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ROUTINE MEASUREMENT OF PLASMA CATECHOLAMINES IN CLINICAL PHARMACOLOGY BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A method for the simultaneous determination of epinephrine, norepinephrine and dopamine in human plasma is described, which combines the advantages of liquid–liquid extraction sample preparation, high-performance liquid chromatography on weak cation-exchange stationary phases and dual-electrode coulometric detection. The limits of quantification are less than 5 pg/ml (at a signal-to-noise ratio > 5) for each analyte. The influence of various experimental parameters (e.g., composition of the mobile phase, pretreatment of the assay buffer, components of the re-extraction system) on the performance of the assay is reported in detail. A number of applications are presented, which demonstrate the quality of the data obtained in terms of sensitivity, reproducibility and significance.

INTRODUCTION

The accurate and precise determination of plasma catecholamines in the low pg/ml range still remains a challenging task, even if modern instrumental methods of chemical analysis are used. It appears from the literature that at present high-performance liquid chromatography (HPLC) with electrochemical detection (ED) is used by most workers. Reversed-phase columns [1–3] are now employed more frequently for the chromatographic separation than are strong cation-exchange materials, which historically were introduced first for catecholamine analysis [4]. The classical aluminium oxide work-up procedure [5,6], however, is still employed more often than are various other isolation techniques, which were successfully applied, e.g. gel permeation chro-

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matography [7], chromatography on cation-exchange cartridges [8], adsorption/desorption on materials such as dihydroxyboryl cellulose [9] or immobilized phenylboronic acid [10] and liquid–liquid extraction [11].

The comparatively low recoveries with alumina stimulated us to evaluate possible alternatives. We have investigated the various steps of a method that combines for the first time the high yield of liquid–liquid extraction with an advantageous HPLC separation on a weak cation-exchange column showing a unique elution order for catecholamines [12]. Peak detection was achieved electrochemically with a coulometric detector. During our experimental work we have identified and solved a number of critical issues, which to our knowledge have not previously been sufficiently emphasized. A full description of the experimental details is given in this paper, together with typical results from methodological–pharmacological studies.

EXPERIMENTAL

Chemicals

Sodium hydroxide, orthophosphoric acid, acetic acid, ammonia, ammonium chloride, *n*-heptane, chloroform, acetonitrile, citric acid and sodium disulphite ($Na_2S_2O_5$) were obtained from Merck (Darmstadt, F.R.G.). Epinephrine, norepinephrine, dopamine, dihydroxybenzylamine hydrobromide and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma (Taufkirchen, F.R.G.). Diphenylboric acid anhydride (DPBA) and tetraoctylammonium bromide (TOAB) were purchased from Fluka (Neu-Ulm, F.R.G.), diphenylboric acid ethanolamine complex (DPB) from Aldrich (Steinheim, F.R.G.), *n*-octanol from Baker (Gross-Gerau, F.R.G.) and *n*-butanol from Riedel-de Haën (Seelze, F.R.G.). All chemicals were used as received if not stated otherwise. Purified water (resistivity 18 MΩ cm) was produced with a Millipore Milli-Q system.

Extraction solvent

The extraction solvent was *n*-heptane–chloroform (75:25, v/v) containing 0.25% (w/v) of TOAB.

Assay buffer

The assay buffer was prepared from 2 *M* ammonia solution and 2 *M* ammonium chloride to give a solution of pH 8.5. To this 2 *M* ammonia–ammonium chloride buffer was added EDTA (0.5%, w/v), followed by DPB (0.2%, w/v). The buffer was stirred until complete dissolution of the solutes was achieved. The solution was then kept in a stoppered flask at 80°C in a waterbath for 8 h.

Stock solutions of catecholamines

Stock solutions of epinephrine (2 ng/ml), norepinephrine (20 and 2 ng/ml), dopamine (2 ng/ml) and dihydroxybenzylamine hydrobromide (10 ng/ml) as internal standard (I.S.) were prepared by stepwise dilution in 0.05 *M* orthophosphoric acid. These working stock solutions were kept at 4°C. The more concentrated stock solutions were kept at −20°C. The working stock solutions were replaced routinely every four weeks. The frozen solutions were then thawed, each mixed thoroughly and diluted to obtain the concentrations required.

Chromatography

The chromatographic system consisted of a Merck-Hitachi Model 655 A-12 pump (E. Merck) and a WISP 710B automatic sample processor, a system controller and a Data Module integrator, all from Millipore-Waters (Eschborn, F.R.G.). Alternatively, for sample injection a Merck-Hitachi Model 655 A-40 autoinjector (E. Merck) was used and peak integration was performed on a TRIO chromatography computing integrator (SES, Nieder-Olm, F.R.G.). The electrochemical detector was an ESA Coulochem Model 5100A with a Model 5011 dual-electrode analytical cell (Biotronik, Maintal, F.R.G.). The detector was operated at a nominal potential of −0.15 V applied to the upstream electrode and a potential of +0.3 V applied to the downstream electrode. Routinely only the signal generated at the second electrode was measured. Separations were carried out on a Bio-Rad Clinical weak cation-exchange column (Bio-Rad Labs., Munich, F.R.G.) kept at 40°C in a column oven (Waters TCM). This stationary phase is a silica gel of 10 μm particle size with chemically bonded carboxyl functional groups. The mobile phase consisted of 85–90% (v/v) of an aqueous buffer and 15–10% (v/v) of acetonitrile. The buffer was prepared from citric acid and orthophosphoric acid (0.035 *M* each), adjusted to pH 6.4 with 1 *M* sodium hydroxide solution. Prior to use, the mobile phase was filtered through a 0.2-μm membrane filter and degassed by sonication under vacuum. The eluent was then delivered to the column at a flow-rate of 1.0 ml/min. During analysis the mobile phase was not recycled.

