Determination of dihydroxyphenylalanine and dihydroxyphenylacetic acid in biological samples by coupled-column liquid chromatography with dual coulometric–amperometric detection

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Abstract: *Adrenaline, noradrenaline, dopamine and dihydroxyphenylalanine in brain* tissue and dihydroxyphenylacetic acid in plasma have been determined by direct *injection of brain homogenates or plasma into a liquid chromatographic system* comprising three columns, one packed with a boronic acid gel and two with reversed-phase material. The catecholamines and dihydroxyphenylalanine were selectively adsorbed on the boronic acid gel and separated by ion-pair chromatography on the reversed-phase columns. DOPAC was determined in a modified system without the addition of ion-pairing reagents. The catechols were detected by coulometry or amperometry with two working electrodes, operated in the oxidative–reductive mode. *The reductive signal was used for quantification. The limits of determination were 0.05* nmol g^{-1} with 40 mg brain tissue and 2 nmol l^{-1} with 0.5 ml plasma for DOPA and *DOPAC respectively. The limit of quantification of DA and DOPAC in urine was about* 0.1 µmol l^{-1}.

Keywords: *Catecholamines, dihydroxyphenylalanine and dihydroxyphenylacetic acid; coupled-column LC; biological samples; coulometric–amperometric detection.*

Introduction

There has been a great interest in the analysis of dihydroxyphenylalanine, catecholamines and their metabolites in the study of neurochemical disorders and the effects of drug treatment. Dihydroxyphenylalanine is decarboxylated by aromatic L-amino acid decarboxylase to form dopamine. Dopamine is metabolized by deamination and/or O-methylation to form 3,4-dihydroxyphenylacetic acid, 3-O-methyldopamine and homovanillic acid. The accumulation of dihydroxyphenylalanine after inhibition of DOPA-decarboxylase activity has been used to study the 'turnover' rate of dopamine in the brains of experimental animals [1]. Endogenous levels of dihydroxyphenylalanine in rat brains are low compared with the catecholamines, nevertheless methods with sufficient sensitivity to detect endogenous levels have been obtained by radioenzymatic techniques [2] and liquid chromatography with electrochemical detection, after isolation on

Sephadex G-10 [3]. Elevated levels have been determined by liquid chromatography with direct injection of brain homogenates [4] or after isolation on alumina [5].

Dihydroxyphenylacetic acid has been determined in biological samples by radio-enzymatic techniques [6, 7], by gas chromatography–mass spectrometry [8, 9] and liquid chromatography with amperometric detection [10–14]. Catechols are usually isolated from tissue homogenates, plasma and urine by adsorption on alumina [11, 12] or complexation with boronic acid gels [15–19] prior to chromatography.

An automated method for the quantification of catecholamines in plasma and urine has been described earlier [20]. A liquid chromatographic system comprising three coupled columns, one packed with a boronic acid gel and two with reversed-phase material, was used.

Methods for the isolation and detection of dihydroxyphenylalanine and dihydroxy-phenylacetic acid by coupled column liquid chromatography have been studied in the present work. Two systems had to be developed since it was not possible to obtain efficient enrichment of carboxylic acids and amines simultaneously on reversed-phase columns. The first system was used for the simultaneous quantification of noradrenaline, adrenaline, dopamine and dihydroxyphenylalanine in rat brain homogenates, or dopamine in urine, and the second for dihydroxyphenylacetic acid in plasma and urine.

Experimental

Chemicals and reagents

Adrenaline bitartrate (A), noradrenaline bitartrate (NA), dopamine hydrochloride (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), 3,4-dihydroxybenzoic acid (DOBA), isoproterenol and reduced glutathione were obtained from Sigma (St Louis, MO, USA).

3,4-Dihydroxyphenylpropionic acid (dihydrocaffeic acid, DOPPA) was obtained from Fluka (Buchs, Switzerland) and N-ethylnoradrenaline hydrochloride was synthesized at the Department of Organic Chemistry, Astra Läkemedel AB, Södertälje, Sweden. Sodium decyl sulphate was obtained from Research Plus Inc (Bayonne & Denville, NJ, USA). Methanol, perchloric acid and buffer substances were all of analytical grade quality. Water was deionized in an Elgstat Spectrum water purification system (Lane End, Buckinghamshire, UK).

Apparatus

The liquid chromatograph involved three six-port pneumatic valves with solenoid interfaces (Autochrom, Milford, MA, USA). The switching events were controlled by an integrator, model PU 4810, with an external controls option (Pye Unicam Ltd, Cambridge, UK). The column switching system was assembled as described previously [20].

