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DETERMINATION OF CATECHOLAMINES IN URINE BY ION-EXCHANGE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A liquid chromatographic method for the determination of free urinary concentrations of epinephrine, norepinephrine and dopamine is presented. For urine samples, pre-purified by adsorption onto alumina, ion-exchange chromatography was, in terms of selectivity, found to be superior to the more widely used reversed-phase chromatography. The column eluates were monitored with an electrochemical detector utilizing a glassy carbon working electrode. The method allows determination of the concentrations in 0.5 ml of normal urine samples with a relative standard deviation below 2%.

INTRODUCTION

Simplified analytical methods for the determination of free urinary catecholamine concentrations are of great interest, and during the last decade continuous efforts have been made to improve these techniques. Liquid chromatographic methods with on-line fluorometric [1—5] or electrochemical detection [6—8] have been developed to achieve better sensitivity and selectivity than given by the previously used trihydroxyindole procedure. Urine is a complex matrix which contains many electroactive and fluorescent substances making pre-purification mandatory. In post-column trihydroxyindole methods the fluorescence of dopamine is weak [2, 4, 5]. In the case of electrochemical detection a single purification step has proved to be insufficient [6—8]. Extensive purification by adsorption onto alumina combined with cation-exchange resin [6], boric acid gel [7] or Sephadex [8] have been used. An improvement was obtained when the catecholamines were extracted using complex formation in alkaline medium between diphenylborate and the catechol group [9]. A method for direct injection of urine using pre-column sample enrichment in a micro liquid chromatographic system has

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been presented [10] and recently a paper reported that purification by alumina adsorption was sufficient when using a sodium gradient for the separation [11].

The pre-purification needed depends on the selectivity of the liquid chromatographic system used for analysis. Reversed-phase C_{18} materials have frequently been used in chromatography of catecholamines. However, for the analysis of urinary catecholamines, microparticulate strong cation-exchange columns appear to be favourable as they do not retain interfering components as is the case for the reversed-phase columns. The sample preparation could then be a simple solvent extraction and adsorption onto alumina to eliminate non-catechols and to concentrate the sample.

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of an LDC mini-pump 711-47 (Riviera Beach, FL, U.S.A.) with a Touzart-Matignon pulse dampener (Vitry-sur-Seine, France), an injection valve (Rheodyne 7125, Berkeley, CA, U.S.A.) with a 60-μl loop, a stainless-steel separation column (150 × 4.5 mm I.D.) and an electrochemical detector [Bioanalytical Systems (BAS) LC 4, West Lafayette, IN, U.S.A.]. The detector was operated at +0.7 V with an Ag/AgCl reference electrode BAS RE 1 and a thin-layer cell BAS TL 5A consisting of a glassy carbon working electrode. A Cenco rotary mixer for 56 tubes (Breda, The Netherlands) was used to rotate the tubes.

Chemicals

Epinephrine (E) and norepinephrine (NE) (hydrogen tartrate form) were obtained from Société des Usines Chimiques (Paris, France) and Österreichische Stickstoffwerke (Linz, Austria), respectively. Dopamine (DA) hydrochloride and reduced glutathione (GSH) were from Sigma (St. Louis, MO, U.S.A.). α-Methyldopamine (MDA) hydrochloride was obtained from Merck Sharp and Dohme (Rahway, NJ, U.S.A.) and tris(hydroxymethyl)aminomethane (Tris), analytical grade, was of Fluka quality (Buchs, Switzerland). Alumina, Woelm neutral, was from Woelm Pharma (Eschwege, F.R.G.) and was prepared according to the method given in ref. 12. 3,5-Dimethylcyclohexyl sulphate (DMCHS) was supplied by the Department of Organic Chemistry, AB Hässle (Mölndal, Sweden). Tetrahydrofuran (THF) and hexane, both of HPLC grade, were purchased from Rathburn Chemicals (Walkerburn, U.K.). Disodium EDTA, methanol, ethyl acetate, sodium hydroxide, acids and buffer substances were all of analytical grade from E. Merck (Darmstadt, F.R.G.).