Sample collection and preparation

Blood samples were collected from healthy volunteers under carefully controlled conditions via an indwelling cannula in a forearm vein. The pre-chilled heparinized tubes, into which the blood was sampled, were spiked with 50 μl of 1.0% (w/v) sodium disulphite solution in water per 5 ml of blood to be taken. After gentle mixing, the samples were immediately centrifuged (4000 *g*, 10 min), the plasma was separated, divided into 2.2-ml aliquots if possible and frozen in a cooling bath (solid carbon dioxide–ethanol). The samples were then stored at −20°C until further analysis.

For thawing prior to analysis, samples were transferred into an ice-bath for

1 h and then kept at room temperature for 15 min. A 2-ml volume of plasma in a polypropylene centrifuge tube was subsequently spiked with 250 ng/ml of internal standard (50 μl, 10 ng/ml) followed by 1 ml of assay buffer. The sample was then briefly mixed and the catecholamines were extracted into 5 ml of extraction solvent on a vortex-type mixer for 2 min. After centrifugation at 4000 *g* for 5 min, 4.5 ml of the organic layer were removed by pipette. *n*-Octanol or *n*-butanol (2 ml) was added to the extract and the analytes were again extracted as above into 150 μl of 0.5 *M* acetic acid.

The organic phase was aspirated off, leaving a volume of approximately 0.5 ml, which was transferred into an Eppendorf reaction vial. To improve phase separation, the vials were briefly centrifuged (3000 *g*, 10 min) and subsequently 100 μl of the aqueous phase were pipetted into a second reaction vial. Finally, the samples were kept under a gentle stream of nitrogen for 20 min at room temperature. Alternatively, the samples were placed in a Speed Vac sample concentrator (Savant Instruments, Vaterstetten, F.R.G.) for 20 min. To the remaining 20 μl of sample, 60 μl of 0.05 *M* orthophosphoric acid were added. A 50–60 μl volume of the sample was used for the HPLC separation and determination.

RESULTS AND DISCUSSION

Chromatographic separation

The weak cation-exchange column operated under the above conditions allowed an excellent separation of epinephrine, norepinephrine, dopamine and the I.S. A test mixture which contained metanephrine and normetanephrine in addition to these compounds produced the chromatogram shown in Fig. 1A. A comparison of the retention times of the analytes analysed separately revealed that epinephrine and normetanephrine coeluted. A second chromatogram (Fig. 1B) shows the catecholamines and the I.S. together with epinine (N-methyldopamine), a non-endogenous catecholamine-like compound.

The most important feature of the separation of catecholamines on the weak cation-exchange material is the elution order, with epinephrine being eluted first. This is in contrast to the results obtained on various strong cation-exchange particles [13–15], where invariably norepinephrine was eluted first, followed by the I.S. and epinephrine, with dopamine as the last peak.

A similar situation is found with most separations on covalently bonded reversed stationary phases. Again, norepinephrine shows the shortest retention time, this time followed by epinephrine and the I.S., with dopamine still eluting behind.

Within the pH range tested (4.0–8.0), the retention times and elution order change significantly with pH when aqueous mobile phases are used (Table I). As an effective pK_a of 6.5 was measured for the stationary phase material (unpublished results), the functional groups can be assumed to be completely de-

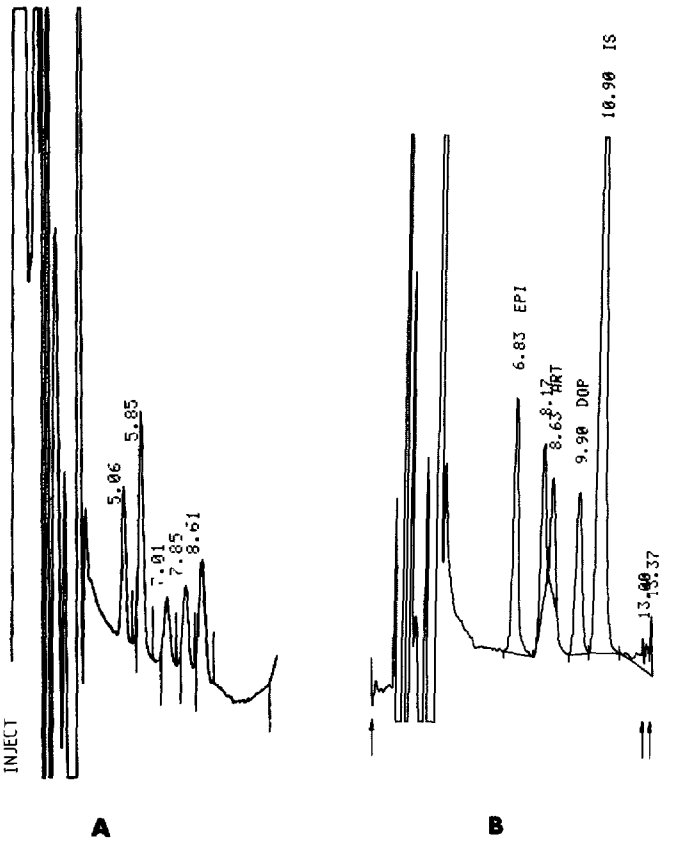


Fig. 1. Separation of catecholamines and related compounds. (A) Peaks: 5.06 min, metanephrine; 5.85 min, epinephrine and normetanephrine; 7.01 min, norepinephrine; 7.85 min, dopamine; 8.61 min, I.S. Mobile phase, 85% orthophosphate-citrate buffer (each 0.030 *M*, pH 6.4) + 15% (v/v) acetonitrile. Sensitivity: 5 nA full-scale. (B) Peaks: EPI = epinephrine; ART = norepinephrine; DOP = dopamine; 8.17 min, epinine. Mobile phase, 90% buffer as in (A) and 10% acetonitrile. Sensitivity: 2.5 nA full scale.

protonated at pH 8. Under these conditions ion exchange dominates the separation and the elution order is essentially identical with that obtained on strong cation-exchange resins.