In the system for DOPAC the second column was replaced by a capillary loop (5.5 m × 0.76 mm), and the last columns consisted of a pre-packed cartridge containing 7-μm Aquapore RP-18, 15 × 3.2 mm i.d. (Brownlee Labs Inc., Cotati CA, USA), coupled in series either with a 100 × 4.6 mm i.d. column, packed with 3-μm Spherisorb ODS-2 (LKB, Bromma, Sweden) or with a 75 × 4.6 mm i.d. column, packed with 3-μm Ultrasphere ODS (Beckman Instruments Inc., Berkeley, CA, USA).

The last column was connected to a coulometric detector, Coulochem model 5100 A, with cells model 5010 or 5011 (Environmental Science Assoc. Inc., Bedford, MA, USA). The mobile phases for the precolumns were delivered by Eldex pumps model 1004-A and

1610-A (Eldex, Menlo Park, CA, USA). The mobile phase for the main column was delivered either with a model 300 pump (Applied Chromatography Systems Ltd., Luton, Bedfordshire, UK) or a LKB 2150 pump (LKB, Bromma, Sweden). The samples were injected with automatic injectors model 1SS-100 (Perkin–Elmer, Norwalk, CT, USA) and model MSI 660 (Kontron, Zürich, Switzerland) for DOPA-DA and DOPAC samples respectively. A diagram of the system for DOPAC is presented in Fig. 1. Brain samples were sonicated with a cell disruptor model B-30 (Branson Sonic Power Co., Danbury, CT, USA).

Figure 1
Column-switching and detection system for DOPAC.
L 1 = 0.5 ml; L 2 = 2.5 ml; L 3 = 25 μl; (for direct injection to the main column) and L 4 = 2.5 ml. C 1 = Boronic acid affinity column; C 2 = guard column and C 3 = main column. CH 1 = Channel 1 and CH 2 = channel 2.
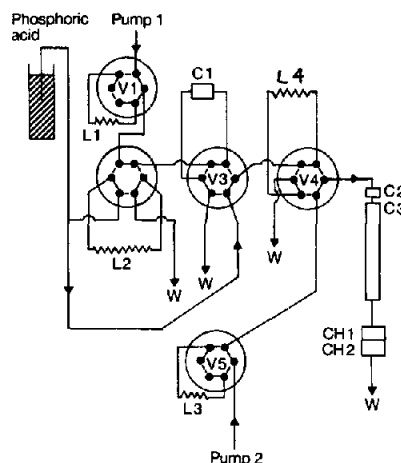
Column switching and chromatographic systems

Boronic acid columns. A polyacrylamide gel substituted with phenylboronic acid (Affi-Gel 601) was used for the isolation of catechols from the biological matrix. The gel was swollen in mobile phase and packed in a precolumn (18 × 3.8 mm i.d., Waters Associates Inc., Milford, MA, USA). The internal diameter was enlarged at both ends in order to accept two wide-pore polymeric filters, obtained from liquid–solid extraction columns (Bond Elut®, Analytichem). A 0.5 mm hole was made in the original filters supplied with the column.

During injection the mobile phase consisted of a phosphate buffer ($\mu = 0.1$, pH 8.0) containing 1 mM EDTA. The flow rate was set to 0.63 ml min^{-1} by pump 1. The catechols were eluted with 0.2 M phosphoric acid, and 0.2 M phosphoric acid with 2 mM decyl sulphate, for DOPAC and DOPA samples, respectively. A step gradient of phosphoric acid solution was generated by means of loop 2 (2 ml) by switching valve 2.

Chromatographic system for catecholamines and DOPA. Brain homogenates or urine (55 μl) was injected directly onto column 1 for 7 min. The catechols were eluted from the boronic acid column (C1) by back-flushing with phosphoric acid for 2.5 min and were enriched as ion pairs with decyl sulphate on a reversed-phase column (C2; 5-μm Supelcolsil LC 18-DB, 20 × 4.6 mm i.d). The mobile phase for column 2 consisted of phosphate buffer ($\mu = 0.1$, pH 2.0) containing 2 mM decyl sulphate during conditioning. The flow rate was set to 1.0 ml min^{-1} by pump 2. The catecholamines and DOPA were eluted from the enrichment column (C2) to the main column (C3) by back flushing with a buffer containing 20% v/v methanol for 0.55 min. These switching events are summarized in Table 1.

