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# DETERMINATION OF EPINEPHRINE, NOREPINEPHRINE AND DOPAMINE IN HUMAN URINE USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH COULOMETRIC DETECTION

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*A relatively simple method for the simultaneous determination of epinephrine, norepinephrine and dopamine in urine is presented. We have used a high-performance liquid chromatography (HPLC) with coulometric electrochemical detection. The urine samples pretreated by a solid-phase extraction were injected directly into the HPLC system and were eluted isocratically on a Discovery HS C18, 250×4 mm i.d., 5 μm, analytical column. The analytical performance of this method is satisfactory: the intra-assay and inter-assay coefficients of variation were below 10 %. Quantitative recoveries from spiked urine samples were in the intervals of 78.2-88.0 % for epinephrine, 80.0-93.0 % for norepinephrine, and 96.4-107.2 % for dopamine. The linear range is 2-200 μg $l^{-1}$, with a detection limit*

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*of 0.6 μg $l^{-1}$ (signal-to-noise ratio = 3) for epinephrine, 5-500 μg $l^{-1}$, with a detection limit of 1.5 μg $l^{-1}$ for norepinephrine, and 20-2000 μg $l^{-1}$, with a detection limit of 6 μg $l^{-1}$ for dopamine.*

## Introduction

Catecholamines epinephrine (E), norepinephrine (NE) and dopamine (D) (Fig. 1) are organic compounds that have a catechol and a side-chain amine. Catecholamines are derived from the amino acid tyrosine. They are sympathomimetic hormones that are released by the adrenal glands in response to stress. They are neurotransmitters at central and peripheral sympathetic nervous system. Epinephrine and norepinephrine are produced mainly by the chromaffin cells of the adrenal medulla and the postganglionic fibers of the sympatic nervous system. Dopamine is mainly produced by cells of the *substantia nigra* in the brain. Catecholamines are important biomarkers for the diagnosis, therapy and prognosis of several neuroendocrine and cardiovascular disorders. Generally, two areas are defined by abnormal catecholamine levels, tumors of adrenal medulla (pheochromocytoma and neuroblastom) and cardiovascular disease [1-3].

Numerous analytical methods for the measurement of E, NE and D in urine have been described. With regard to the presence of the other metabolites with similar structures and properties, it is necessary to use purification steps prior to the determination. At present, solid phase extraction (SPE) is most widely used sample preparation methodology for the extraction and purification of catecholamines. SPE is often time-consuming and a source of errors. To achieve satisfactory recovery of catecholamines, different washing and eluting procedures must be investigated. Several types of stationary phases can be used for SPE procedures, such as strong or weak cation exchange [4], $C_8$, $C_{18}$ or $C_{30}$ [5-7], alumina [8] and phenylboronic acid [1,9] phases. Several internal standards, such as 3,4-dihydroxybenzylamine (DHBA) [10-12], *N*-methyldopamine [13], isoproterenol [14,15], norphenylephrine [16], 4-hydroxy-3-methoxybenzylamine [17], and 4-methoxytyramine [18,19], have been used for the quantitation of catecholamines. Most frequently used internal standard is DHBA. Different HPLC methods have been described, mainly with electrochemical (ECD) [20-24], fluorescence [25-29], chemiluminescence [5, 17, 30] and mass spectrometry [31-35] detection. HPLC with ECD using either amperometric or coulometric electrodes can measure E, NE and D directly. Moreover, these techniques avoid typical problems associated with derivatization procedures. In spite of the fact that many types of chromatographic columns are available at present, the reversed-phase columns are still the standard choice. Cation-exchange columns can also be used. They have a lower performance than reversed-phase columns. Beyond that, cation-exchange columns are selective [13,36,37]. In the case of use of reversed-

phase columns, the addition of ion-pairing agents to mobile phase is commonly used in order to improve the chromatographic separation of catecholamines.

In this paper, a sensitive HPLC with coulometric electrochemical detection for simultaneous determination of E, NE and D using DHBA as an internal standard and a relative rapid and accuracy SPE procedure is described. Moreover, this method is inexpensive and applicable to routine analyses.