At pH 6.4 only approximately 50% of the ion-exchange sites are available. As a consequence, the retention times are generally decreased. Furthermore, secondary effects become visible. For the less polar molecules hydrophobic interactions superimposed on ion-exchange retention determine the elution order (retention times: dopamine < epinine, normetanephrine < metanephrine), while the ability to form hydrogen bonds seems to be more important with the

TABLE I

RETENTION TIMES OF CATECHOLAMINES AND RELATED COMPOUNDS

A = Orthophosphate-citrate buffer (0.03 and 0.035 *M*, respectively, pH 4.0); B_1 = buffer as A, pH 6.4; B_2 = 85% buffer as A, pH 6.4, +15% acetonitrile; C = buffer as A, pH 8.0.

Compound	Retention time (min)			
	A	B_1	B_2	C
Epinephrine	4.6	6.5	6.1	8.0
Norepinephrine	4.9	6.7	7.2	7.2
Dopamine	5.5	8.5	8.4	10.3
I.S.	5.6	8.6	9.1	9.4
Epinine	5.5	10.0	7.4	15.5
Metanephrine	5.0	7.7	5.4	7.6
Normetanephrine	5.0	6.9	6.4	7.6

homologues epinephrine and norepinephrine, where epinephrine is now eluted prior to norepinephrine.

This concept is supported by the retention results with a mobile phase containing 15% (v/v) of acetonitrile. Now the influence of hydrophobic interactions between the analytes and the stationary phase is markedly reduced and N- or O-methylation reduce the retention by decreasing the number of hydrogen atoms which can form hydrogen bonds.

The presence of an additional hydroxyl group in the alkyl side-chain contributes to a decreased retention in all instances. Probably the increase in polarity in the molecules is prevailing. Despite the importance of secondary effects, ion exchange is still the basic mechanism at pH 6.4. It can be shown that with the pH held constant, retention times are increased with decreasing sodium ion concentrations in the mobile phase, thus indicating displacement chromatography. The elution order is not affected by using different sodium ion concentrations.

At pH 4.0, the retention times are further decreased and for practical purposes separation ceases. At the same time, only a very slight influence of the sodium ion concentration on the retention remains.

It is obvious that for measurements in the low picogram range, such as with unstimulated endogenous concentrations of epinephrine and dopamine, the peaks must be kept as narrow as possible. Although the ideal system, which would have epinephrine and dopamine eluting prior to norepinephrine and the I.S. has to our knowledge not yet been discovered, the chromatography on weak cation exchangers appears to be the best approximation available at the present time.

An additional advantage of the chromatographic separation at pH 6.4 is the enhanced detector response for epinephrine versus norepinephrine and dopa-

mine. This effect is due to an increase in the apparent number of electrons available for the electrochemical oxidation of epinephrine [16,17].

Extraction and re-extraction

The pivotal reagent for the selective extraction of catecholamines is the ammonia–ammonium chloride buffer containing EDTA and DPB. Initially chromatograms of spiked aqueous samples after work-up displayed an extra peak, which could be shown to arise from the assay buffer (Fig. 2). Incubation of the buffer solution at 80°C for 5 h removed this signal completely. The decay followed (pseudo-)first-order kinetics and was significantly slower at 60°C. The nature of this compound remains unclear; its concentration seemed to vary from batch to batch.