Table 1
Column-switching procedure used for the analysis of amines and DOPA

Time after injection (min)	Switch of valve No.	Event
0.0	1	Sample is injected on the boronic acid column with buffer pH 8.0 for 7 min
7.0	—	Start of integrator
7.02	2	Catechols are eluted from the boronic acid column with 0.2 M phosphoric acid for 3 min
7.5	3	Column 1 and 2 are connected in series and the amines are enriched as ion pairs with decyl sulphate on column 2
9.5	3, Reset	Column 1 and 2 are disconnected from each other
9.55	4	The sample is back-flushed from the enrichment column to the main column with methanol-buffer (20:80, v/v)
10.0	2, Reset	The boronic acid column is conditioned with buffer pH 8.0
10.1	4, Reset	The elution from the enrichment column is completed. The catecholamines are separated as ion pairs with decyl sulphate on the main column. The enrichment column is conditioned with buffer pH 2.0 containing 2 mM decyl sulphate
17.5	End	Calculation and report
19.0	Next sample is injected	

A reversed-phase column (C3; 3-μm Microsphere ODS, 100 × 4.6 mm i.d.) was used for separation of the different catecholamines and DOPA as ion-pairs with decyl sulphate. The mobile phase consisted of a phosphate buffer ($\mu = 0.1$, pH 2.5)–methanol (80:20, v/v) containing 2 mM decyl sulphate. The flow rate was set to 1.0 ml min^{-1} by pump 3.

Chromatographic system for DOPAC. Plasma (0.5 ml) or urine (20 μl) was injected directly onto column 1 for 2.5 min. The catechols were eluted from column 1 by back-flushing with phosphoric acid for 3 min and the fraction containing catechols was trapped in loop 4 containing phosphoric acid. The acidified solution in loop 4 was injected onto the main column (C2 + C3; 7-μm Aquapore RP-18, 15 × 3.2 mm i.d. + 3-μm Sperisorb ODS-2, 100 × 4.6 mm i.d.) for enrichment and separation of acidic catechols. The mobile phase consisted of phosphate buffer ($\mu = 0.1$, pH 2.5)–methanol (90:10, v/v), and was pumped with pump 2 at a flow rate of 1 ml min^{-1}. The switching events are summarized in Table 2.

Coulometric detection. The coulometric cells consisted of two separate working electrodes coupled in series. Model 5010, which contains two coulometric working electrodes, was used for detecting catecholamines and DOPA with channels one and two (CH1 and CH2) operated at +0.30 V and −0.07 V versus the reference electrodes

Table 2
Column-switching procedure used for the analysis of DOPAC

Time after injection (min)	Switch of valve No.	Event
0.0	1	Sample is injected on the boronic acid column with buffer pH 8.0 for 2.5 min
2.5	2	Catechols are eluted from the boronic acid column with 0.2 M phosphoric acid for 3 min
2.55	3	Catechols eluting from the boronic acid column are collected in a capillary loop
5.50	3, Reset	Elution is completed
5.52	4	The acidic solution in loop 4 is injected and acidic catechols are enriched on the top of the guard column
6.50	2, Reset	The boronic acid column is conditioned with buffer pH 8.0
8.30	4, Reset	The enrichment is completed and separation continues with an acidic mobile phase containing 10% v/v methanol
17	End	Calculation and report
17	Next sample is injected	

respectively. Model 5011, which contains one coulometric and one amperometric electrode, was used for detecting DOPAC with CH1 and CH2 operated at +0.40 V and −0.20 V versus the reference electrodes respectively. The reductive signal on channel 2 was used for determination in both systems.

Recoveries in the column-switching system. The switching system was assembled as described for DOPAC above. The mobile phase composition for the main column was adjusted for separation of DOPAC, DOPA and the catecholamines. The mobile phase consisted of phosphate buffer ($\mu = 0.1$, pH 2.5)–methanol (87:13, v/v) with 0.9 mM octyl sulphate. The recoveries of the different catechols in the switching system were determined by comparison of peak areas obtained by injections to the main column.

Sample collection and storage

Venous blood samples were collected in heparinized Venoject® tubes (Leuvren, Belgium). The blood was centrifuged and the plasma stored in polypropylene tubes at −70°C. Urine samples were acidified with hydrochloric acid to pH 2.5–3.5 and stored in polypropylene tubes at −20°C. Rat brain samples (striatum or accumbens) were stored in alumina foils at −70°C.

Sample preparation

Brain samples were weighed in polycarbonate tubes on an analytical balance, and sonicated for 20–25 s in 0.1 M perchloric acid (1 ml/100 mg tissue) with 2.6 mM metabisulphite and 2 nM internal standard (EtNA). The homogenate was centrifuged at 2000 *g* for 5 min. The supernatant was transferred to another tube and the pH was

adjusted to 3.5 ± 0.5 with 0.5 M sodium dihydrogen phosphate. The samples were centrifuged and 55 μl of the supernatant was injected for determination of catecholamines and elevated DOPA levels. Low concentrations of endogenous DOPA levels were determined after adjustment of pH to 7.5 ± 0.1 and injection of 0.8–1.0 ml within 1 h after pH adjustment.

