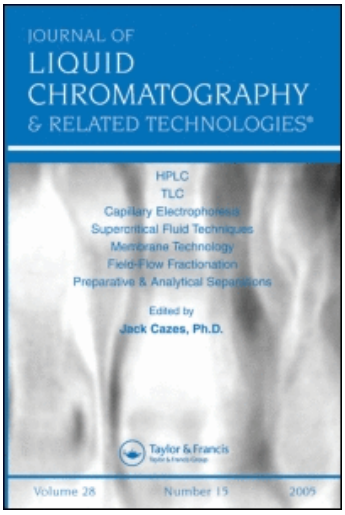

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Determination of Plasma Norepinephrine and Epinephrine by High Performance Liquid Chromatography Using a Two-Column System and an Electrochemical Detector

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DETERMINATION OF PLASMA NOREPINEPHRINE AND EPINEPHRINE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY USING A TWO-COLUMN SYSTEM AND AN ELECTROCHEMICAL DETECTOR

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ABSTRACT

A method for the simultaneous plasma norepinephrine (NE) and epinephrine (E) determination by reversed-phase ion-pair liquid chromatography with electrochemical detection has been developed. Catecholamines were extracted from a 4 ml plasma sample using an alumina adsorption procedure. A two-pump, two-injection valve, two-column system allowed both to detect plasma NE and E with a good sensitivity due to large injected volumes of extract without any electrochemical detector disturbance and to eliminate uric acid and dopa the low k' of which would prevent the NE detection. Using this method, NE and E would be detected in respective injected amounts down to 30 and 50 picograms. Plasma NE and E determinations were found to be linear in the range of 288 to 788 pg/ml and 24 to 274 pg/ml respectively. The reproducibility, expressed as the coefficients of variation, varied from 2.1 % for NE to 10.8 % for E.

INTRODUCTION

The determination of the plasma catecholamines norepinephrine (NE) and epinephrine (E) can be useful both in clinical studies

when a pheochromocytoma is suspected (1, 2) and in physiopathological and pharmacological studies (3, 4, 5, 6, 7, 8, 9, 10).

The detection of low plasma levels of NE (less than 500 picograms per milliliter) and E (less than 100 pg/ml) in healthy individuals requires the use of techniques involving extremely sensitive procedures, such as gas chromatography/mass spectrometry (11, 12) or radioenzymology (13, 14).

High performance liquid chromatography (HPLC), appeared in recent years as being a new efficient method for the separation of catecholamines and their derivatives (15, 16). If numerous techniques dealing with the assay of catecholamines in tissues or urines have been published, few authors have described the assay of plasma catecholamines by HPLC. Two detection methods have been used in plasma : fluorimetric and electrochemical. Fluorimetric detection has been employed after products had been derivatized with orthophthalaldehyde (17) or transformed into trihydroxyindole derivatives (18). Electrochemical detection which seemed to be an accurate method in order to measure catecholamines concentrations has been used by Hallman et al (19), Hjendahl et al (20) and Allenmark et al (21) together with ion exchange HPLC.

Like Davis et al (22), we have chosen to use a reversed-phase ion-pairing chromatography technique. This method allows us to modify many parameters : pH of the mobile phase, ionic strength, type and number of carbon atoms in the n-alkyl chains, concentration of the counter-ion, in order to improve the selectivity of the system.

A major problem which remains unanswered is the close elution of uric acid and NE, the capacity factor (k') of which are similar. The method we report allows us to overcome these problems using two injection valves and two columns one of which is placed in a loop position.

EXPERIMENTAL

Instrumentation

The chromatographic system (scheme 1) consisted of - two pumps : Waters 6000 A (Waters Assoc. Inc., Milford, MA., U.S.A.) - two injection valves : Rheodyne 7125 and 7110 (Rheodyne, Santa Rosa, U.S.A.) equipped, the first (I) with a 250 µl loop, the other (II) with a short column placed in loop position - a thin layer electrochemical detector : Eldec 102 (Chromatofield, Chateauneuf-les-Martigues, France) provided with a glassy carbon electrode maintained at a + 0.75 V potential vs a Ag/AgCl reference electrode. The detector output was connected to a recorder : Kontron W + W 610 (W.W. Electronic Inc., Basel, Switzerland).