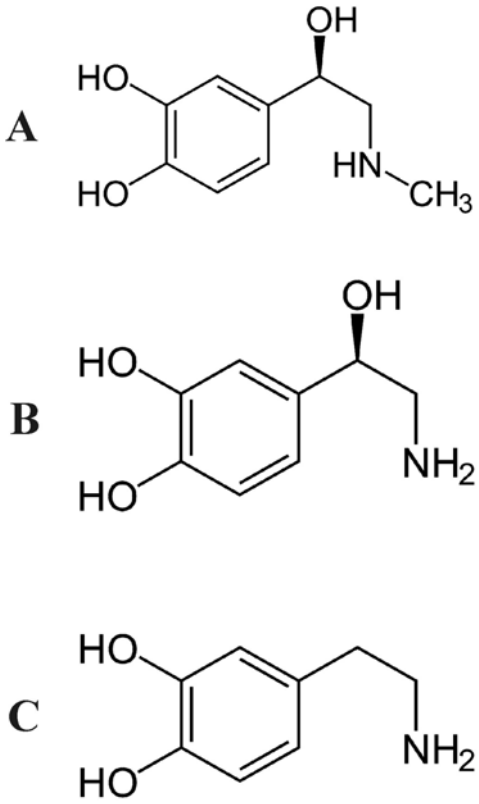


Fig. 1 Structures of epinephrine (A), norepinephrine (B) and dopamine (C)

## Materials and Methods

### Reagents and Chemicals

Epinephrine, norepinephrine, dopamine, 3,4-dihydroxybenzylamine, sodium hydrogenphosphate, sodium dihydrogenphosphate, potassium hydrogenphosphate, potassium dihydrogenphosphate, sodium acetate, ammonium acetate, orthophosphoric acid, hydrochloric acid, acetic acid, perchloric acid, sodium citrate dihydrate, and 1-octanesulfonic acid sodium salt were obtained from Sigma Chemical Company (St. Louis, MO, USA). HPLC gradient grade ethanol was from Merck (Darmstadt, Germany). All the other chemicals were of analytical grade. Lyophilized urine endocrine controls (normal and pathological ranges) were from

Chromsystems (Munich, Germany). Epinephrine, norepinephrine, dopamine, and 3,4-dihydroxybenzylamine were prepared daily in 0.1 mol $l^{-1}$ hydrochloric acid and stored at 4 °C until used.

## Instrumentation

Chromatographic analysis was performed with a liquid chromatograph equipped with an LC-10ADvp solvent delivery system, a CTO-10ASvp column oven (Shimadzu, Kyoto, Japan), a cooled autosampler, Model 542 and Coulochem® II electrochemical detector (ESA Laboratories, Inc., Chelmsford, MA, USA). Data were collected digitally using Clarity chromatography software (DataApex, Prague, the Czech Republic).

## Subject and Samples

Samples of urine were obtained from a group of donors ($n$ = 20, 10 women in the age 22-31 years, mean age 25 years, and 10 men in the age 23-33 years, mean age 27 years). None of studied subjects exhibited renal, hepatic, gastrointestinal, pulmonary or oncological disease.

## Sample Preparation

DHBA was used as an internal standard. Twenty-four-hour or first morning urine samples, protected against daylight, were acidified with 6 mol $l^{-1}$ hydrochloric acid (10 µl per 1 ml of urine). For the analysis of urine, 10 µl of the internal standard DHBA solution in 0.1 mol $l^{-1}$ of hydrochloric acid (≈ 10 mg $l^{-1}$) was pipetted into a well-capped 1.5ml polypropylene tube (Thermo Fisher Scientific, Pardubice, the Czech Republic). One milliliter of urine was added and the content mixed vigorously on a vortex mixer for 60 s. The pH was adjusted to 6.5 ± 0.5 with 5 mol $l^{-1}$ ammonium acetate (≈ 60 µl). The pH was verified using pH paper and a digital pH-meter. Thus treated urine samples were applied on the SPE columns.