Urine and plasma samples were thawed, mixed and centrifuged. Urinary dopamine was determined after addition of internal standard solution (0.95 + 0.05) and injection of 55 μl. Urinary DOPAC was determined after dilution with internal standard solution (1 + 2) and injection of 20 μl. DOPAC in plasma was determined after the addition of internal standard solution (0.95 + 0.05) and injection of 0.5 ml.

Standard solutions for calibration

The catecholamines and DOPA were dissolved and diluted with 0.1 M percholoric acid containing sodium metabisulphite (2.6 mM). DOPAC and DOBA were dissolved and diluted with citrate buffer ($\mu = 0.1$, pH 4.0), with reduced glutathione (2 mM) and EDTA (2 mM). The standard solutions were dispensed in polypropylene tubes and frozen at $-20°C$.

Aliquots of the standards were thawed daily and diluted. DOPA-standards were diluted in phosphate buffer, ($\mu = 0.1$, pH 3.7) containing 2.6 mM metabisulphite. DOPAC standards were diluted in citrate buffer, $\mu = 0.1$, pH 4, with 1 mM glutathione.

The internal standard solution (DOBA) for plasma was diluted in a buffer containing sodium dihydrogenphosphate (50 mM), glutathione (40 mM) and EDTA (40 mM) pH 5.3.

Results and Discussion

Boronic acid columns

Immobilized boronic acid is commercially available bonded to gels as well as to silica. Boronate has high affinity for cis-diols, e.g. catechols, carbohydrates, ribonucleotides and ribonucleosides. Affi-Gel 601 consists of phenylboronic acid bound to Bio-Gel P6, a polyacrylamide gel with an exclusion limit of 6000 daltons. The gel separates components both by size exclusion and complexation with boronic acid.

The distribution of NA, DA and DOPAC between Affi-Gel 601 and phosphate buffers of different pH was studied in a previous work [20]. The distribution ratio (D) at pH 8.04 was 115 and 37.5 for the amines and DOPAC respectively. A non-specific distribution with $D \approx 1.4$ was obtained at pH <4.

The recoveries of DOPAC, DOPA, DA and isoproterenol on the boronate column were determined (Fig. 2). The different catechols were retained according to their net charge at pH 8 (DA > DOPA > DOPAC). The distribution of the catechols to the gel is high, but a continuous loss occurs after injection due to low chromatographic efficiency. An attempt was made to estimate the number of theoretical plates by comparing observed and calculated elution curves for DOPAC (Fig. 3). The correlation between found and calculated elution curves was poor which might be explained by slow equilibrium during gel chromatography.

A decrease of the flow rate from 0.63 to 0.40 ml min^{-1} increased DOPAC recoveries by 1–2% units.

Plasma proteins and most of the other matrix components with non-specific distribution to the gel were eluted in the first millilitre of buffer, while all catechols were
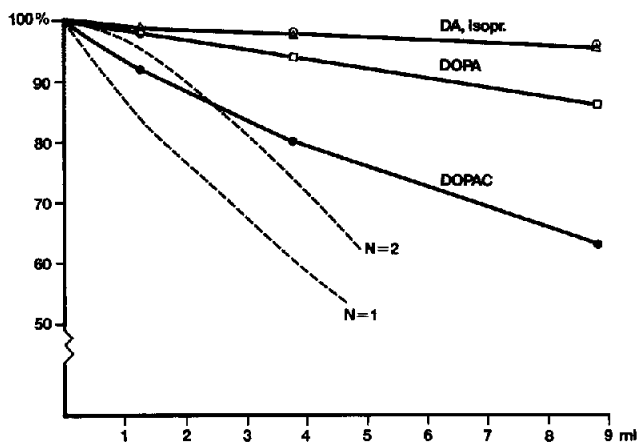
Figure 2
Break-through curves for DOPAC, DOPA, DA and isoproterenol on Affi-Gel 601 at pH 8.0 and comparison with calculated elution profiles for DOPAC. A distribution ratio of 37.5 for DOPAC, and an estimated phase volume ratio of 3 (gel volume/interstitial volume) was used for the calculation of elution curves with one and two theoretical plates. The recoveries were determined by comparing peak areas after injection of a buffer standard to the switching system and main column, respectively.

retained to >90%. The catechols were eluted to the next column in small volume of an acidic buffer.

Enrichment columns

Amines and acids are retained on reversed-phase columns under entirely different conditions. Catecholamines are usually separated as ion-pairs with alkyl sulphates or sulphonates, whilst carboxylic acids are separated in their uncharged form at an acidic pH, or as ion-pairs with lipophilic amines at neutral pH.