The two stainless steel columns we used (a short column : 4.6 × 35 mm and an analytical column : 4.6 × 150 mm) were packed under pressure by a slurry packing technique with Nucleosil C18, 5 µm (Macherey-Nagel, Düren, F.R.G.) according to the method described by Coq (23).

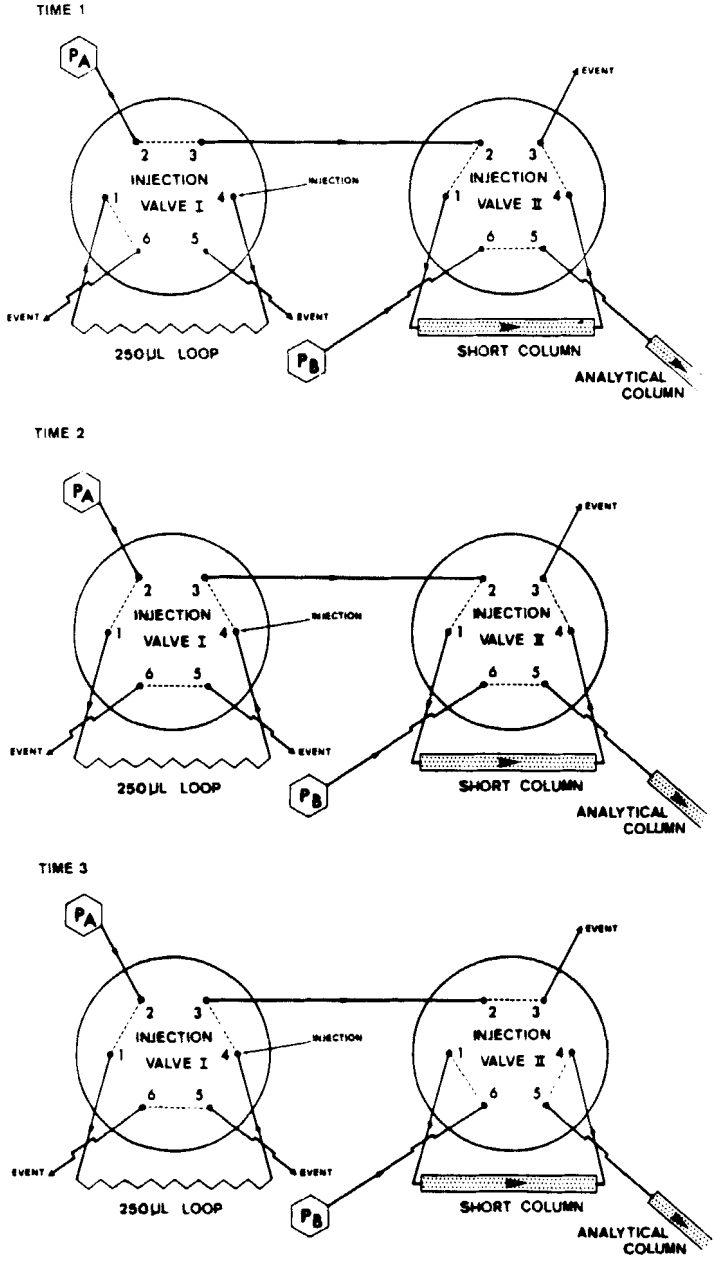
A pulse damper (Touzart-Matignon, Vitry-sur-Seine, France) was inserted between the pump and the injection valve in order to decrease the background.

The 250 µl syringe we used was fitted with a teflon piston nozzle (Hamilton, Bonaduz, Switzerland).

The detector was mounted within its proper Faraday cage. The detector, the column and the metal capillaries were grounded.

Chemicals and solvents

Catecholamines (NE, E, dopamine (DA), epinine), their precursors (phenylalanine, tyrosine, phenylethylamine, tyramine, dopa), their derivatives (3,4,5,trihydroxyphenylethylamine, metanephrine, normetanephrine, vanilmandelic acid, homovanillic acid) as well as the internal standard (dihydroxybenzylamine (DHBA)) were provided by Sigma (Sigma Chemical Co., Saint-Louis, MO., U.S.A.).