Analytical procedure

Fresh urine samples were adjusted to pH 2 with hydrochloric acid (5 mol/l) before freezing. Frozen samples were thawed, homogenized by shaking and centrifuged. Urine to which the internal standard MDA (4 μmol/l urine) had been added was mixed for a few seconds with two volumes of ethyl acetate followed by one volume of hexane, the organic solvents being aspirated. Aqueous phase corresponding to 0.5 ml of urine was transferred into a 4-ml

conical centrifuge tube; 50 μl of GSH (0.05 mol/l), 50 μl of EDTA (0.3 mol/l, pH 7) and 20 mg of alumina were added. Then 300 μl of Tris buffer (1 mol/l, pH 8.6), or the volume giving a pH of 8.5, were added while vortexing the tube, which was then rotated in a rotary mixer for 10—20 min. The aqueous phase was discarded and the alumina was washed three times by mixing for a few seconds with an EDTA solution (3 mmol/l, pH 7). The tube was centrifuged, whereupon the amines were eluted from the alumina by vortexing for 1 min with 250 μl of perchloric acid (0.2 mol/l). The tube was stored frozen (—20°C) and thawed just before injection of 50 μl onto the column. Each series of analyses also included reference samples of 40 pmol of the catecholamines and 2 nmol of the internal standard.

Chromatographic system

The separation column was packed with Nucleosil SA (strong cation-exchange), 5-μm average particle size, from Macherey-Nagel (Düren, F.R.G.). The packing was performed upwards at 450 bar with methanol as slurry medium and eluent (see ref. 13). The mobile phase was a citrate buffer (I = 0.15, pH 5.0) containing 7% THF. The composition of the buffer was so-

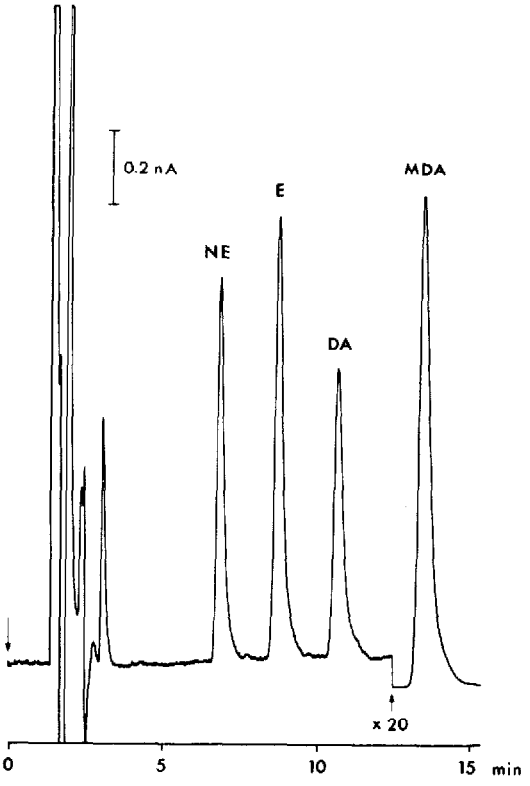

Fig. 1. Chromatogram of a reference sample, worked up according to the analytical procedure, containing 40 pmol each of norepinephrine (NE), epinephrine (E) and dopamine (DA), and 2 nmol of α-methyldopamine (MDA). A 50-μl sample was injected. Stationary phase: Nucleosil 5 SA, 5 μm. Mobile phase: citrate buffer (I = 0.15, pH 5.0) containing 7% THF. Potential: +0.7 V.

dium hydroxide (98.4 mmol/l) and citric acid (52.2 mmol/l). Deionized water, which had been passed through a Milli-Q reagent-grade water system (Millipore, Bedford, MA, U.S.A.), was used. The mobile phase was degassed by vacuum filtering through a 0.45-μm MF-Millipore filter. The flow-rate was 1.0 ml/min. The detector cell was kept in a Faraday cage to avoid disturbances.