Attempts were made to obtain simultaneous enrichment of amines (NA, A, DA), amino acids (DOPA) and carboxylic acids (DOPAC) on reversed-phase columns, but suitable conditions were not found because the addition of alkyl sulphates to the mobile phase decreased the retention of DOPAC. DOPA was well retained as an ion-pair with decyl sulphate at an acidic pH, and DOPA can be enriched together with the catecholamines in line with previous work [20]. DOPAC was retained with a capacity factor of about 40 on ODS columns at acidic conditions (phosphate buffer pH 2.5).

Main columns

The catecholamines and DOPA were separated as ion pairs with decyl sulphate at an acidic pH. The retention of DOPA decreased with increasing pH, while the catecholamines were unaffected, thus the acidity of the mobile phase can be used to control the selectivity for separation of catecholamines and DOPA. The chromatographic system gave complete separation of the catecholamines and DOPA in brain homogenates from rats, pretreated with the decarboxylase inhibitior NSD-1015, and provided the possibility of determining endogenous levels (Fig. 3). The same system was also used for determination of DA in urine (Fig. 4). A chromatographic peak corresponding to DOPA was detected in urine, but it was not completely resolved from the background in some samples.
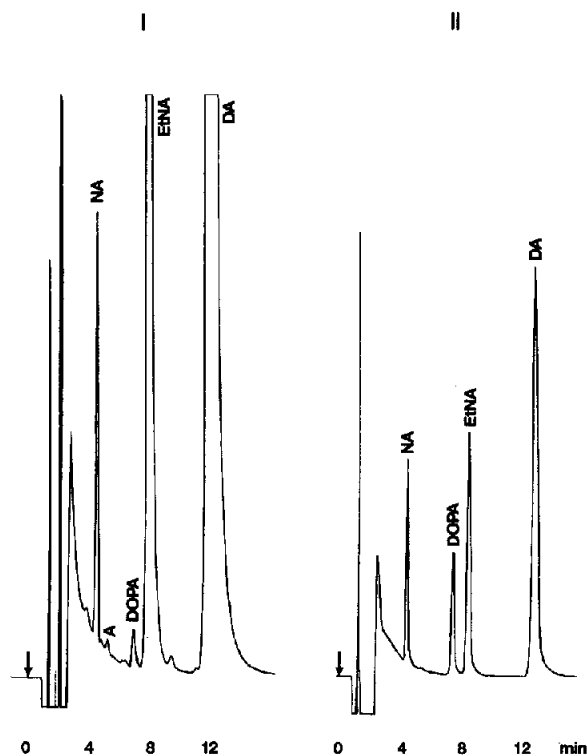
Figure 3

Chromatogram obtained with a normal rat striatum (I) with NA, 0.79 nmol g^{-1}; A $\approx$ 0.03 nmol g^{-1}; DOPA, 0.13 nmol g^{-1}; DA, 78 nmol g^{-1}, and a rat accumbens (II), pretreated by DOPA-decarboxylase inhibition. NA, 8.5 nmol g^{-1}; DOPA, 8.2 nmol g^{-1} and DA, 44 nmol g^{-1}. Injection volumes: I, 0.8 ml and II, 55 μl, respectively.

Two reversed-phase columns were tested for separation of acidic catechols; 3-μm Ultrasphere ODS (Beckman) and 3 μm Spherisorb ODS-2, (LKB). Both columns gave suitable selectivity for separation of DOPAC from the background, but the Spherisorb column gave about 70% higher retention compared with the Ultrasphere column (Table 3). The Spherisorb column gave higher retention during enrichment and was used for determination of DOPAC in plasma, by column switching (Fig. 5), while the Ultrasphere column was used for determination of DOPAC in urine by direct injections (Fig. 6).

Column switching

The catechols were injected on to the boronic acid column with phosphate buffer pH 8 and desorbed by back-flushing with 0.2 M phosphoric acid. A step pH gradient from pH 8 to <2 was formed during elution and desorption was observed to begin at pH 5.5–6.5 in the order DOPAC > DOPA > NA, A, DA, EtNA. An efficient enrichment of catecholamines from aqueous solutions was obtained in a broad pH range on reversed-phase columns equilibrated with decyl sulphate, whilst an acidic pH was required for enrichment of DOPA.