Scheme 1. - Diagram of the HPLC system showing the three steps of the chromatography.

1 mg/ml standard solutions were prepared in 0.1 M $HClO_4$ and stored at + 4°C for a month, in brown glass flasks. 10 ng/ml working solutions (8 ng/ml for DHBA) were obtained daily by dilution of the standard solutions with 0.1 M $HClO_4$. The heptane sulfonic acid sodium salt was provided by Eastman Kodak (Eastman Kodak, Rochester, NY, U.S.A.), the disodium ethylenediaminetetraacetate (EDAT Na_2) and the acid type alumina were provided by Fluka (Fluka, Buchs, Switzerland). Alumina was reactivated before use according to the Anton and Sayre technique (24). 1 M tris buffer (pH = 8.6) and sodium metabisulfite were from Merck (Merck, Darmstadt, F.R.G.).

Chromatographic conditions

The mobile phase we used consisted of 15 g/l phosphate buffer (NaH_2PO_4,$2H_2O$), 300 mg/l of heptane sulphonate and 200 mg/l of EDTA. The pH was ajusted to 4.2 with 3 M phosphoric acid. 5% (Vol%) acetonitrile was used as an organic modifier of this mobile phase. The mixture was filtered through a 0.45 µm filter then degassed in an ultrasonic bath.

The elution was carried out at ambient temperature. They were cleaned every day using 60 ml of methanol. The electronic system was left continuously under tension, the cell put to counterbalance for at least 90 minutes before each manipulation.

Two pumps (P_A and P_B) delivered independently the same mobile phase which was thus divided into two parts (MP_A and MP_B). The flow rates of MP_A and MP_B were identical (1 ml/min). The usual positions "load" and "inject" on valve II (Rheodyne 7110) were inverted and so were the entry numbers (2←→6, 3←→5) thus allowing the flow in the short column to be always in the same way (whatever the position of the valve might be). Valve I (Rheodyne 7125) was used according to the operating instructions.

The chromatography was carried out over three periods (scheme 1) :

1. Valve I and valve II were placed in the "load" position. The loop was filled with 200 µl of eluate containing catecholamines. During this period, MP_A flowed through the short column and MP_B through the analytical column.
2. Valve I was placed in the "inject" position while valve II remained in the "load" position. Thus, catecholamines in the loop eluate were sent through the short column, while MP_B was flowing through the analytical column.
3. After a 60 second elution through the short column, valve II was placed in the "inject" position. So catecholamines were eluted by MP_B from the short column into the analytical column. MP_A was discarded.

Sample preparation

Polystyrene tubes containing 100 µl of the following mixture : heparine (50 mg/ml) and metabisulfite (25 mg/ml) were kept in an ice bath.

In fasting volunteers asked to remain in the supine position, an arm vein was catheterized. 20 min after the veinipuncture a 10 ml blood sample was drawn using a heparinized syringe. Blood was immediately transfered into the polystyrene tubes.

The tubes were centrifuged at + 4°C ; the plasma was separated from blood cells and stored in polystyrene tubes at + 4°C if the assay was to be done within the following 12 hours. If the assay was done later, plasma was stored at - 20°C for a maximum of 7 days.

Extraction was done at room temperature by adsorption on alumina according to the classic method without deproteinization. In 10 ml polystyrene tubes, to 4 ml of plasma were added 500 µl of 1 M HCl tris buffer and 200 µl of DHBA (8 ng/ml) used as an internal standard. The mixture was homogenized for 15 sec (final pH = 8.6) and 75 mg of alumina was added. The tubes were capped and placed on a reciprocal shaker for 15 min and then spun down at 1000g for 5 min. The supernatant was discarded and alumina was

washed three times with bidistilled water. The washings were removed by aspiration. Following the last wash, alumina was aspirated to near dryness. Catecholamines were eluted from the alumina by 250 µl of 0.1 M $HClO_4$. All the eluate was transfered to a small Eppendorf conic tube, spun for 1 min (8000g) and stored in the dark at + 4°C until the assay. A 200 µl eluate sample was injected in the chromatographic system.