Quantitative evaluation

The ratio between the peak height of the catecholamine and the internal standard for the sample was compared with the median value of the corresponding ratios for the reference samples.

RESULTS AND DISCUSSION

Chromatography

Ion-exchange chromatography was used for the separation of the catecholamines in urine, the mobile phase being citrate buffer with THF as organic modifier. Chromatograms from injections of a reference sample and a urine sample, worked up according to the analytical procedure, are shown in Figs. 1 and 2 respectively. If reversed-phase chromatography was used the isolation by adsorption onto alumina was insufficient. This can be seen in Fig. 3, which shows a chromatogram for the same sample as in Fig. 2.

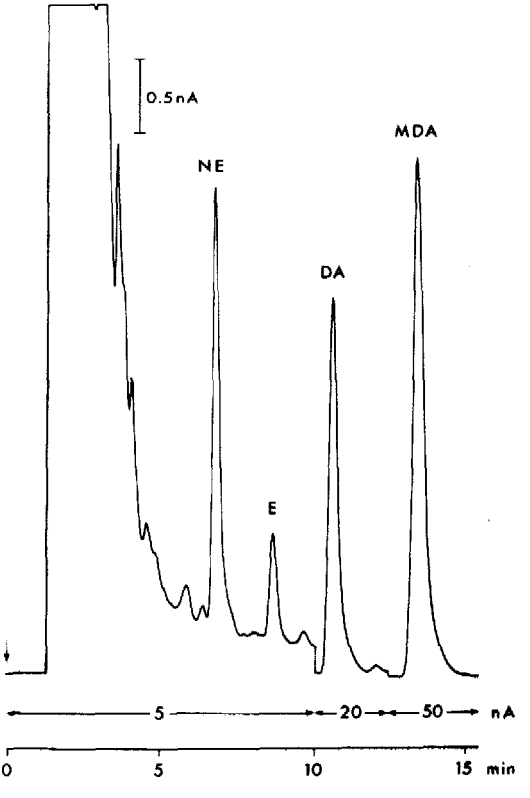

Fig. 2. Chromatogram from 0.5 ml of urine, containing 103 pmol of norepinephrine (NE), 25 pmol of epinephrine (E) and 488 pmol of dopamine (DA). The chromatographic conditions were the same as in Fig. 1.

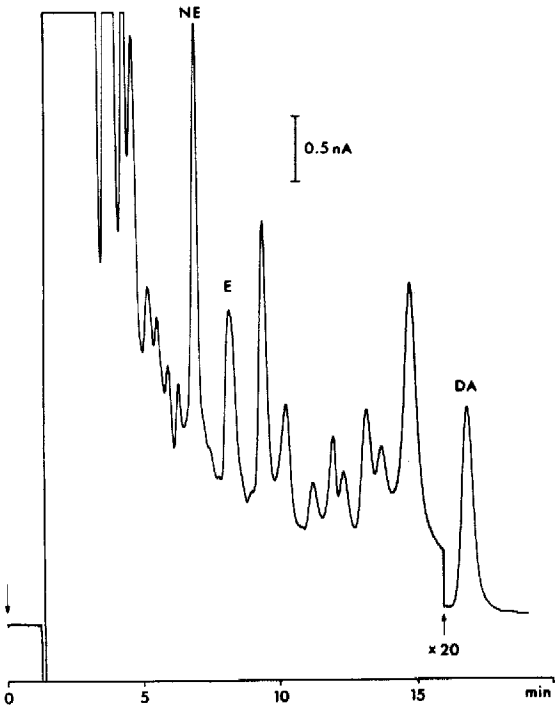

Fig. 3. Chromatogram of the same sample as in Fig. 2, injected onto a reversed-phase system. A 50-µl sample was injected. Stationary phase: LiChrosorb RP-18, 5 µm. Mobile phase: citrate buffer (I = 0.1, pH 5.0) containing 2% THF and DMCHS (4.2 nmol/l). Potential: +0.7 V.