An alternative way to obtain an assay buffer free from contaminants present

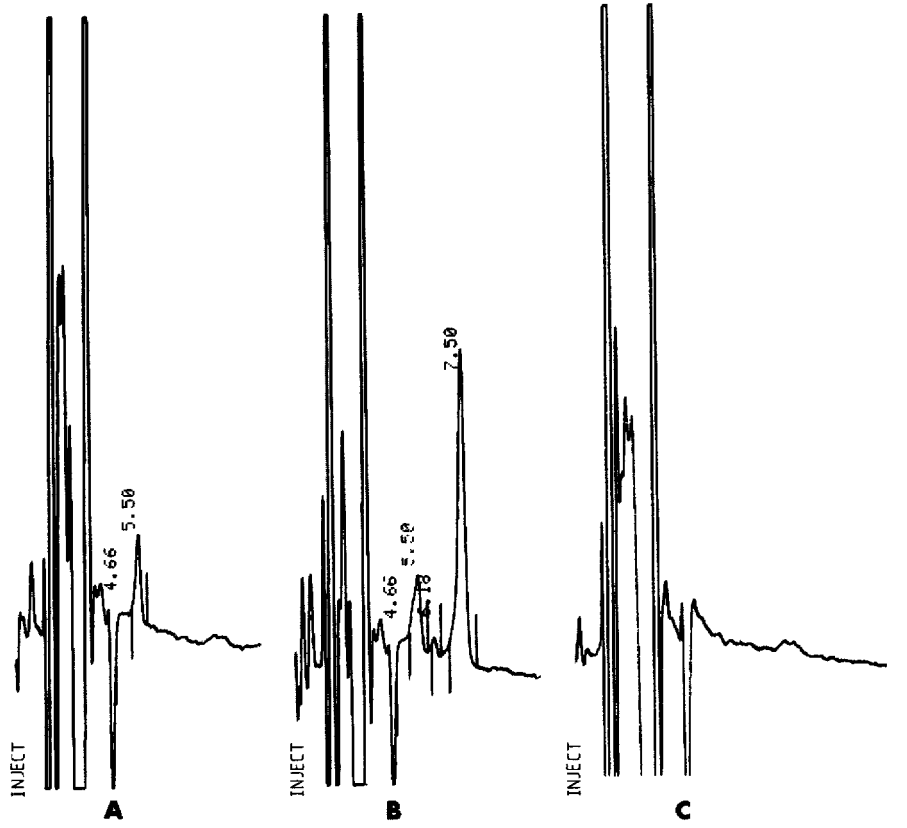


Fig. 2. Interference from diphenylboric acid. (A) Reagent blank with interfering peak. (B) Peaks: 5.5 min, interfering peak obscuring 10 pg/ml epinephrine; 6.18 min, 10 pg/ml norepinephrine; 7.5 min, 125 pg/ml I.S. (C) Reagent blank after incubation of buffer. Chromatographic conditions as under Experimental. Sensitivity: 10 nA full scale.

in DPB is the use of diphenylboric acid anhydride (DBPA). This compound is dissolved in the assay buffer with hydrolysis even more slowly than is DBP.

Whereas the other reagents used for the extraction of the catecholamines into the organic phase did not present any experimental obstacles, the *n*-octanol necessary for an optimum yield in the re-extraction occasionally gave rise to a negative chromatographic signal which showed an unpredictable retention time on different columns. In Fig. 3A this signal can be observed between epinephrine and norepinephrine. On other occasions this disturbance occurred between dopamine and the internal standard, thus obscuring small dopamine peaks. As *n*-octanol cannot be omitted without a significant decrease in recoveries, it was eventually replaced with *n*-butanol, which was found not to affect the efficiency of the re-extraction step (Fig. 3B). The only marked difference between these two additives is a decrease in the volume of the aqueous phase after re-extraction from 110 µl with *n*-octanol to 50 µl with *n*-butanol. This effect is probably due to the greater hydrophobicity of *n*-octanol, as *n*-

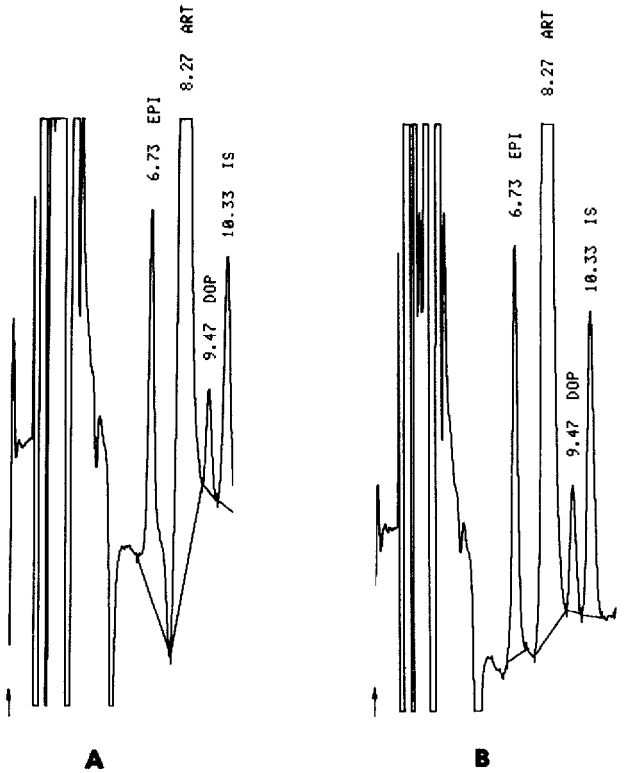


Fig. 3. Octanol baseline disturbance. (A) Sharp negative peak with *n*-octanol. (B) No disturbance after work-up with *n*-butanol. Each sample contained 50 pg/ml epinephrine and dopamine, 500 pg/ml norepinephrine and 125 pg/ml I.S. Sensitivity: 2 nA full scale.

propanol completely inhibits the formation of an aqueous phase. Other attempts, e.g., pretreatment of *n*-octanol with water, acetic acid or hydrogen peroxide, did not produce the desired result.

For the re-extraction of the analytes, 0.5 *M* acetic acid proved to be the best choice. With 0.5 *M* perchloric acid, a broad, tailing front was seen in the chromatograms, with only very small peaks for the analytes, indicating low recoveries. Citric acid and trichloroacetic acid were not tested as these compounds on injection produced a pronounced negative peak near the retention time of the analytes. Orthophosphoric acid (0.05 *M*) did not re-extract the catecholamines from the organic solvent.

The acid used in the re-extraction not only must recover the analytes in high yield, but also the resulting aqueous phase on injection must be compatible with the mobile phase. This is achieved through partial neutralization of the acid by residues of the assay buffer present in the organic solvent. If the analytes are chromatographed as solutions in untreated 0.5 *M* acetic acid, the resulting chromatograms show severe peak splitting and a decrease in retention times. Obviously 0.5 *M* acetic acid is a stronger eluent than the mobile phase and on passing through the column a sample with excess of acetic acid causes irregular non-equilibrium conditions.