## Solid-Phase Extraction

We have used SPE procedure for removal of interfering compounds. For SPE, a Discovery DSC-SCX SPE tube (Supelco, Bellefonte, PA, USA) was used with a bed weight of 100 mg and a column volume of 1 ml (cation-exchange resin, a polymerically bonded benzene sulfonic acid, counter ion is $H^+$). The column was

washed stepwise (by 1 ml) with 3 ml of deionized water, then equilibrated stepwise (by 1 ml) with 2 ml of 1 mol $l^{-1}$ ammonium acetate and finally again washed stepwise (by 1 ml) with 2 ml of deionized water at a flow-rate of about 1 ml $min^{-1}$. Then the urine sample was applied on the equilibrated column. The SPE procedure was performed according to the sequence shown in Table I. Collected effluent was filtered through a nylon filter (pore size 0.20 μm, 4 mm diameter, Supelco) and transferred into 0.2ml amber glass vial.

Table I The SPE procedure

| | |
|---|---|
| The column washed with deionized water (3×1 ml) | - |
| The column equilibrated with ammonium acetate (1 mol $l^{-1}$) (2×1 ml) | - |
| The column washed with deionized water (2×1 ml) | - |
| Treated urine sample applied to the column | Discard effluent |
| The column washed with deionized water (2×1 ml) | Discard effluent |
| Catecholamines eluted with perchloric acid (1.0 mol $l^{-1}$) (200 μl) | - |

## Chromatographic Analysis

The chromatographic analysis of E, NE, D and DHBA was accomplished using an isocratic elution on a Discovery HS $C_{18}$, 250×4 mm i.d., 5 μm analytical column that fitted with a Discovery HS $C_{18}$, 4×4 mm i.d., 5 μm guard column (Supelco) at 37 °C. Other columns were also used in this study: an LiChroCart 250×4 mm i.d., Purospher Star RP-18e, 5 μm (Merck, Darmstadt, Germany) and a Supelcosil LC-SCX, 250×4.6 mm i.d., 5 μm (Supelco). The mobile phase (pH 3.00 ± 0.05) was a mixture of 2.5 % ethanol in a solution of EDTA (0.1 g $l^{-1}$), 1-octanesulfonic acid (0.1 g $l^{-1}$) and sodium citrate (25 mmol $l^{-1}$). The flow rate was kept constant at 0.8 ml $min^{-1}$. The column was equilibrated at a flow rate of 0.8 ml $min^{-1}$ at least 20 h. E, NE, D and DHBA were detected following HPLC separation with a Coulochem® II detector equipped with a dual analytical cell (Model 5010) and a guard cell (Model 5020). The guard cell was connected in line before the autosampler and used to remove oxidizable impurities in the mobile phase. The dual analytical cell contains two flow-through porous graphite, four counter and four reference electrodes in series. Reference electrode was the α-hydrogen/palladium electrode. A carbon filter was placed before the guard cell and between the autosampler and the analytical column, a PEEK filter between the analytical column and the analytical cell. For optimum detection of E, NE, D and DHBA, the electrode potentials for the guard cell, E1 and E2, were set at +300 mV,

–20 mV and 150 mV, respectively. Gain ranges were 1 μA and 100 nA. The hydrodynamic voltammogram analysis was performed to optimize conditions for the accurate determination of E, NE, D and DHBA. It was carried out by injection of 10 μl of E, NE, D and DHBA (10 μg $l^{-1}$) and measuring the current produced by E, NE, D and DHBA at the electrodes. Before injection of the first sample, the potential at the electrodes (except the guard cell and E1) was increased stepwise from +0 mV in 50 mV increments to the final working potential (+150 mV), and the HPLC system was equilibrated with the mobile phase at a flow rate of 0.8 ml $min^{-1}$ for approximately 3 h. When not running samples overnight; the flow rate of a mobile phase was set at 0.1 ml $min^{-1}$ with guard and analytical cells voltages +300 mV, +0 and +50 mV, respectively. After about 50 injections, the electrode potentials were set first at +600 mV, then at –400 mV, at each electrode for 1 h with the mobile phase at a flow rate of 0.8 ml $min^{-1}$, followed by 20-min water rinse and by 60-min ethanol rinse at a flow rate of 0.8 ml $min^{-1}$ (electrodes off). This procedure was used to remove impurities from electrodes to achieve electrode sensitivity and baseline stability.

