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DETERMINATION OF URINARY AND PLASMA DIHYDROXYPHENYLALANINE BY COUPLED-COLUMN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH C_8 AND C_{18} STATIONARY PHASES

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SUMMARY

This paper describes a rapid, simple and sensitive method for measurement of L-dihydroxyphenylalanine (DOPA) in biological fluids. The method uses a two step frontal-chromatographic clean-up, followed by coupled minibore HPLC with electrochemical detection. The use of phenylboronate removes the peaks that interfere with the detection of DOPA. Inclusion of [^{14}C]DOPA corrects for any variability in recovery.

INTRODUCTION

A significant amount of L-dihydroxyphenylalanine (DOPA) is normally present in the circulation[1]. The source of this amino acid and its metabolic fate are unclear at present. DOPA is synthesized by hydroxylation of tyrosine and thus acts as an important intermediate in the pathway of catecholamine biosynthesis. DOPA is partly cleared by the kidney, where it appears to be an important precursor for urinary dopmaine[2]. In addition, DOPA also penetrates the blood brain barrier and serves as an additional precursor reservoir for brain catecholamine synthesis. Thus, the study of plasma and urinary DOPA should contribute to our understanding of both normal physiology and pathological states.

Plasma levels of DOPA, though substantially higher than those of circulating catecholamines, have been difficult to measure. Radioenzymatic assays involve conversion of DOPA to dopamine, and the intrinsic dopamine must be subtracted from the final value[1,3,4]. Since dopamine is present in significant concentrations in urine, this subtraction method can produce substantial errors in the determination of the concentration of DOPA in urine. Gas chromatographic methods have been described[5,6], but these involve the inconvenience of multiple derivatization.

High-performance liquid chromatography (HPLC) with electrochemical detection (ED) provides the necessary sensitivity[7]. However, untreated plasma or urine contains many extraneous compounds that interfere with the determination of DOPA. We have developed a rapid two-step procedure for the clean-up of urine and plasma for DOPA estimation. The concentration of DOPA in the purified eluate is

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determined by using a coupled minibore column and ED. Radioactive [^{14}C]DOPA is added to the urine or plasma sample to correct for losses during the process of purification.

MATERIALS AND METHODS

Sample preparations

Urine from each subject is collected and pooled for 24 h (due to diurnal variation in excretion). As preservatives, 20 ml of 6 *N* hydrochloric acid and 20 mg of ascorbic acid are added to the urine, which is stored at 0 – 4°C. Two ml of urine is taken for analysis and 10^4 dpm of [^{14}C]DOPA (New England Nuclear) is added to monitor recovery at each step. The urine is brought to pH 1.0–1.2 with about 0.8 ml of 2 *N* hydrochloric acid.

The first clean-up step removes the majority of interfering urochromes. A coupled column is prepared by dry-packing a 3-ml syringe (Monoject) with 20-μm porosity frit supports with 0.5 ml benzenesulphonic acid-derivatized silica (SCX) on the top and 0.5 ml methyl-derivatized silica (C_1) on the bottom (both from Analytichem). This column is primed with 5 ml of methanol, followed by 5 ml of 0.2 *N* hydrochloric acid. The urine 2.8 ml and two bed volumes of 0.2 *N* hydrochloric acid wash are collected by plunger pressure and pooled in a graduated test tube. The cationic urochromes are retained on the SCX phase and hydrophobic urochromes on the C_1 phase.

This combined extract is brought to pH 7.5–7.7 with 1.5 ml of 2 *M* Tris, and one fourth of this volume is taken for further processing. A 1-ml syringe with 20-μm porosity frit supports is dry-packed with 0.1 ml (0.2 gram) of phenylboronate silica (Analytichem) and is primed with 1 ml of methanol and 1 ml of 0.2 *M* Tris. The urine is passed through this column, followed by successive washes of 2 ml methanol and 1 ml of 0.1 m*M* Tris (pH 7.5) to remove residual urinary contaminants. A low dead-volume 0.45-μm filter (Millipore SJHV 0.45 μm) is fitted on the lower end of the boronate column and the covalently bound DOPA is eluted with 0.3 ml of 0.1 *N* hydrochloric acid into a volumetric Reactivial. The boronate column can be recycled ten to fifteen times by repriming with the same reagents. The clear acidic eluate is stable at −20°C for over six months.