As the samples contained an internal standard, catecholamines concentrations were calculated by measuring the following ratio : height of catecholamine peak/height of internal standard peak, and comparing that to the mean of equivalent ratios obtained from two aqueous extracts containing known amounts of catecholamines (250 pg of NE and E) assayed following the same procedure.

RESULTS

As most of the perchloric acid was eliminated before it entered the analytical column, the k' we measured were rough estimates : 0.9 (NE), 1.7 (E), 2.3 (DHBA), 4.1 (DA). (Fig. 1a, 1b).

Linearity

Figure 2 shows the linearity plots when injecting increasing amounts of each catecholamine as pure solutions (from 50 pg to 1 ng). The plots indicate that linearity was good up to 1 ng.

Figures 3 and 4 show linearity plots obtained after extraction from aqueous solutions of catecholamines and plasmas charged with NE and E. No linearity discrepancy appeared in plasma concentrations ranging from 288 to 788 pg/ml for NE and from 24 to 274 pg/ml for E.

Reproducibility (table 1)

It was tested by repeated measurements of NE and E in two different plasmas at a 15 day-interval. Five assays were done in the first plasma and seven in the second.

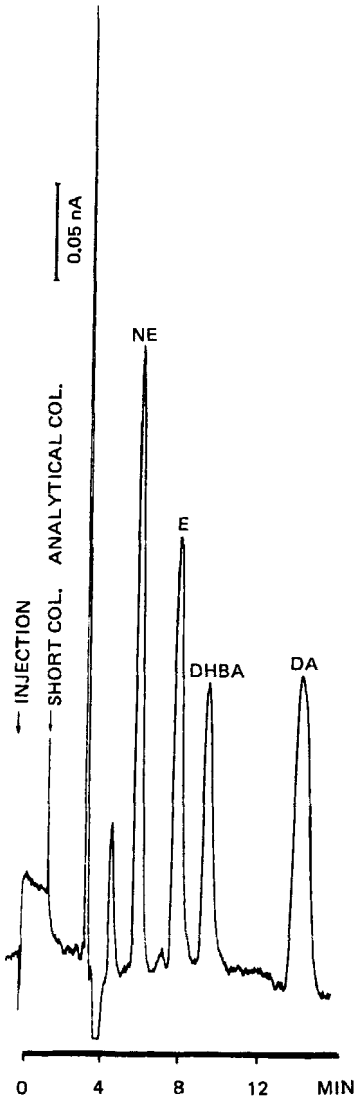


FIG. 1A

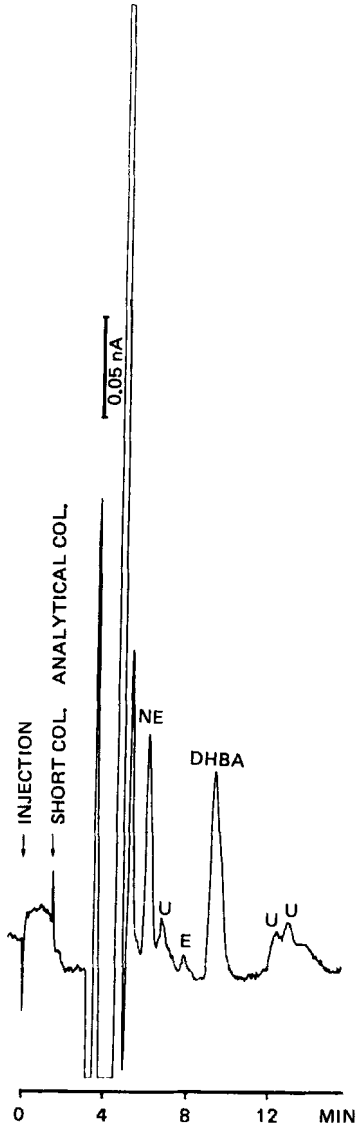


FIG. 1B

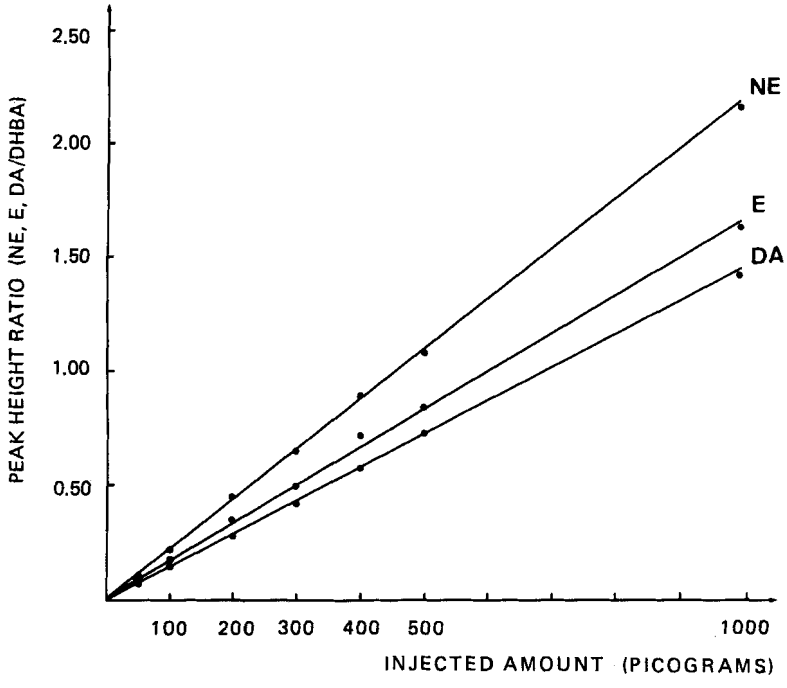


Figure 2. - Standard curves obtained after chromatography of increasing amounts of catecholamines (NE, E, DA) and 800 picograms of internal standard (DHBA) in 0.1 M $HClO_4$.

Figure 1. - a. Injection of 1 ng of each catecholamine and 0.8 ng of internal standard, in 200 ul of 0.1 M $HClO_4$.

b. Chromatogram of catecholamines obtained from 4 ml of human plasma to which 200 ul of a 8 ng/ml DHBA solution were added. This sample was extracted with alumina eluated by 250 ul of 0.1 M $HClO_4$ and a 200 ul eluate was injected. The NE and E peaks are respectively equivalent to 230 and 33 pg/ml of plasma. (U = unknown peak).

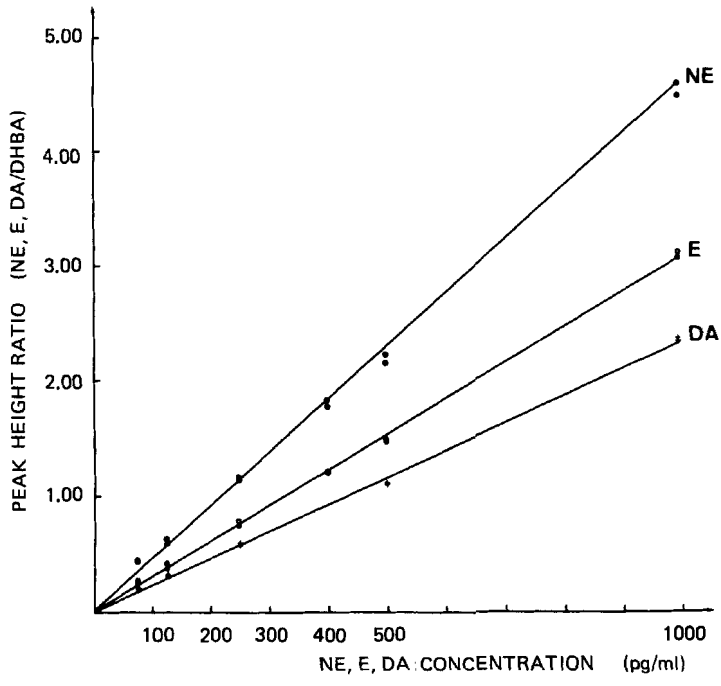


Figure 3. - Standard curves obtained after extraction and chromatography of NE, E, DA at different concentrations in aqueous solutions.

Detection limit

A study of the applied voltage between 0.5 and 0.8 volts has shown that the best signal-to-noise compromise (S/N) was reached at 0.75 volts. Under these conditions, the detection limit defined as the amount of catecholamines leading to a S/N ratio of 5 was 30 pg of NE and 50 pg of E.

