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SIMULTANEOUS MEASUREMENT OF URINARY AND PLASMA NOREPINEPHRINE, EPINEPHRINE, DOPAMINE, DIHYDROXYPHENYLALANINE, AND DIHYDROXYPHENYLACETIC ACID BY COUPLED-COLUMN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON C_8 AND C_{18} STATIONARY PHASES

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SUMMARY

This paper describes a simple and sensitive method for measuring norepinephrine, epinephrine, dopamine, dihydroxyphenylalanine, and dihydroxyphenylacetic acid in urine and plasma. This method involves a two-stage frontal-chromatographic clean-up, followed by coupled minibore high-performance liquid chromatography with electrochemical detection. The use of phenylboronate instead of alumina yields recoveries in the range of 80–90% for all the catecholamines, dihydroxyphenylalanine and dihydroxyphenylacetic acid without interferences. The interassay coefficient of variation for the measurement of these compounds was less than 12%.

INTRODUCTION

Catecholamines are important neurotransmitters and circulating hormones. Investigations into the synthesis and metabolism of these monoamines has been facilitated by the advent of high-performance liquid chromatography (HPLC) with electrochemical detection (ED). HPLC–ED possesses adequate sensitivity and specificity for measuring catecholamines and their metabolites in brain tissue. For biological samples, such as urine or plasma, a purification step is necessary to remove potential interfering compounds and increase the specificity. This purification step has been performed mainly on activated alumina[1]. The selective adsorption of *cis*-diols on alumina yields purified samples for analysis. However, this process introduces variability[2] and is relatively time-consuming.

A rapid two-stage method is described for the clean-up of urine and plasma samples for the estimation of norepinephrine (NE), epinephrine (E), dopamine (DA), dihydroxyphenylalanine (DOPA), and dihydroxyphenylacetic acid (DOPAC). The concentration of these compounds in purified eluate is determined by the use of 2.1 mm I.D. coupled-column (C_8 in series with C_{18}) HPLC and ED. ^{14}C-Labeled NE, E, DA, DOPA and DOPAC are added to the samples to correct for losses during the process of purification.

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MATERIALS AND METHODS

Sample preparation

Urine from each subject is collected and pooled for 24 h (to correct for diurnal variations). As preservative, 20 ml of 6 *N* hydrochloric acid are added to the urine, which is stored at 0–4°C during collection. For analysis 2 ml of the pooled urine sample are used. To monitor recovery 10^4 dpm each of the radioactive ^{14}C-catecholamines, DOPA and DOPAC (New England Nuclear, Boston, MA, U.S.A.) in 10 μl of 0.2 *N* acetic acid is added to the 2-ml urine sample and then acidified to pH 1.0–1.2 with 0.8 ml of 2 *N* hydrochloric acid.

The majority of interfering urochromes are removed by the first step, described here: a coupled column is made by dry-packing a 3-ml syringe (Monoject, Sherwood Medical, St. Louis, MO, U.S.A.) with a 20-μm porosity frit support with 0.5 ml methyl-derivatized silica (C_1) on the bottom and 0.5 ml benzenesulphonic acid-derivatized silica (SCX) on the top (both from Analytichem, Harbour City, CA, U.S.A.). This is primed with 5 ml of methanol, followed by 5 ml of 0.2 *N* hydrochloric acid. The 2.8 ml of urine is passed through the column by plunger pressure followed by 1 ml of 0.3 *N* hydrochloric acid to wash the column. The wash is pooled with the urine in a graduated test tube. The SCX phase retains the cationic urochromes, while the hydrophobic urochromes are trapped inthe C_1 phase.