For 1 ml of plasma, 30 mg of sodium meta bisulfite (Baker) and 10^4 dpm of [^{14}C]DOPA are added. Plasma proteins are precipitated by adding 0.1 ml of ice-cold 2 *N* perchloric acid and mixing vigorously. After 20 min of incubation at 4°C, suspended solids are removed by centrifugation at 16,000 *g* for 20 min. To 0.55 ml of the clear supernatant 0.45 ml of 2 *M* Tris is added to bring the pH to 7.5–7.7.

DOPA is bound to and then eluted from the phenylboronate column, as described for the preparation of urinary samples. DOPA (Sigma) for standard was dissolved in 0.1 *N* hydrochloric acid.

HPLC instrumentation

The HPLC system consists of a Waters 6000A pump with microflow modifications, a Waters U6K injector, a LC-4B electrochemical detector (Bioanalytical Systems) and a Linear strip-chart recorder. To enhance the lower limit of detection, a minibore (2.1 mm I.D.) Brownlee column, consisting of 3 cm C_{18} and 22 cm C_8

reversed-phase (5-μm particle size) cartridges in series, is used. Additionally, the TL-5A glassy carbon detector cube is modified by using the low-volume stainless-steel cell top, which enhances the peak separation. The detector potential is set at 700 mV.

Chromatography

The optimal flow-rate was found to be 150 μl/min. The mobile phase consists of 50 m*M* citric acid–sodium acetate buffer (pH 2.35) to which 0.2 m*M* disodium EDTA is added to reduce the detector noise. No organic modifier or ion-pairing reagent is used. A 10-μl volume of the eluate is injected into the column for quantitation.

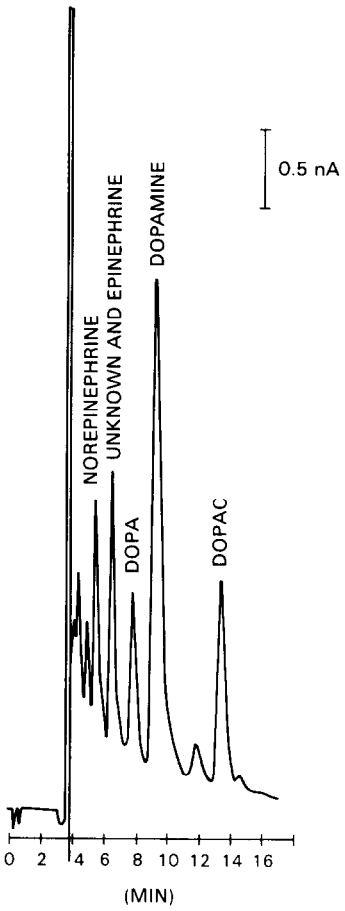
Quantitation

A standard curve is constructed for each assay by using different amounts of DOPA added to 1-ml aliquots of plasma or 2-ml aliquots of urine, which are then carried through the entire procedure. The amount of DOPA used covers the expected range of DOPA in the samples to be assayed. The concentration of DOPA in plasma or urine sample is determined by extrapolating the peak height to the constructed standard curve of DOPA. This is then multiplied by a dilution factor of 60 and by percentage recoveries to obtain the concentration DOPA in the sample.

RESULTS

A clearly separated peak, representing DOPA, is seen 7.6 min after injecting the DOPA standard in 0.1 *N* hydrochloric acid. In preliminary studies we found that an alumina extraction of urine did not give satisfactory results due to background interference from urochromes. Using the two-step extraction procedure described, a clearly separated peak of DOPA is eluted at 7.6 min with k' 1.3, along with other catecholamines and dihydroxyphenyl acetic acid (Fig. 1). With plasma samples, similar results are obtained but without evidence of a dopamine peak. The standard curve is linear throughout the range tested (20 pg to 50 ng). A portion of this curve is shown in Fig. 2. To examine the efficiency of our extraction procedure, we estimated the recovery of DOPA by using [^{14}C]DOPA. The recovery was 96 $\pm$ 2% (mean $\pm$ S.D.) (n = 10) for the first step of purification. The recovery for the second step of purification was 80 $\pm$ 2% (n = 10) for urine and 84 $\pm$ 3% (n = 6) for plasma. The minimum detectable amount of DOPA in urine and plasma was 10–15 pg. The purity of the DOPA was tested by several methods: (1) no peak was detected when the urine or plasma sample was preincubated with L-aromatic amino acid decarboxylase (Sigma) (Fig. 3); (2) the peak height was increased in direct proportion to exogenously added DOPA (Fig. 4); (3) The [^{14}C]DOPA was eluted with the electrochemically detected peak of DOPA. Table I shows the DOPA values in urine and plasma of healthy volunteers.