The final sample concentration step was introduced in order to remove any traces of organic solvents after the extraction–re-extraction process. During the concentration procedure the samples must not be allowed to become completely dry, as then serious losses of compounds will occur and with plasma samples very broad, slowly tailing front peaks were observed.

Analytical limits, recoveries and calibration

The absolute limit of detection of the HPLC system was assessed by injecting decreasing amounts of the analytes onto the column. A 1-pg amount of epinephrine can be reliably detected by the system, and for norepinephrine and dopamine, 2.5 pg could be measured. The signal-to-noise ratio was better than 5 : 1 in these experiments. The overall recoveries of the work-up procedure for the analytes were measured with the aid of aqueous samples spiked at concentrations ranging from 5 to 50 pg/ml for epinephrine and dopamine and from 50 to 500 pg/ml for norepinephrine. The samples were analysed as described but without the final concentration step. Recoveries were calculated by comparing the peak heights of spiked samples with those of known amounts injected directly. The recoveries were $88 \pm 4\%$ (range 83–95%; $n = 11$) for epinephrine, $93 \pm 2\%$ (range 92–96%; $n = 11$) for norepinephrine and $84 \pm 4\%$ (range 79–87%; $n = 10$) for dopamine. In the concentration ranges examined, no influence of concentration on recoveries was seen.

The recoveries found for epinephrine and dopamine are slightly lower than those reported previously [11,18]. However, in that work concentrations in the ng/ml range were used in the recovery experiments. On the basis of the

sensitivity investigation and the recoveries, lower limits of quantification of 1.3 pg/ml for epinephrine, 3.0 pg/ml for norepinephrine and 3.3 pg/ml for dopamine were calculated. For routine measurements, calibration samples containing all three analytes were prepared daily and analysed together with the test samples. The calibration range was 5–100 pg/ml for epinephrine and dopamine and 50–1000 pg/ml for norepinephrine. Regression analysis with weighting was used for the daily evaluation of the results, the reciprocals of the concentrations being chosen as weights. The calibration results recorded within a period of four weeks during the completion of a project showed on average coefficients of variation of 10% for each analyte over the whole calibration range.

The performance of the assay system was monitored by the inclusion of control samples. These samples were spiked with known concentrations, then aliquoted, frozen and kept together with the test samples. A random selection of controls were analysed daily. The results of the control analyses are given in Table II. Although in general the data confirm the accuracy and precision of the assay method, epinephrine measurements at the 50 pg/ml level appear to be negatively biased. In our opinion, this indicates an error in the preparation of the stock solution of the 50 pg/ml control batch rather than a malfunction of the analytical procedure.

Calibration samples and controls were prepared in water. Some workers recommend the use of catecholamine-free plasma for calibration purposes. In our experience, the purification of plasma is tedious and erratic. However, we found that the calibration functions for the catecholamines have identical slopes in plasma and water. In order to confirm this result, pooled human plasma was

TABLE II

CONCENTRATION RESULTS FOR CONTROL SAMPLES

The different number of replicates is a consequence of the random selection.

Compound	Concentration (pg/ml)	
	Added	Found
Epinephrine	5	4.4, 5.3, 4.8, 6.6, 6.9, 4.8
	20	22, 16, 21, 21, 20, 22
	50	43, 40, 43, 45, 39, 45, 40
Norepinephrine	50	47, 43, 62, 50, 55, 52
	100	110, 99, 116, 93, 93
	200	266, 244, 208, 227, 233
Dopamine	5	4.7, 3.4, 4.2, 9.2[a]
	20	18, 19, 19, 17, 22, 19, 18, 16, 17
	50	45, 33, 43, 49, 44

[a]Contamination was present in the reagent blank.

TABLE III

CATECHOLAMINE CONCENTRATIONS IN POOLED PLASMA WITHOUT AND WITH ADDITION OF KNOWN CONCENTRATIONS

Compound	Concentration (pg/ml)		
	Pool (mean, $n=3$)	Added	Found (mean, $n=3$)
Epinephrine	I 16	10	27
	II 9	50	68
		10	19
		20	30
Norepinephrine	I 352	50	403
		100	460
	II 264	50	310
		100	358
Dopamine	I 11	10	22
		20	34
	II 8	10	20
		20	29

analysed with and without the addition of known concentrations of the analytes. The concentrations found in the pool samples are detailed in Table III. Fig. 4 shows a chromatogram of pool II prior to and after spiking together with a calibration sample. The values found are in good agreement with the concentrations expected, thus demonstrating that aqueous calibration samples can be used for the determination of catecholamines in plasma.

Sample preparation, stability, assay interference

As mentioned under Experimental, plasma samples were collected with addition of sodium disulphite. This antioxidant was also added to the calibration samples and controls [20 μl of 1.0% (w/v) solution per 2 ml]. Ascorbic acid cannot be used because it is extracted together with the analytes and gives rise to a large front peak, so that sensitive detection of the catecholamines is impossible. With sodium disulphite such an effect is not observed, but a decrease in the recovery of the internal standard was noted after addition of 50 μl of 10% sodium disulphite solution, whereas epinephrine and norepinephrine were not affected.

In order to control temperature effects as far as possible, plasma samples were thawed at 0°C and calibration/control samples were prepared at this temperature. In any case repeated freezing–thawing cycles of samples or stock solutions should be avoided. Consequently, plasma samples from clinical trials were aliquoted prior to freezing. When kept frozen at −20°C, samples are stable for at least three months.