The pooled eluate is adjusted to pH 7.2–7.5 with 1.6 ml of 2 *M* Tris (pH 10.5) and 1.5 ml of this mixture is used for subsequent steps. A 1-ml syringe with a 20-μm porosity frit support is dry-packed with 0.2 ml (0.4 g) of phenylboronate silica (Analytichem) and is primed with 1 ml of methanol and 1 ml of 0.3 *M* Tris (pH 9.4). The 1.5 ml of the eluate is passed through the column, followed by successive washes of 2 ml methanol and 1 ml of 0.1 m*M* bis-Tris (pH 7.5) (Sigma, St. Louis, MO, U.S.A.). This probably removes the residual contamination from urochromes and minimizes the solvent front on HPLC. A small-dead-volume 0.45-μm filter (Millipore SJHV, Millipore Corp., Milford, MA, U.S.A.) is fitted to the lower end of the boronate column, and the covalently bound catecholamines, DOPA and DOPAC, are eluted with 0.3 ml of 0.1 *N* hydrochloric acid into a volumetric Reactivial (Pierce, Rockford, IL, U.S.A.). The boronate column can be recycled 5–10 times by repriming with 2 ml of methanol and 1 ml of 0.3 *M* Tris (pH 9.4). After 5–10 uses, the recovery of catecholamines decreases below 50%. Between runs, the boronate column is kept stored in methanol. The acid eluate is stable at −20°C for six months.

For 2 ml of heparinized venous plasma 30 mg of sodium metabisulfite (J. T. Baker, Phillipsburg, NJ, U.S.A.) and 10^4 dpm of ^{14}C-catecholamines, DOPA and DOPAC, in 10 μl of 0.2 *N* acetic acid is added. Then 0.2 ml of ice-cold 2 *N* perchloric acid is added with vigorous mixing to precipitate the plaslma proteins. After 20 min of incubation at 4°C, the precipitate formed is removed by centrifugation at 16000 *g* for 20 min. To 1.1 ml of the clear supernatant 0.9 ml of 2 *M* Tris (pH 10.5) is added to bring the pH to 7.2–7.5.

Catecholamines are bound to and then eluted from the phenylboronate column, as described under the preparation of urine samples. The 300 μl of 0.1 *N* hydrochloric acid eluate is frozen to −20°C and then evaporated to dryness in a Savant (Hicksville, NY, U.S.A.) vacuum centrifuge in 10–15 min. The residue is reconstituted in 20 μl of ice-cold 0.05 *N* hydrochloric acid. NE, E, DA, DOPA (all from

Sigma) and DOPAC (Aldrich, Milwaukee, WI, U.S.A.) as standards were dissolved in ice-cold 0.1 *N* hydrochloric acid.

HPLC instrumentation

The system consisted of a Waters (Milford, MA, U.S.A.) 6000A pump with microflow modifications, a Waters U6K injector with a 20-μl injection loop, a LC-4B electrochemical detector (Bioanalytical Systems, West Lafayette, IN, U.S.A.) and an Omniscribe (Houston Instruments, Houston, TX, U.S.A.) strip-chart recorder. To enhance the lower limit of detection, a minibore (2.1 mm I.D.) Brownlee column (Santa Clara, CA, U.S.A.) consisting of a 3-cm C_{18} and 22-cm C_8 reversed-phase (5-μm particle size) cartridge in series was used. Additionally, all the connecting tubing was kept as short as possible, and the TL-5A glassy carbon detector cube was modified with the low-volume stainless-steel auxiliary top (BAS/MF 1018), all of which enhanced the peak separation. The detector was operated at a potential of +700 mV *vs.* Ag/Agcl.

Chromatography

The optimal flow-rate was found to be 160 μl/min. The mobile phase consisted of 55 m*M* citric acid–sodium acetate buffer (2 ml of 1 *M* sodium acetate added to 750 ml of 55 m*M* citric acid) (pH 2.35) to which 0.35 m*M* disodium EDTA is added to reduce the detector noise. No ion-pairing reagents or organic modifiers were used. A 10-μl volume of the eluate was injected into the column for quantitation using a Hamilton 50-μl syringe (Hamilton, Co., Reno, NV, U.S.A.). When the column was not in use the mobile phase was recycled through the column to keep the chromatographic conditions stable.