Several HPLC parameteres were examined, including pH and ionic strength of the mobile phase and HPLC column characteristics, to determine their influence on enhancing peak separation without significantly prolonging the analysis time for each sample. When a 25-cm C_8 cartridge was used, the separation of DOPA from the other peaks was poor (Fig. 5). A coupled (3 cm of C_{18} with 22 cm of C_8) 25-cm



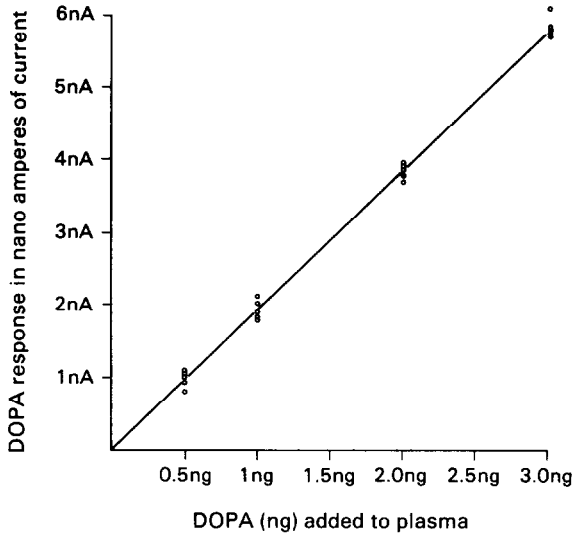


Fig. 1. Chromatogram of DOPA standard in 0.1 *N* hydrochloric acid. A clearly separated peak, representing DOPA with a k' of 1.3 is seen. DOPAC = dihydroxyphenyl acetic acid. Coupled column, 3 cm C_{18} and 22 cm C_8, in series.

Fig. 2. The standard curve for DOPA, constructed by adding four different concentrations of DOPA to 2 ml of urine or 1 ml of plasma and carrying them through the entire procedure.

cartridge was therefore utilized for all analyses. Using this column, a sample can be injected every 15 min. Minor changes in pH of the mobile phase were found to have a major effect on the retention characteristics of DOPA (Table II). The pH selected for the mobile phase did not significantly alter the peak charcteristics, even after six months of continuous use.

DISCUSSION

This procedure is dependent on an effective removal of the many interfering urinary compounds that vitiate HPLC analysis. The urochromes that interfere are removed by the first purification step. A low pH column equilibration and wash are mandatory for maximizing the recovery of DOPA. The second step of purification