### Additional Analyses

Urine creatinine was measured with the set Creatinine Flex® by standard procedure using an automatic biochemistry analyzer Dimension® RxL Max® (Siemens Healthcare Diagnostic Ltd., Deerfield, IL, USA).

### Statistical Analysis

The data are presented as median ± IQR (interquartile range; the difference between the upper and lower quartiles). Regression analysis was carried out using the least squares method (software SigmaStat for Windows, Version 3.5, Systat Software Inc., Point Richmond, CA, USA).

## Results and Discussion

### The Effectiveness of Solid-Phase Extraction, Stability

We have tested the effectiveness of SPE procedure. The volume, flow rate and composition of solutions were studied with the aim of removing the interferences with minimum loss of E, NE, D and DHBA. The volumes assayed were from 0.5 to 2.0 ml at a flow rate of 0.5-2.0 ml $min^{-1}$. The optimum volume and flow rate were 1.0 ml and 1 ml $min^{-1}$, respectively. The column was equilibrated with ammonium acetate (1.0 mol $l^{-1}$, pH about 6.5); it has similar composition as the

treated urine. Different mixtures of ethanol and water were assayed within clean-up step. Single deionized water provided the best results, removing the interferences and causing practically no loss of E, NE, D and DHBA. A solution of 1.0 mol $l^{-1}$ perchloric acid provided the best elution of analytes. The E, NE and D content in samples extracted using SPE was stable at 4 °C for at least 24 hours.

## HPLC Assay of Epinephrine, Norepinephrine and Dopamine

E, NE, D and DHBA were separated on a reverse-phase column using an isocratic system of ethanol and sodium citrate and an ion pair reagent. Three different columns, two of them of $C_{18}$ type and one of them of cation-exchange type, were tested in the research: a Discovery HS $C_{18}$, an LiChroCart 250×4 mm i.d., Purospher Star RP-18e, 5 μm and a Supelcosil LC-SCX, 250×4.6 mm i.d., 5 μm. The Discovery HS $C_{18}$ column and ion pairing chromatography were selected for further experiments. The classic reverse-phase HPLC is based upon the non-polar, hydrophobic interaction between non-polar molecules and non-polar stationary phase. Catecholamines contain ionic components; therefore they are too polar to be retained in a reverse-phase mode. In such situations, ion pairing reagent selectively increases the retention of charged analytes. Routinely, alkyl sulfonate with 5-12 carbon atoms is used. The higher number of carbon atoms in the alkyl sulfonate, the stronger the retention effect on the catecholamines. The mobile phase was optimized in order to obtain the best separation of the analytes in the shortest time. Standard solutions of E, NE, D and internal standard as well as urine samples were used for studying the mobile phase composition. Several eluents (different buffers and ion-pairing reagent containing ethanol) were tested. We prefer the use of an eco-friendly mobile phase, because the organic solvents such as acetonitrile and methanol are considered as significant pollutants. Optimization of the separation was obtained after studying the effect of sodium hydrogenphosphate, sodium dihydrogenphosphate, potassium hydrogenphosphate, potassium dihydrogenphosphate, sodium acetate, ammonium acetate and sodium citrate concentration (from 5.0 to 100.0 mmol $l^{-1}$), 1-octanesulfonic acid concentration (from 0.05 to 1.00 g $l^{-1}$), EDTA concentration (0.01 to 0.20 g $l^{-1}$) and ethanol concentration. The retention behavior was studied in dependence of pH value of the mobile phase in the range of 2.5-6.5. The optimal pH 3.0 was chosen for the best separation and detection of catecholamines. Column temperature was changed from 25 to 45 °C. Optimal temperature interval was from 35 to 40 °C. The best results were obtained for the conditions as described in "Chromatographic Analysis". The criteria were resolution, electrode sensitivity, baseline stability and the analysis speed. HPLC chromatogram of E, NE and D in human urine is shown in Fig. 2.