Quantitation

A standard curve was constructed for each assay from different amounts of NE, E, DA, DOPA and DOPAC added to 2-ml aliquots of plasma or 2-ml aliquots of urine as standard additions, and these are then carried through the entire procedure. The amount of standard added covers the range from 20 pg to 50 ng. The concentration of NE, E, DA, DOPA or DOPAC in plasma or urine sample is determined by extrapolating the peak height to the constructed standard curve of each compound.

RESULTS

When a mixture consisting of all the catecholamine standards in 0.1 *N* hydrochloric acid was injected, NE was eluted at 5.5 min, E at 6.4 min, DOPA at 7.6 min, DA at 9.2 min, and DOPAC at 13.4 min. In preliminary studies it was found that alumina extraction of urine did not give satisfactory results due to interference from urochromes that eluted with the solvent front on chromatography. The two-stage extraction permits the separation of NE ($k' = 0.5$), E ($k' = 0.83$), DOPA ($k' = 1.11$), DA ($k' = 1.5$), and DOPAC ($k' = 2.72$) (Fig. 1). With plasma samples, similar results are obtained, the DA peak being slightly smaller than the E peak. The standard curve was linear throughout the range tested (20 pg to 50 ng). To examine the efficiency of the extraction steps, the recoveries of radiolabelled NE, E, DA, DOPA,

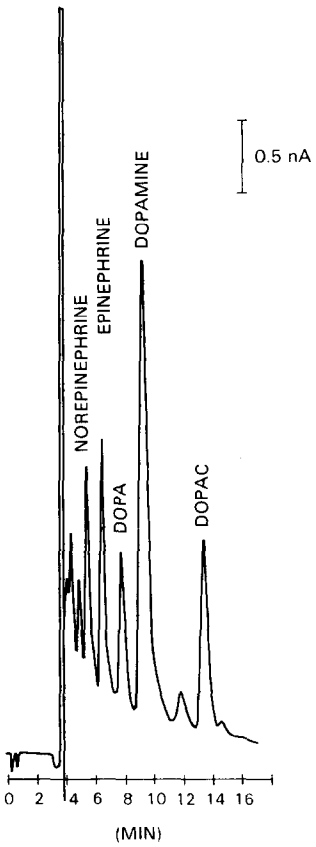

Fig. 1. Chromatogram of a random urine sample showing the various catecholamine, DOPA and DOPAC peaks. Coupled column, 3 cm C_{18} followed by 22 cm C_8.

TABLE I

SENSITIVITY AND PRECISION OF THE ASSAY

	nA of current per nanogram	*Sensitivity (pg)**	*Concentration in plasma** (pg/ml)*	*Inter-assay coefficient of variation (%)****
Norepinephrine	2.48	20–25	208 ± 34	8
Epinephrine	2.8	15–20	42 ± 26	7
Dopamine	1.4	40–50	39 ± 20	12
Dihydroxyphenylalanine	1.8	30–35	2400 ± 401	7
Dihydroxyphenylacetic acid	1.6	35–40	1380 ± 367	9

* Picograms of compound needed to produce a signal-to-noise ratio of 2.

** Heparinized plasma from 12 adults (6 males and 6 females) 22–46 years old supine with indwelling catheter for 30 min, 2 h after a carbohydrate breakfast (mean ± 1 S.D.).

*** Calculated from the peak height for 100-pg standards added for six different plasmas and done on six different assays (days).

and DOPAC were determined. The recoveries were: NE 86 ± 2%, E 87 ± 3%, DOPA 96 ± 2%, DA 94 ± 3%, and DOPAC 90 ± 2% (mean ± 1 S.D.) (n = 10) for the first stage of purification. The recoveries for the second stage were NE 82 ± 3%, E 80 ± 2%, DOPA 84 ± 3%, DA 86 ± 4%, and DOPAC 81 ± 2% (n = 10). The values were similar for both urine and plasma. The sensitivity and precision of the assay are given in Table I. The purity of the catecholamine peaks was tested by several methods: (1) no NE, E, DA or DOPA peaks were detected when the urine or plasma sample was incubated with L-aromatic amino acid decarboxylase and catechol-O-methyl transferase (Fig. 2); (2) the peak heights were increased in direct proportion to exogenously added NE, E, DOPA, DA, and DOPAC (Fig. 3); (3) the radiolabelled NE, E, DOPA, DA, and DOPAC were eluted with the corresponding electrochemically detected peaks. This was demonstrated by collecting 20-μl aliquots of the outflow from the electrochemical detector and counting it in a Beckman 3701 liquid scintillation counter.