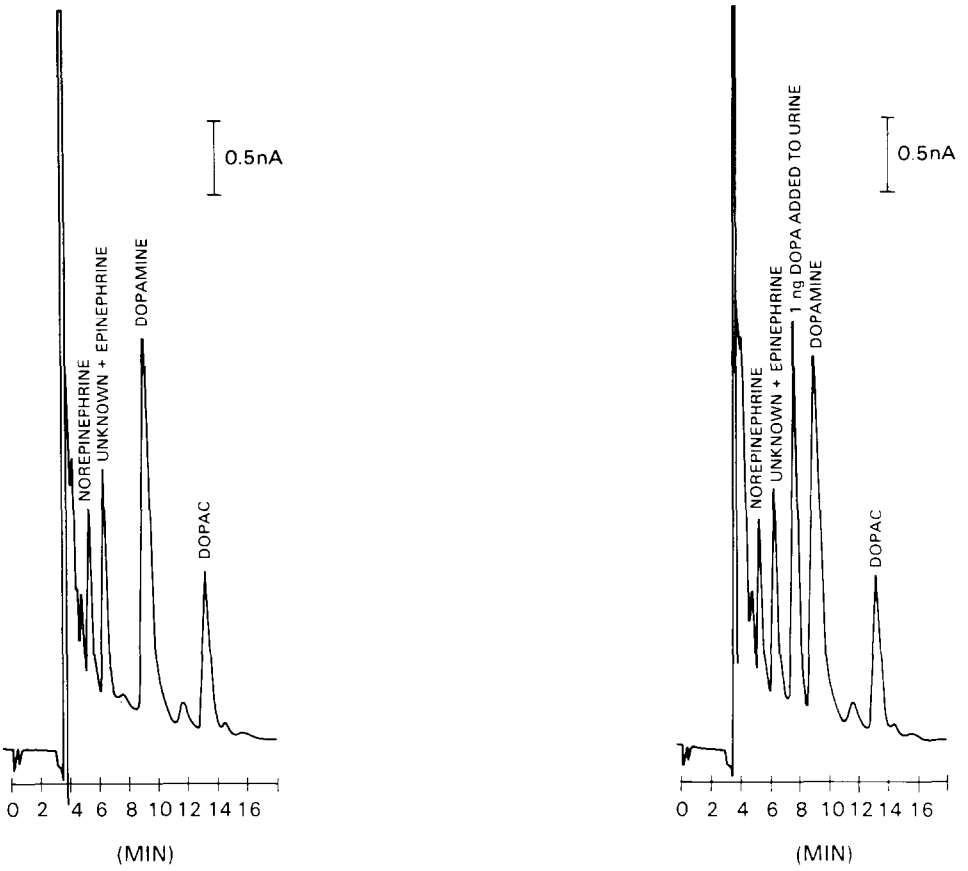


Fig. 3. Absence of peak representing DOPA after treatment of the urine sample with L-aromatic amino acid decarboxylase.

Fig. 4. The increase in the height of the peak representing DOPA after the addition of 1 ng of internal standard to a urine sample.

depends on covalent bonding of the adjacent diol functionalities of DOPA. These bonds are broken in acidic medium and DOPA is eluted from the column. This procedure has all the advantages of the use of alumina without some of its drawbacks. The recovery is uniformly high, residual interferences are low and the tedious mixing

TABLE I

DOPA CONCENTRATIONS IN HUMAN BIOLOGICAL FLUIDS

Urine: first morning specimen from seven adult men and women, 22–50 years, fasting overnight. Values in parenthesis represent $\mu g/g$ of creatinine. Plasma: heparinized plasma from seven adult men and women, 22–50 years, supine with indwelling venous catheter for 30 min, 3 h after a carbohydrate breakfast.

Sample	*DOPA concentration (ng/ml)*
Urine	59.6 ± 9.7 (43.4 ± 4.2)
Plasma	2.12 ± 0.31

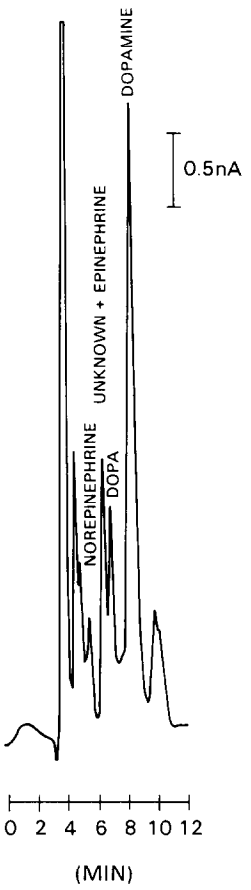


Fig. 5. Effect of substituting 3 cm of C_{18} by 3 cm of C_8 on the retention time of DOPA (25 cm of C_8). Note also the poor separation of other, related biogenic amines.

TABLE II

THE EFFECT OF pH AND IONIC STRENGTH OF MOBILE PHASE ON RETENTION OF DOPA ON 3 cm OF C_{18} AND 22 cm C_8 REVERSED-PHASE COLUMN IN SERIES

Capacity factor (k')	*Molarity* (mM)*	*Capacity factor (k')*	*pH***
1.30	50	1.30	2.35
1.25	100	1.24	2.40
1.19	150	1.18	2.45
1.14	200	1.10	2.5

* The molarity of the mobile phase was increased while keeping the pH at 2.35.