A number of compounds were tested for their potential to interfere in the

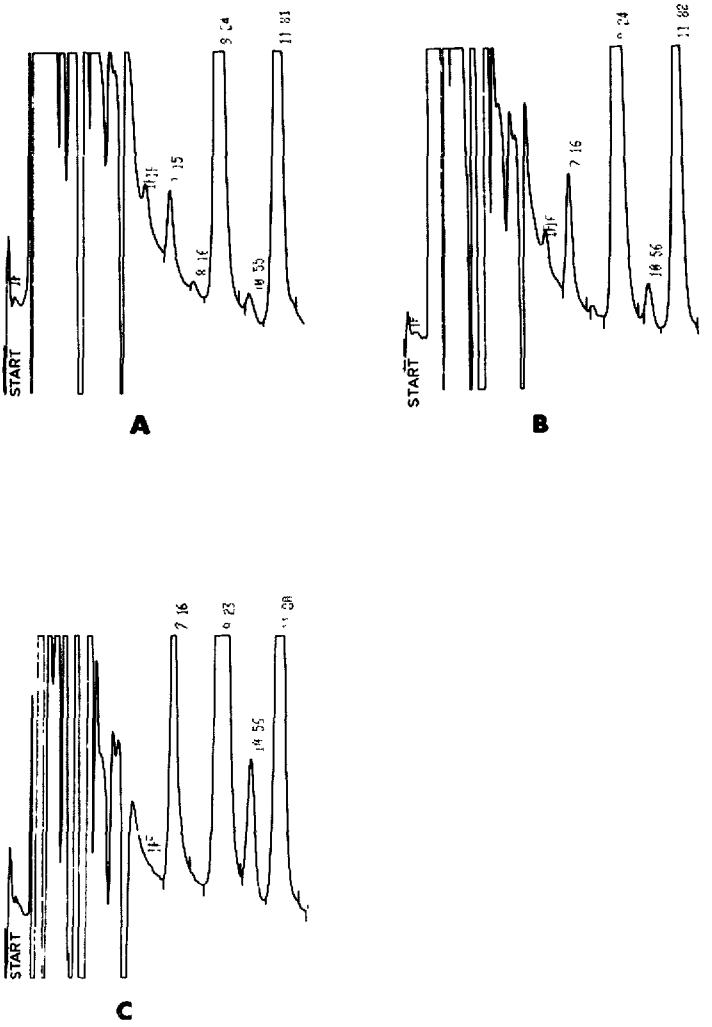


Fig. 4. Pool plasma samples. (A) Sample from pool II. Peaks: 7.15 min, epinephrine (7.1 pg/ml); 9.24 min, norepinephrine (242 pg/ml); 10.55 min, dopamine (7.9 pg/ml); 11.81 min, I.S. (B) Sample from pool II with catecholamines added. Peaks: 7.16 min, epinephrine (18.5 pg/ml with 10 pg/ml added); 9.24 min, norepinephrine (308 pg/ml with 50 pg/ml added); 10.56 min, dopamine (16.7 pg/ml with 10 pg/ml added); 11.82 min, I.S. (C) Calibration sample in water. Peaks: 7.16 min, epinephrine (50 pg/ml); 9.23 min, norepinephrine (500 pg/ml); 10.56 min, dopamine (50 pg/ml); 11.82 min, I.S. Sensitivity: 2.5 nA full scale.

assay. It has already been mentioned that normetanephrine coelutes with epinephrine. However, this substance is only poorly extracted from aqueous samples under the experimental conditions used. Therefore, only concentrations above 500 ng/ml normetanephrine would cause biased epinephrine results.

