L-enantiomers after a single oral dose of 500 mg of DL-*threo*-DOPS ranged from 0.4 (in plasma after 30 min) to 33 (in a urine sample after 30–36 h).

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