The ion-exchange columns used have shown both efficiency and long-term stability. However, the quality of different batches of the ion-exchange packing material was found to vary. The variability of the retention properties from one batch to another could in most cases be overcome by modifying the ionic strength of the mobile phase. A few batches were not usable at all, since they either did not retain the catecholamines or retained interfering substances. An example of the latter is shown in Fig. 4, where the sample injected was the same as in Fig. 2.

Methanol was originally used as organic modifier in the mobile phase, but in some urines other sample components interfered, which resulted in falsely high values. With THF this was avoided and epinephrine was better separated from dopamine without an increase of the total elution time. Several mobile phases with varying contents of methanol, THF, acetonitrile or a combination of two of these were tested.

Detection

The effect on the detector response of varying contents of methanol or THF in citrate buffer was investigated (Fig. 5). The separation column was substituted with a PTFE coil to eliminate the difference in retention properties of the column for the various contents of organic modifier. The response decreased with increasing content of organic solvent, more rapidly

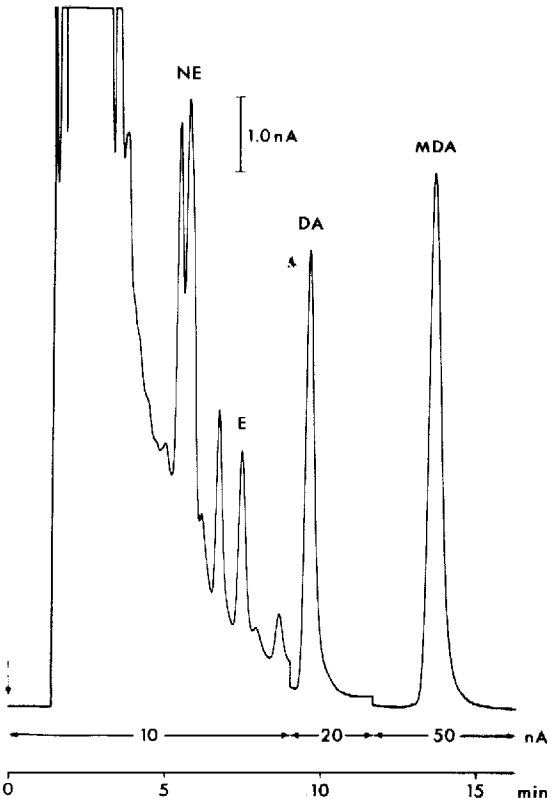

Fig. 4. Separation on an ion-exchange column retaining interfering substances. The urine sample injected and the chromatographic conditions were the same as in Fig. 2.

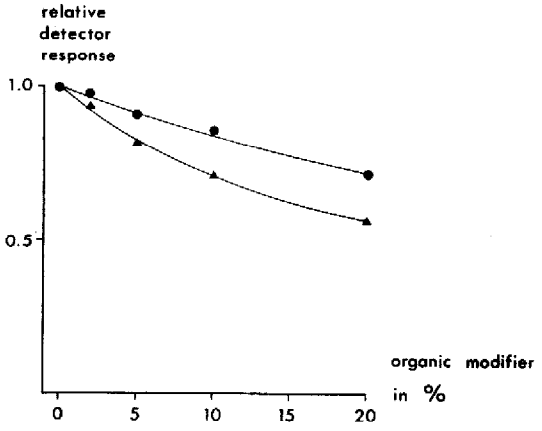

Fig. 5. Detector response versus addition of methanol or THF in citrate buffer (I = 0.1, pH 5.0) as mobile phase. (●), Methanol; (▲), THF.

for THF than for methanol. The loss of response was, however, compensated by increased column efficiency. The admixtures of methanol and THF used, 20 and 7% respectively, gave about the same response.

Accuracy, recovery and coefficient of variation

Extraction of the urine with ethyl acetate before adsorption onto alumina was not needed for most of the urines tested. However, the extraction resulted in a smaller front peak and for some urines in the removal of small interfering peaks, which is why it is recommended. Chromatograms of such a urine sample before and after extraction are shown in Figs. 6 and 2, respectively. The extraction with hexane removed the residual ethyl acetate dissolved in the aqueous phase.