Recovery (table 2)

This study was done after extraction of each catecholamine (250 pg/ml) and of the internal standard (400 pg/ml) from a 4 ml aqueous solution. Four extracts were recovered. In order to compare the peak heights of the extracts, we injected an amount

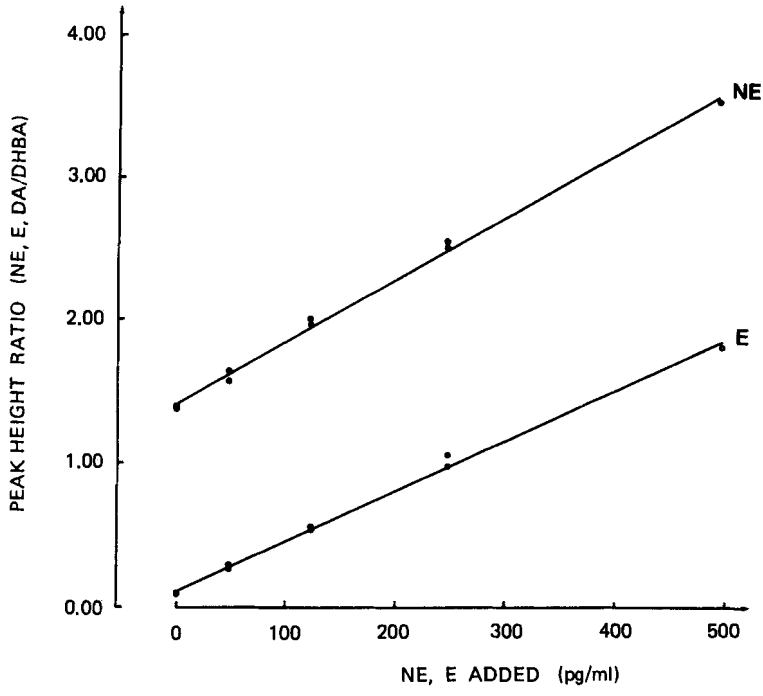


Figure 4. - Standard curves obtained after extraction and chromatography of a human plasma charge with NE and E. The plasma sample contained 288 and 24 pg/ml of NE and E respectively before addition.

TABLE 1

Reproducibility of the Method (Extraction, Chromatography)

	Extracts							Mean	Coefficient of variation (percentage)
	1	2	3	4	5	6	7		
Plasma I[a]									
NE (pg/ml)	258	260	256	256	271			260	2.4
E (pg/ml)	128	109	100	122	103			112	10.8
Plasma II[a]									
NE (pg/ml)	274	261	276	270	272	279	268	272	2.1
E (pg/ml)	47	43	43	50	37	43	43	43	9.2

a - Blood was collected without taking special precaution (subjects were not resting or lying before sampling, blood was drawn by veinipunture and not through an indwelling catheter).

TABLE 2

Recovery Percentage of Catecholamines and Internal Standard after Extraction and Chromatography

Compounds		Peak height after extraction (mm)				Mean of peak height without extraction (mm) (mean of 3 injections)	Recovery % (±2 SD)
		1	2	3	4		
NE	(800 pg)	62	67	62	67	113	57.3 ± 5.1
E	(800 pg)	41	43	38	45	74	56.1 ± 8.6
DA	(800 pg)	33	33	28	37	60	54.8 ±12.3
DHBA	(1280 pg)	50	54	49	53	97	53.1 ± 4.9

of catecholamines and internal standard equivalent to a 100 % yield. Taking into account the fact that only a 200 µl volume out of the 250 µl eluate was injected, an amount of 800 pg of each catecholamine and 120 pg of internal standard was injected. The recovery percentage was calculated by measuring the peak height ratio (53.1 - 57.3 %).

Conservation of catecholamines and interference (tables 3, 4)

The samples of catecholamines in 0.1 M $HClO_4$ were divided into four portions. Two samples were processed after 4.5 hours of storage at + 4°C either in the dark or exposed to daylight ; two other samples were processed after 2.5 hours of storage at room temperature either in the dark or exposed to daylight. No significant difference was noted (table 3) between the results of stored samples.

Table 4 illustrates that in no case was a detectable interference between catecholamine or internal standard and tested endogenous compounds.