The β-blocking agent atenolol could not be detected electrochemically on

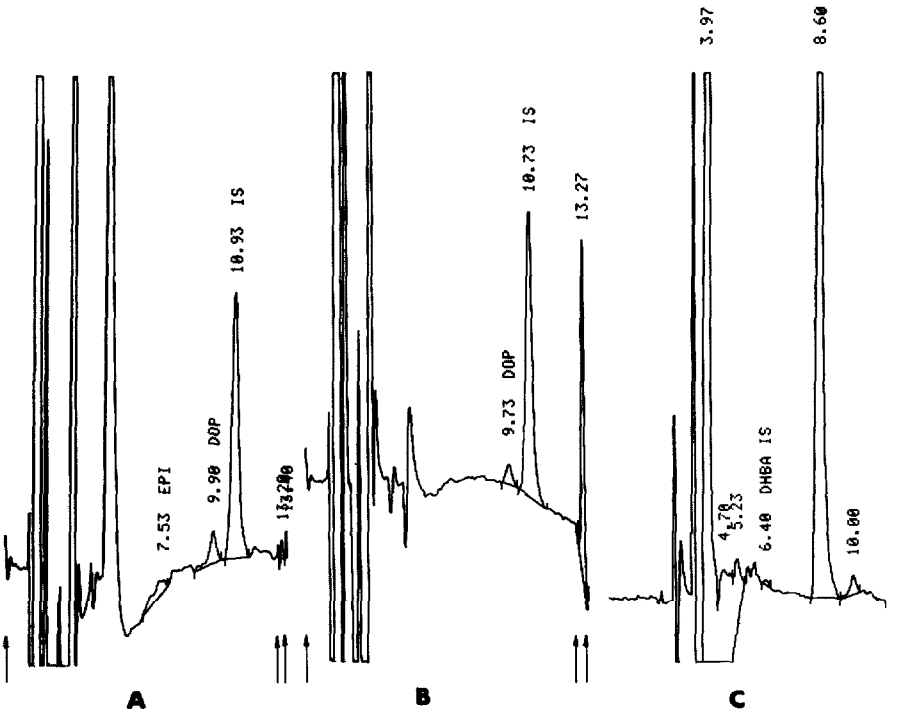
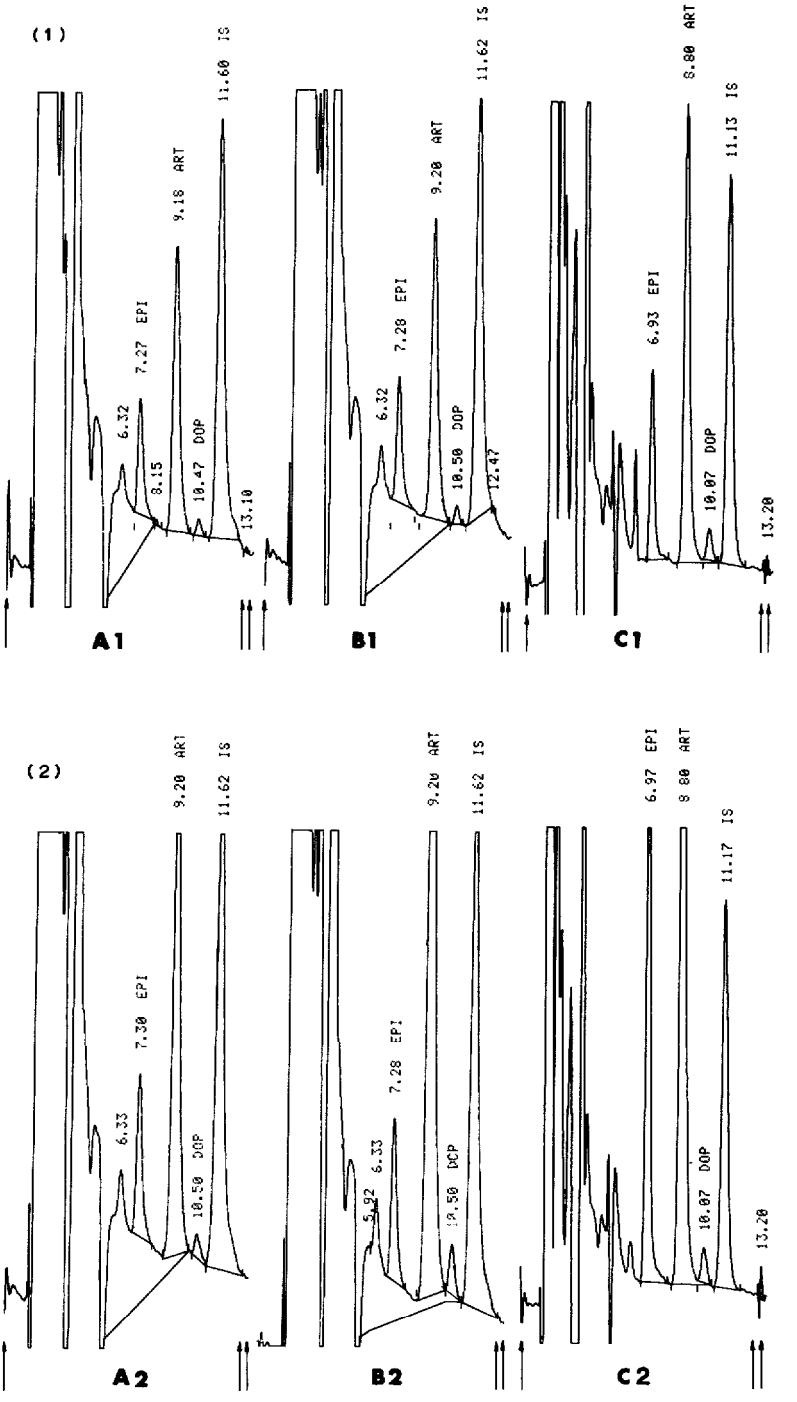


Fig. 5. Dopamine monosulphate in the catecholamine assay. (A) 1 ng/ml dopamine sulphate after work-up of a freshly prepared solution. Peaks: 9.90 min, dopamine; 10.93 min, I.S. (B) 1 ng/ml dopamine sulphate kept for 8 h at pH 8.5 prior to work-up. Peaks: 9.73 min, dopamine; 10.73 min, I.S. The chromatographic conditions were as described under Experimental. Sensitivity: 2.5 nA full scale. (C) 500 ng/ml dopamine sulphate after 24 h incubation with glucuronidase/sulphatase. Peak: 8.60 min, dopamine. Direct injection of 50 μl of the reaction mixture. Column, 25 cm $\times$ 4.6 mm I.D. packed with 10 μm μBondapak C_{18} (Waters Assoc.). Mobile phase: 94% citrate–acetate buffer (0.09 and 0.13 *M*, respectively, pH 4.7) + 6% (v/v) acetonitrile. Metrohm amperometric detector. Sensitivity: 25 nA full scale.

injection into the HPLC system, whereas the dopamine agonist SK&F 101468, an indole derivative, produced an interfering peak after work-up when 500 ng/ml were subjected to analysis. At 100 ng/ml no interference was seen. As this compound is typically administered in doses that give rise to maximum plasma concentrations below 50 ng/ml, no practical difficulty was seen.