** The pH of the mobile phase was changed while keeping the ionic strength at 50 m*M*.

required for binding of catechols to alumina is obviated. One urine sample can be processed up to the HPLC step within 5 min by using gentle suction or plunger pressure. Using a suitable vacuum manifold (Vac-Elute from Analytichem), ten samples can be processed in 30 min.

Other methods have been described recently, involving a clean-up prior to HPLC. In most cases adsorption on alumina, followed by acid elution is required. We have found that, for urine samples, alumina adsorption alone did not provide adequate cleaning prior to HPLC injection. The use of SCX matrix in conjunction with alumina has been described[8]. A rapid two- or three-step cleanup by C_{18} and other silica matrices has also been developed[9]. However, for cleaning the urine prior to the determination of DOPA, all of these methods produce low, variable recoveries. We observed a more uniform recovery by replacing the C_{18} matrix with C_1 and by removal of the cations in urine with SCX. More recently, boronate gels have been employed for sample preparation[10–12]. This functionality confers the advantage of high specificity: in neutral or basic medium a stable, bivalent covalent bond is formed between the immobilized boronate ion and the *vicinal* diol groups on the catechol molecules, and they are readily eluted by acid[13]. The gels previously used required slow flow-rates, since they are compressible at elevated pressure. For this reason, the availability of a rigid, silica-derived boronate matrix, useable at high flow-rates and having good binding ability has the advantage of permitting a rapid passage of sample with excellent recoveries.

The mobile phase was optimized by careful examination of all major variables. It was found that low ionic strength and low pH both improved the separation of DOPA from other peaks. A good compromise between resolution and duration of chromatography was achieved by using coupled columns. The significant effect of even minor (0.5-unit) alterations in pH demonstrates that an additional mechanism of separation is significant for these coupled columns. The major effect of alkane stationary phases in aqueous media is hydrophobic interactions with solvent–analyte partitioning. The effect of varying the hydrocarbon chain length indicates that this mechanism is a major component in affecting the retention characteristics of the catechols tested.

The effect of H^+ is presumably due to polar interaction with the silanol groups of the matrix[14]. It is known that even after exhaustive endcapping, steric hinderance precludes the derivatization of all silanol groups. The interactions with these residual groups were quite beneficial in permitting modulation of separation characteristics without recourse to ion-pairing reagents[15]. This additional selective parameter confers an advantage in situations where coupled-column chromatography can be utilized.

REFERENCES

1 G. A. Johnson, J. M. Green and R. Kupiecki, *Clin. Chem.*, 24 (1978) 1927.
2 M. J. Brown and D. J. Allison, *Br. J. Clin. Pharmacol.*, 12 (1981) 251.
3 G. Zurcher and M. DaPrada, *J. Neurochem.*, 33 (1979) 631.
4 M. J. Brown and C. T. Dollery, *Br. J. Clin. Pharmacol.*, 11 (1981) 79.
5 Y. Mizuno, *Clin. Chim. Acta.*, 74 (1977) 11.
6 C. Hansson, G. Agrup, H. Rorsman, A.-M. Rosengren and E. Rosengren, *J. Chromatogr.*, 162 (1979)

7 A. Shum, M. J. Sole and G. R. van Loon, *J. Chromatogr.*, 228 (1982) 123.
8 G. S. Mayer and R. E. Shoup, *J. Chromatogr.*, 255 (1983) 533.
9 B.-M. Eriksson, E. Gustafsson and B.-A. Persson, *J. Chromatogr.*, 278 (1983) 255.
10 K. N. Frayn and P. F. Maycock, *Clin. Chem.*, 28 (1983) 1426.
11 D. S. Goldstein, *J. Chromatogr.*, 275 (1983) 174.
12 B. Kagedal and A. Petterson, *Clin. Chem.*, 29 (1983) 2031.
13 L. Hansson, M. Glad and C. Hansson, *J. Chromatogr.*, 265 (1983) 37.
14 H. Svendsen and T. Greibrokk, *J. Chromatogr.*, 212 (1981) 153.
15 J. Wagner, P. Vitali, M. G. Palfreyman, M. Zarika and S. Huot, *J. Neurochem.*, 38 (1982) 1241.