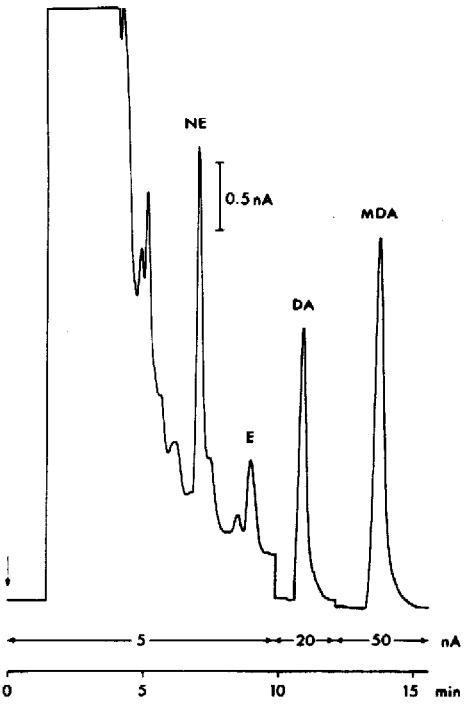

Fig. 6. Chromatogram of a sample not extracted with ethyl acetate. The urine used and the chromatographic conditions were the same as in Fig. 2.

Ten urine samples and a reference solution were monitored at two different potentials. The quotients between the peak heights of the catecholamines at the two potentials were the same in the urine samples and in the reference solution, which indicated that there were no interfering peaks.

It was observed that the internal standard MDA was less stable than the other catecholamines. In 0.01 mol/l perchloric acid solution MDA was unstable when stored in the refrigerator (+4°C). A peak that eluted at the same time as epinephrine appeared after a few days. To make sure there was no decomposition of the MDA in the worked-up samples, the MDA solution was assayed in parallel to the urine samples. No interfering peak had appeared after storing the samples for one month at −20°C.

Standard curves of norepinephrine, epinephrine and dopamine with and without urine were co-linear for each of the catecholamines, thus quantification could be performed from aqueous reference samples. The absolute re-

covery of the latter was earlier determined to be 82—92% [12]. The intra-assay coefficients of variation were 1.5% for norepinephrine, 1.1% for epinephrine and 0.6% for dopamine, when performing the analysis on twelve replicates of a urine sample.

If higher sensitivity than that obtained by the analytical procedure is required, a urine volume of 2 ml and an elution volume of 150 μl can be used without any decrease in recovery. The linearity of the method was tested for concentrations up to ten times that of an average urine sample.

Application

Urine samples collected over a 24-h period from five healthy persons were analysed. The urine from each 8-h period was collected in 10 ml of hydrochloric acid (3 mol/l), to maintain a final pH between 2 and 3, and was stored at −70°C before being analysed. The results are summarized in Table I. During the period covering sleep there was a decrease in the concentrations of epinephrine and norepinephrine, which is in agreement with results by others [14, 15]. The amounts excreted in the urine during 24 h showed great interindividual variations and are within the range of values reported for normal human urines [1, 4, 7, 8, 16].

TABLE I

MEAN URINARY EXCRETION OF CATECHOLAMINES OVER 8-h PERIODS IN FIVE HEALTHY PERSONS

Hours	Amount excreted (nmol ± S.D.)		
	NE	E	DA
07—15	69.4 ± 24	19.3 ± 11.4	399 ± 143
15—23	70.5 ± 6.2	20.9 ± 5.4	476 ± 109
23—07	39.4 ± 11.3	3.7 ± 1.8	485 ± 125

CONCLUSION

We have found this method to be a simple and accurate procedure for the determination of free urinary catecholamines. Ion-exchange chromatographic columns appeared to be more selective than reversed-phase columns and the pre-purification of urine could be simplified.

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