Proper selection of applied electrode potentials is critical for accurate, interference-free measurement of catecholamines in urine. The analysis of the E,

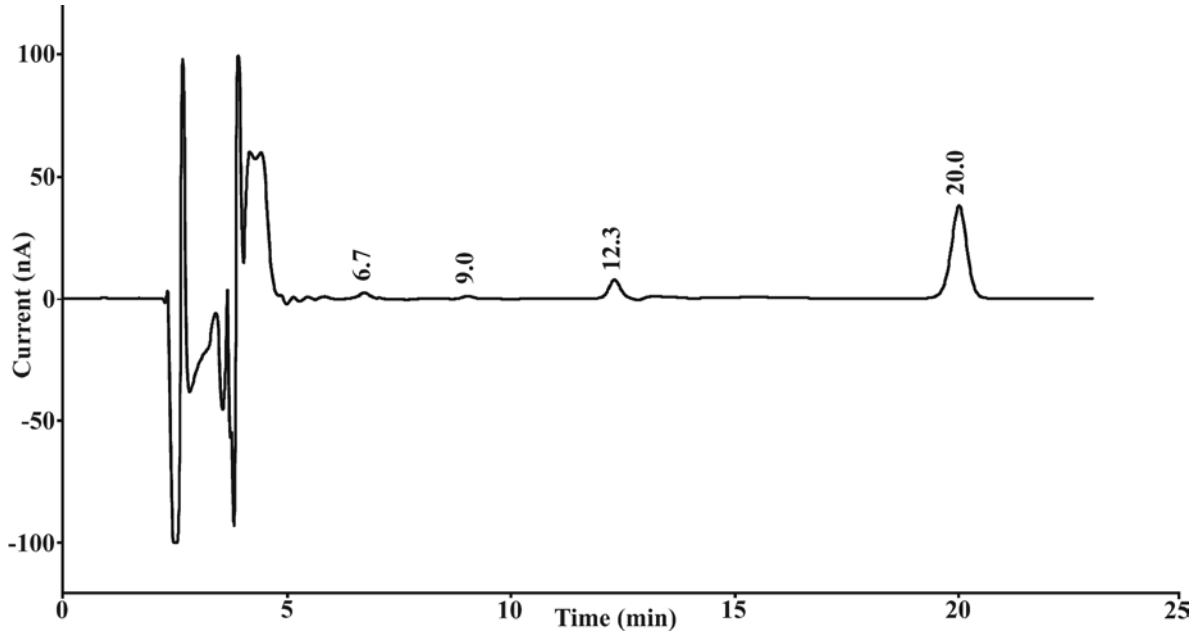


Fig. 2 Chromatogram of norepinephrine (28 μg $l^{-1}$), epinephrine (11 μg $l^{-1}$) and dopamine (282 μg $l^{-1}$) in human urine. Peaks: norepinephrine (6.7 min), epinephrine (9.0 min) and dopamine (20.0 min). Peak (12.3 min) corresponds to 3,4-dihydroxybenzylamine (internal standard). HPLC conditions: isocratic elution (2.5 % ethanol in 0.1g $l^{-1}$ EDTA, 0.1g $l^{-1}$ 1-octanesulfonic acid, 25 mmol $l^{-1}$ sodium citrate, pH 3.00 ± 0.05). Stationary phase was an analytical column Discovery HS $C_{18}$, 250×4 mm i.d., 5 μm that fitted with guard column Discovery HS $C_{18}$, 4×4 mm i.d., 5 μm. The flow-rate was kept constant at 0.8 ml $min^{-1}$ and separation ran at 37 °C. The detection was electrochemical ($E_G$ = +300 mV, $E_1$ = –20 mV, $E_2$ = +150 mV)