Isoprenaline was extracted together with the catecholamines and coeluted with epinephrine. A separation of both compounds was achieved when a mobile phase consisting of buffer (as described)–acetonitrile (95:5, v/v) was used. The separation of dopamine and the internal standard was no longer complete with the modified mobile phase. No isoprenaline was detected, however, in plasma samples taken 60 min after a 16-μg dose of isoprenaline. The cate-


(1)
6.32
7.27 EPI
8.15
9.18 ART
10.47 DOP
11.60 IS
13.10
A1
6.32
7.28 EPI
9.20 ART
10.50 DOP
11.62 IS
12.47
B1
6.93 EPI
8.80 ART
10.07 DOP
11.13 IS
13.20
C1
(2)
6.33
7.30 EPI
9.20 ART
10.50 DOP
11.62 IS
A2
5.92
6.33
7.28 EPI
9.20 ART
11.62 IS
B2
6.97 EPI
8.80 ART
10.07 DOP
11.17 IS
13.20
C2

cholamine metabolite dihydroxyphenylacetic acid (DOPAC) did not produce a chromatographic signal on injection. The diphenylborate interference reported by other workers [18] was not observed.

In order to investigate the stability of catecholamine sulphate metabolites under the assay conditions, dopamine was derivatized according to the procedure described previously [19]. Even though the monosulphate could not be prepared completely free from dopamine, after 8 h in the assay buffer at room temperature no further dopamine was generated by hydrolysis (Fig. 5). In dilute acid (0.05 *M* orthophosphoric acid) slow hydrolysis was observed, whereas on addition of glucuronidase/sulphatase rapid cleavage of the semiester occurred with the formation of dopamine. We therefore conclude that the sulphate conjugates of the catecholamines do not interfere in our assay method.

Applications in clinical pharmacology

This method for the determination of epinephrine, norepinephrine and dopamine has been successfully used in numerous pharmacological studies for several years. The pharmacological aspects of these investigations have been reported elsewhere [20,21] or will be published separately. A few examples should suffice here to demonstrate the performance of the assay with real samples. Fig. 6 shows chromatograms of six plasma samples from a 26-year-old healthy male volunteer, who was studied in the fasting state on three different occasions at the same time of the day. On two study days a placebo was administered and on the third occasion a single oral dose of 50 mg atenolol was given. The subject was studied after supine rest of at least 60 min to establish a baseline followed by 15 min sitting or a 5-min isometric handgrip test (IHG). The catecholamine concentrations under baseline conditions were well reproduced. For epinephrine 26 pg/ml were measured on day A, 28 pg/ml on day B and 30 pg/ml on day C. The norepinephrine concentrations found were 114 pg/ml on day A, 120 pg/ml on day B and 107 pg/ml on day C. The dopamine results were 6 pg/ml on day A, 7 pg/ml on day B and 8 pg/ml on day C. The postural

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Fig. 6. (1) Plasma samples from clinical studies, subject No. 3, after supine rest for at least 60 min, before dosing. (A1) First study day, placebo. Peaks: 7.27 min, epinephrine; 9.18 min, norepinephrine; 10.47 min, dopamine; 11.60 min, I.S. (B1) Second study day, atenolol. Peaks: 7.28 min, epinephrine; 9.20 min, norepinephrine; 10.50 min, dopamine; 11.62 min, I.S. (C1) Third study day, placebo. Peaks: 6.93 min, epinephrine; 8.8 min, norepinephrine; 10.07 min, dopamine; 11.13 min, I.S. (2) Plasma samples from clinical studies, subject No. 3, after stress test. (A2) First study day, placebo, after 15 min sitting. Peaks: 7.30 min, epinephrine; 9.20 min, norepinephrine; 10.50 min, dopamine; 11.62 min, I.S. (B2) Second study day, atenolol, after 15 min sitting. Peaks: 7.28 min, epinephrine; 9.20 min, norepinephrine; 10.50 min, dopamine; 11.62 min, I.S. (C2) Third study day, placebo, after 5 min IHG. Peaks: 6.97 min, epinephrine; 8.80 min, norepinephrine; 10.07 min, dopamine; 11.17 min, I.S. Chromatograms C1 and C2 were recorded after replacement of the column and detector cell with new parts. Concentrations of I.S.: (A) and (B) 250 pg/ml; (C) 125 pg/ml. The chromatographic conditions were as described under Experimental. Sensitivity: (A) and (B) 4 nA full scale; (C) 2.5 nA full scale.

change from a supine to a sitting position under placebo was accompanied by an increased norepinephrine concentration. The epinephrine and dopamine concentrations were not affected (day A, sitting: epinephrine 29 pg/ml, norepinephrine 178 pg/ml, dopamine 7 pg/ml). The postural norepinephrine response was significantly enhanced when atenolol was administered (day B, sitting: epinephrine 29 pg/ml, norepinephrine 315 pg/ml, dopamine 18 pg/ml). Under these conditions an increase in the dopamine concentrations was also observed. After the isometric handgrip test a marked increase in the epinephrine level was recorded, whereas dopamine remained unchanged. The norepinephrine concentrations were more increased than after sitting (day C, IHG: epinephrine 84 pg/ml, norepinephrine 211 pg/ml, dopamine 9 pg/ml).

These examples show further that a very sensitive assay method is required if dopamine plasma concentrations are to be measured. For the determination of epinephrine a limit of quantification of 10 pg/ml may be sufficient in most instances. However, we have encountered a few subjects who displayed epinephrine concentrations consistently below 10 pg/ml and occasionally even below 5 pg/ml.

The combination of liquid–liquid extraction for sample preparation with chromatographic separation of the analytes on weak cation-exchange materials monitored with a coulometric detector has proved to be a useful and reliable tool for the determination of catecholamines present at physiological concentrations.

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