DISCUSSION

During our first chromatographic studies of catecholamines, we used two detection methods : flucrimetric (direct or after post

TABLE 3

Conditions of Conservation of Catecholamines

Conditions of conservation[a]	Peak height of each compound (mm)[b]		
	NE	E	DA
4.5 hours (+ 4°C, darkness) [c]	49	31	25
4.5 hours (+ 4°C, light)	50	31.5	25
2.5 hours (room temperature, darkness)	48	28	25
2.5 hours (room temperature, light)	50	30	25

a - Concentrations are close to those in the eluate (2,5 ng/ml).
b - Injection of 200 µl of a 2,5 ng/ml solution of each catecholamine in 0.1 M $HClO_4$.
c - Considered as ideal conditions of conservation.

column derivatization with orthophthalaldehyde) or electrochemical. Then, we chose electrochemical detection which appeared to be one of the most effective method, thus confirming results obtained by Yui (25).

Using this method, the most effective stationary phase was Nucleosil C18, 5 µm (26).

At the beginning of our work, the mobile phase pH was set at 5.0 in order to facilitate the oxydation of catecholamines so as to enhance the detector response (16). Later as we used a different batch of the same stationary phase, we observed a band spreading and so had to set the pH at 4.2. This pH induces a loss of ionization of OH and above all facilitates the protonation of the amine, thus improving the efficiency of the counterion. Consequently, we had to slightly increase the amount of acetonitrile (5 %).

During these tests, we noticed that when using an aliquot of 4 ml and an eluate of 250 µl, the best extraction yield needed 75 mg of alumina.

Among the electro active compounds extracted together with catecholamines were uric acid and dopa. This lack of selectivity

TABLE 4

Interference from Endogenous compounds

Compounds	Capacity factors	Extraction with alumina[a]	Detection in our HPLC system
Norepinephrine	0.9	+	+
Epinephrine	1.7	+	+
Dopamine	4.1	+	+
Dihydroxybenzylamine	2.3	+	+
Dopa	no peak		-
Epinine	7.1	-	-
Homovanillic acid	no peak		-
Metanephrine	6.4	-	-
Normetanephrine	4.7	-	-
Phenylalanine	no peak		-
Phenylethylamine	no peak		-
Tyramine	no peak		-
Tyrosine	no peak		-
3,4,5 trihydroxy-phenylethylamine	2.3	-	-
Vanilmandelic acid	no peak		-

a - Concentration of each compound = 2 ng/ml

due to the alumina extraction is a major problem which could induce interferences during chromatography. Effectively, the close k' of uric acid and NE as well as the destabilization of the detector due to the injection of 200 µl of 0.1 M $HClO_4$ impede the detection of NE and E. As the retention time of uric acid did not seem to be modified by the use of various counter-ions and as a double extraction by ion exchange and alumina adsorption (22) could lead to more inaccuracy and increase the length of the assay we used the chromatographic system previously described. This

system uses a preset optimal elution time of 60 seconds in the short column and a 1 ml/min flow. Thus uric acid and 0.1 M $HClO_4$ are eliminated and the analytical column is protected. The mixture which is injected in the analytical column is very similar to the mobile phase, so the detector is less disturbed. All this procedure permits to inject a large volume.

Of course this large volume leads to a slight band spreading and to a loss of resolution. Yet chromatograms are quite satisfactory as peaks are perfectly resolved.

One of the drawbacks of this method using ion-pairing is the loss of efficiency which could limit the detection of E.

We applied this method to the detection of catecholamines in ten volunteers. We measured NE concentrations ranging from 137 to 553 pg/ml and E concentrations ranging from 23 to 100 pg/ml. These results are similar to those previously published (9, 22, 27). We were not able to detect plasma DA which is conjugated as sulfates or glucuronides in a considerable proportion (28). Though a recent study has shown a good stability of catecholamines in plasma (29) we prefered not to store plasma samples more than a week at - 20°C.

We have presented a reliable method permitting the simultaneous assay of plasma norepinephrine and epinephrine. We think this method is useful, first as a chemical tool using reversed-phase ion-pair liquid chromatography with electrochemical detection, second as a clinical tool in order to establish some difficult diagnosis and third as a research tool in physiology and pharmacology.

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