When the acidic gradient was transferred without delay to a small reversed-phase column for enrichment, the catecholamines were well retained, whilst 20% of DOPA
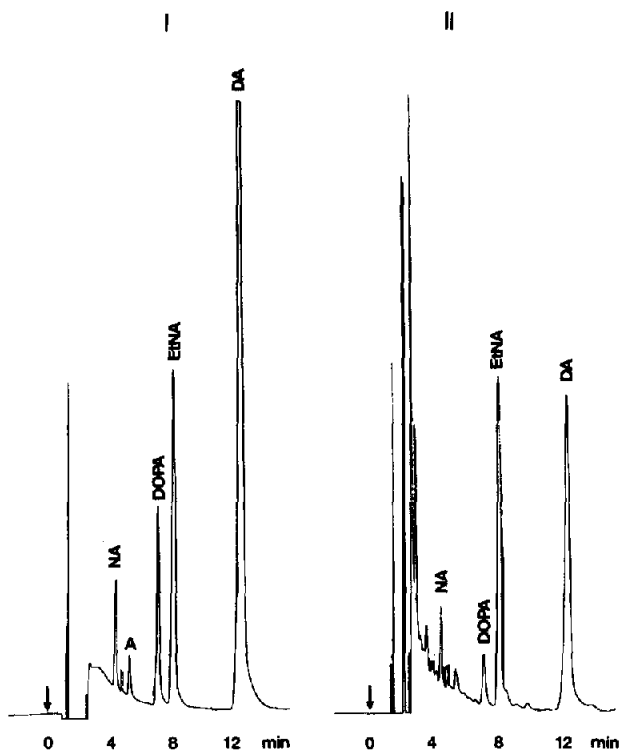
Figure 4
Chromatograms obtained by injection of a buffer standard (I) and a urine sample (II). Injection volume: 55 μl.
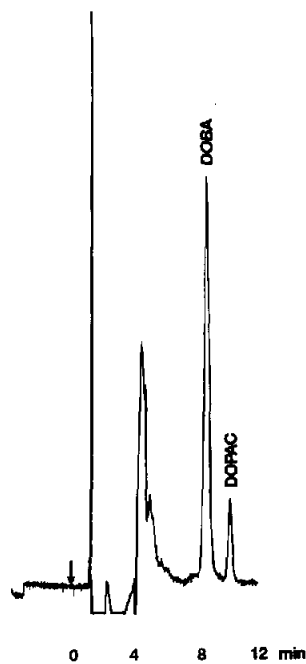The conditions were as described in the text for catecholamines and DOPA. Urinary DA, 2.5 μM.

Figure 5
Chromatogram obtained by injection of 0.5 ml
normal plasma. DOPAC, 19 nmol l^{-1}. The
conditions were as described in the text for DOPAC
in plasma.

Table 3
Capacity factors of acidic catechols measured using reversed-
phase columns

Catechol	k_1'	σ (μl)	σ^*(μl)	k_2'
DOBA	5.8	72	126	3.4
DOPAC	7.7	85	111	4.3
DOPPA	16	151	164	8.8

Capacity factors were determined with a mobile phase con-
taining phosphate buffer, (μ = 0.1, pH 2.5)–methanol (90:10,
v/v). k_1' and k_2' = capacity factors determined on Sperisorb ODS-2
and Ultrasphere ODS, respectively, σ and σ^* = Standard
deviation of peak volumes on Spherisorb without and with
column switching respectively.

Figure 6
Chromatograms obtained by direct injection of a
urine sample on 3-μm Ultrasphere ODS (75 × 4.6
mm i.d.) with coulometric detection at +0.40 V (Ch
1) and amperometric detection at −0.20 V (Ch 2).
Injection volume: 20 μl, DOPAC, 6.3 μM.
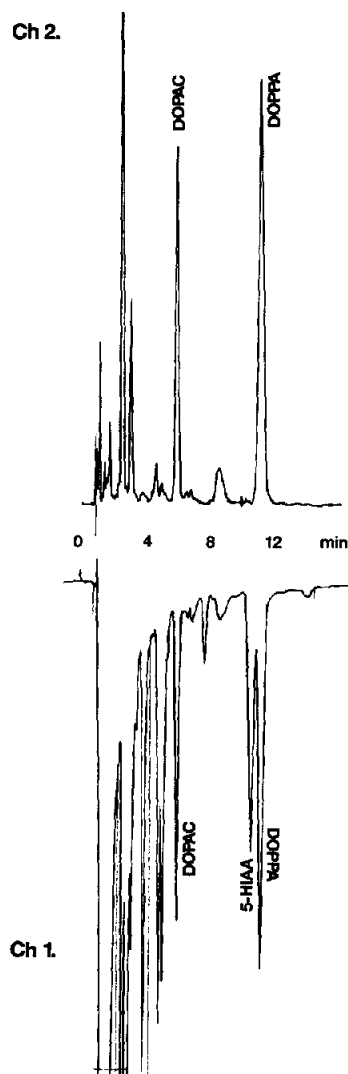
and most of DOPAC was lost due to break-through. The carboxylic acids DOPA and DOPAC were only retained on the enrichment column at pH <4 and break-through occurred at the beginning of the elution gradient. DOPAC was eluted first from the boronic acid column and the retention of DOPAC was low on columns saturated with decyl sulphate. These differences in chromatographic properties between amines, amino acids and carboxylic acids necessitated the development of two different systems.