Several chromatographic parameters were examined, including pH and ionic strength of the mobile phase and LC column characteristics, to determine their role

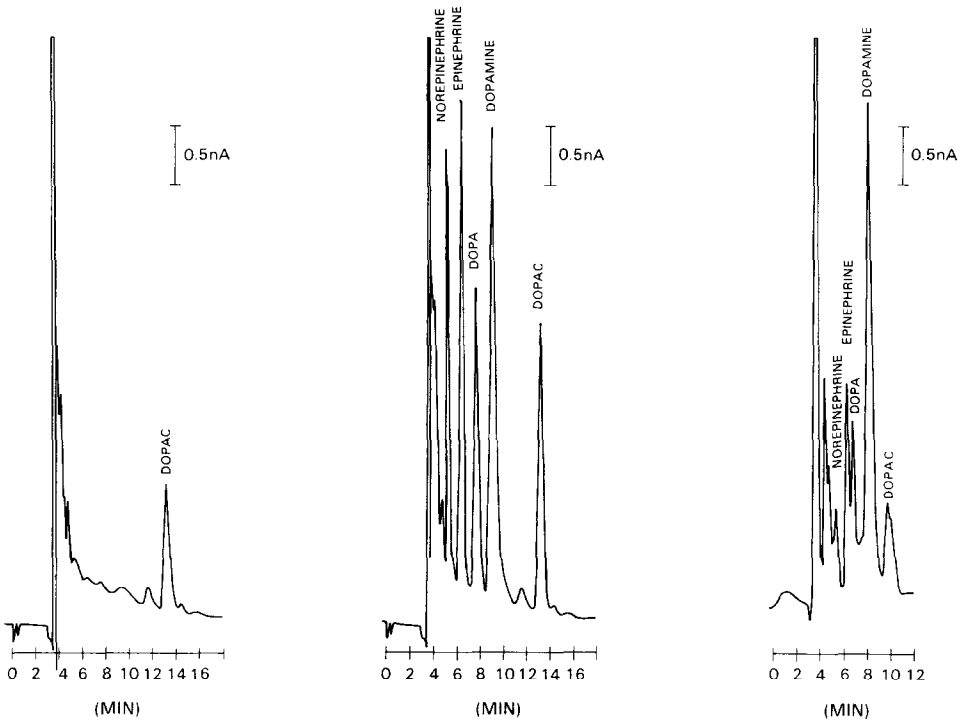

Fig. 2. Absence of peaks representing norepinephrine, epinephrine, dopamine, and DOPA after treatment of urine sample with catechol-O-methyl transferase and L-aromatic amino acid decarboxylase.

Fig. 3. The increase in the peak heights after adding 2 ng each of norepinephrine, epinephrine, DOPA, dopamine, and DOPAC to a urine sample.

Fig. 4. Effect of substituting a 3-cm column of C_{18} by a 3-cm column of c_8 (coupled with a 22-cm column of C_8) on the retention time of catechols.