The recoveries and band broadening of the final systems were determined by comparison with direct injections. The recovery of DOPA was increased to 80% ± 1 S.D. ($n = 8$) by adjustment of the switching events (Table 2). The first portion of the acid gradient (0.3 ml, pH 8–5) was eluted to waste and the following fraction of the gradient (containing DOPA and catecholamines) was transferred to the enrichment column, which had been conditioned previously with buffer at pH 2.0 containing 2 mM decyl sulphate.

The catecholamines and DOPA were transferred to the main column by back-flushing with a buffer containing 20% v/v methanol (Table 2).

About 10% of DOPA was lost during injection and 10% during elution from the boronic acid column. Band broadening effects in the column-switching system were low and comparable with those obtained in the previous work [20].

DOPAC was eluted from the boronic acid column to a capillary loop filled with 0.2 M phosphoric acid. The acidity of the eluate was adjusted for enrichment by dispersion with acid in the loop. The eluate was then injected onto the main column for enrichment of acidic catechols and separation with a mobile phase containing 10% v/v methanol.

About 12% of DOPAC was lost during injection and clean-up on the boronic acid column (Table 4).

The total band broadening in the system was the sum of contributions from the boronic acid column, enrichment column and main separation column. The major part of DOPAC was eluted from the boronic acid column in a volume of about 1 ml. DOPAC was enriched and eluted with capacity factors of 40 and 6.65 respectively, an enrichment factor of 6 thus being obtained. The peak width of DOPAC increased by 30% after column switching (Table 3, Fig. 7) and the efficiency decreased from 83 000 to 52 000 plates m^{-1}.

Sample and system stability

The catechols were stable for at least 16 h at ambient temperature (22°C) when stored at pH 3.5–4 in urine or buffer solutions containing metabisulphite (2 mM) or reduced

Table 4
Relative and absolute recoveries determined from plasma

Substance	n	Absolute recovery* (%)	Relative recovery† (%)
DOBA	6	93	94
DOPAC	6	88	95
DOPPA	6	86	90

*Determined by comparison of peak areas obtained after standard addition to plasma and injection of standards onto the column switching system and main column respectively.

†Determined by comparison of peak heights after standard addition and injection of buffer standards to the column switching system.
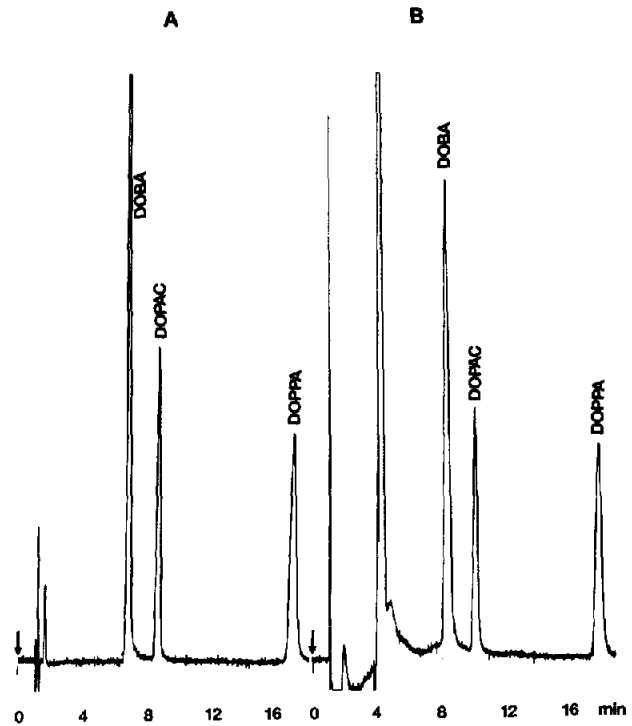
Figure 7
Comparison between chromatograms obtained by injection of a standard solution for plasma samples into (A) the main column: and (B) the switching system. The conditions were as described in the text for DOPAC in plasma. Injection volume: 25 μl, DOBA, 46.4 pmol; DOPAC, 23.5 pmol and DOPPA, 35.3 pmol.
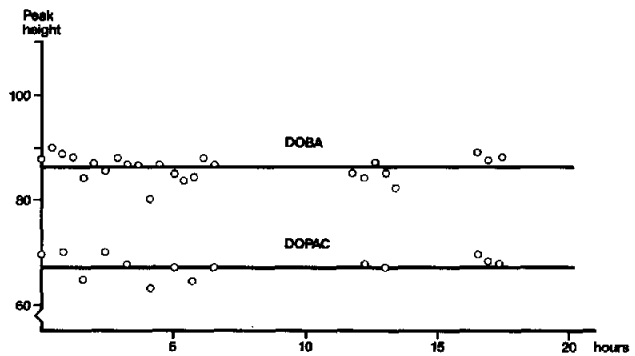

Figure 8
Stability of the column switching system for DOPAC. A 20 μl sample of normal urine was analysed by repeated injections with an automatic injector.

glutathione (1 mM) (Fig. 8). The stability of acidic catechols in plasma buffered to pH 8.5 and 7.5, containing antioxidant (glutathione, 1 mM) and metal masking agent (EDTA, 2 mM), was checked by storage overnight. The acidic catechols were unstable at pH 8.5, but stable for at least 12 h at pH 7.5 (Table 5). The decomposition rate increased in the order DOBA > DOPAC > DOPPA, also it was found that the internal standard did not compensate completely for the effects of decomposition. The pH of blood plasma increases from 7.4 to 8.5–9 upon storage, due to a loss of carbon dioxide, so that it is important to buffer the samples after thawing to avoid decomposition. Cooling of the sample tray in the automatic injector may further improve the sample stability [21].

The chromatographic systems are stable and permit the use of an automatic injector (Fig. 8). The back pressure of the reversed-phase pre-column increased slowly during analysis of plasma samples and the pre-column had to be replaced after 50–100 injections. The boronic acid columns gave reproducible recoveries for large numbers of samples and it was difficult to define a 'life time' for such columns. These columns are easily repacked within 10 min and are normally replaced after about 200 injections as an extra precaution.

Table 5
Stability of DOPAC in plasma. Additions: Glutathione 1 mM and EDTA 2 mM

| Plasma pH | Storage time (h) | n | % Remaining | | |
			DOBA	DOPAC	DOPPA
8.5	16	3	65	50	44
7.5	16	3	102	100	96
7.5	48	2	99	33	10

Accuracy and precision

The response ratio at channels two and one (reduction/oxidation) was determined for DOPAC in standard solutions, plasma and urine, to test the peak purity. The response ratios were 0.602 ± 0.005 S.D. ($n = 4$), 0.562 ± 0.021 S.D. ($n = 20$) and 0.598 ± 0.016 S.D. ($n = 5$) for standard, plasma and urine respectively. The response ratio for DOPAC in plasma was 7% lower compared with the standard, due to interference by a compound which was not reduced at -0.15 V. The interference was also detected as a shoulder on the oxidative signal but was not evident on the reductive signal. The internal standard (DOBA) was disturbed by the chromatographic background in urine samples and urinary DOPAC was quantified without internal standard. The main column alone gave complete separation of DOPAC in urine (Fig. 6) and no interference was detected by comparing with column switching and detection at two different potentials.

3,4-Dihydroxyphenylpropionic acid (DOPPA) was detected in both plasma and urine. This compound has been identified in urine [22] and plasma [23]. Goldstein et al. [23] proposed that DOPPA originates from caffeic acid in coffee. It is known that dihydrocaffeic acid (DOPPA) can be produced from caffeic acid by human intestinal bacteria [24].

The identity of DOPA in rat brain after DOPA-decarboxylase inhibition was verified by variation of the pH of the mobile phase and by comparison of response ratios. The response ratios were 0.719 ± 0.036 S.D. ($n = 8$) and 0.709 ± 0.006 S.D. ($n = 8$) for standards and brain homogenates respectively.

Table 6
Results of an investigation of the intra-assay precision

Sample	Substance	Concentration	RSD (%)	n
55 μ spiked	NA	0.36 μM	1.5	8
brain homogenate	A	0.15 μM	1.1	8
	DOPA	1.10 μM	1.1	8
	DA	6.1 μM	1.3	8
20 μl urine	DOPAC	2.6 μM	3.6	9
0.5 ml plasma	DOPAC	11 nM	4.3	9

The decyl sulphate and pH gradients formed during column switching disturbed the baseline between EtNA and DA on the oxidative signal (CH 1) while the reductive signal (CH 2) was unaffected. The endogenous DOPA levels in brain were low (0.03–0.12 nmol g^{-1}) and comparable with the results obtained by radioenzymatic techniques [2].

The ruggedness of the DOPA-system was tested by injection of other sample matrixes, such as plasma, urine and cerebrospinal fluid. DOPA was well resolved from the background in plasma and CSF, but disturbed in some urine samples. DOPA can probably be determined in urine after minor modifications of the chromatographic conditions. The present system for separating catecholamines and DOPA may be combined with post-column derivatization and fluorimetric detection of NA and A, as described previously [20], and this is currently under investigation.

The precision (relative standard deviation R.S.D.) for the determination of DOPAC in plasma and urine was in the range of 2–4% (Table 6). The limit of determination with 0.5 ml plasma was 2 nmol l^{-1}. NA, DA and elevated DOPA levels in brain tissue and DA in urine were determined with a precision of 1–2% (Table 6). The limit of determination of DOPA in brain tissue was about 1 pmol/sample.

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