TABLE II

EFFECT OF pH AND IONIC STRENGTH OF MOBILE PHASE ON THE CAPACITY FACTORS (k') OF NE, E, DOPA, DA, AND DOPAC ON 3 cm OF C_{18} AND 22 cm OF C_8 REVERSED-PHASE COLUMNS, COUPLED IN SERIES

Ionic strength (mM)	*pH*	*NE*	*E*	*DOPA*	*DA*	*DOPAC*
55	2.35	0.50	0.83	1.11	1.50	2.72
105	2.35	0.46	0.78	1.05	1.54	2.61
155	2.35	0.42	0.73	0.99	1.35	2.49
55	2.35	0.5	0.83	1.11	1.50	2.72
55	2.40	0.44	0.76	1.04	1.41	2.65
55	2.45	0.37	0.68	0.97	1.30	2.57

in enhancing peak separation without significantly increasing the analysis time for each sample. When a 25-cm C_8 cartridge was used, the resolution of compounds was poor (Fig. 4). A coupled (3 cm of C_{18} followed by 22 cm of C_8) 25-cm cartridge was therefore used for all the determinations. Using this column, a sample can be injected every 15 min. Small changes in pH of the mobile phase had a major effect on the retention charactristics of the catechols (Table II). The acidic pH selected for the mobile phase did not significantly alter the peak retention characteristics, even after six months of continuous use.

DISCUSSION

The success of this method depends on the effective removal of the interfering compounds that are present in plasma or urine and would vitiate the HPLC determination. The first stage of clean-up removes the urochromes that are present in urine. The column should be equilibrated at a low pH, and then a wash is necessary for optimizing the recovery of catechols. The second stage of purification depends on covalent bonding of the adjacent diol functionalities of catecholamines, DOPA, or DOPAC with boronic acid. These bonds are broken in acid medium and the compounds are eluted from the column. This step has all the advantages of the use of alumina without its major drawbacks which are relatively lower recoveries (60–65%), long mixing and equilibration time for adsorption of the catechols and inability to remove the interfering urochromes. In contrast, the recovery using this method is uniformly high, and residual interferences are low, even after concentration of the eluate. Moreover, the long mixing time needed to bind the catechols to alumina is eliminated. A urine sample can be prepared for HPLC in 5 min by using gentle suction or plunger pressure. When a suitable vacuum manifold is used (Vac-Elute from Analytichem) 10 samples can be processed in 30 min.

Most of the other methods that have been described[3–6] use adsorption on alumina and acid elution as a clean-up procedure. It was found that for urine samples, adsorption on alumina alone did not provide sufficient purification. The use of SCX matrix for precleaning prior to alumina adsorption has been previously described[7]. A rapid 2- or 3-step clean-up on C_{18} and other silica matrices has been developed[8].

However, none of these methods could remove all the interfering peaks, particularly from urine samples. In addition, for urine samples, a high degree of variability in recoveries was noted in these methods. A more uniform recovery was achieved by replacing the C_{18} matrix with C_1 and by removal of the cations in urine with SCX. Recently, boronate gels have been introduced for sample preparation[9,10]. This functionality offers the advantage of high specificity. At basic or neutral pH, stable covalent bonds are formed between the immobilized boronate ion and the vicinal diol groups on the catechol molecules. These bonds are easily broken in acid pH and the catechols are readily eluted[11]. The boronate gels previously used were difficult to use, because they were readily compressed by increased pressure and, therefore, the flow-rates had to be carefully controlled. For this reason, the availability of a rigid silica-derived boronate matrix usable at high flow-rates and having a high binding capacity is advantageous, because it permits rapid passage of sample with high recoveries.

The mobile phase was optimized with respect to all major variables. Both low ionic strength and low pH improved the resolution of catecholamine, DOPA, and DOPAC peaks. Use of C_{18} alone required long analysis times. A good compromise between resolution and analysis time was achieved by the use of coupled columns. Even relatively small changes (0.5 unit) in pH had significant effects, suggesting that an additional mechanism of separation may be operative in these coupled columns. The major effect of alkane stationary phases in aqueous media is hydrophobic interaction 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 examined.

The effect of pH is probably due to polar interaction with silanol groups of the matrix[12]. It is known that even after exhaustive endcapping, steric hindrance prevents the derivatization of all the silanol groups. The interactions with these residual groups were useful in allowing modulation of separation characteristics without the use of ion-pairing reagents[13]. This additional selective parameter is advantageous in situations where coupled-column chromatography may be used.

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