NE, D and DHBA voltammograms (Fig. 3) suggest that the hydroxyl group of catecholamines is oxidized by the electrode at potentials above +50 mV. The potential of second electrode (E2) was set near the potential at which the E, NE, D and DHBA voltammograms reached the plateau providing maximum sensitivity. At this electrode, the catecholamines and internal standard are oxidized and quantified, virtually free of interferences. On the other hand, the potential first electrode (E1) was set near the potential at which the catecholamines and internal standard practically are not oxidized (–20 mV). The guard cell was set at potential +150 mV above that of detector 2 to oxidize the oxidizable impurities in the mobile phase.

Precision of E, NE and D analysis for urine samples are in Tables II-IV. To determine the within-day precision, the urine samples were analyzed ten times in the same day under the same conditions. The coefficients of variation were below 10 %. The spike recoveries, obtained after the SPE procedure, ranged between 78.2-88.0 % for E, 80.0-93.0 % for NE and 96.4-107.2 % for D (Tables II-IV). The calibration curve (9-point for a determination of analytical parameters and 7-point

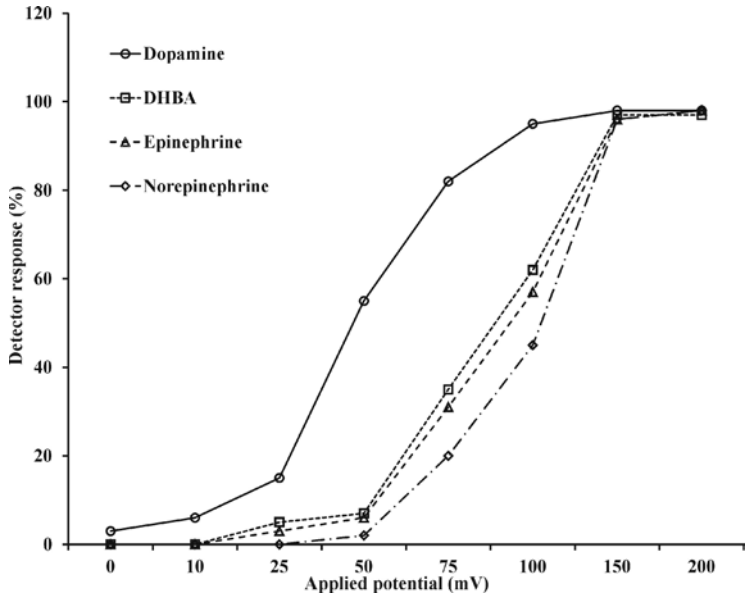


Fig. 3 The hydrodynamic voltammogram generated by repeated injections of epinephrine, norepinephrine, dopamine and DBHA. The voltammogram was developed by plotting relative peak current produced by injection of catecholamines and DBHA (10 μg $l^{-1}$) at various oxidation potentials

Table II Precision and recovery of epinephrine determination in urine

| (A) Precision (within-day) | | |
|---|---|---|
| *N* | Mean ± *SD*, μg $l^{-1}$ | *CV*, % |
| 10 | 14.5 ± 1.1 | 7.6 |
| 10 | 27.9 ± 1.4 | 5 |
| (B) Recovery | | |
| Added, μg $l^{-1}$ | Observed, μg $l^{-1}$ [a)] | Recovery, % |
| 5.5 | 4.3 ± 0.5 | 78.2 |
| 11 | 8.7 ± 0.9 | 79.1 |
| 22 | 17.4 ± 1.8 | 79.1 |
| 33 | 28.1 ± 2.5 | 85.2 |
| 44 | 38.7 ± 3.2 | 88 |
| - | Mean | 81.9 ± 4.4 |
| - | *CV* | 5.4 |

[a)] Mean of triplicate assays is recorded

Table III Precision and recovery of norepinephrine determination in urine

| (A) Precision (within-day) | | |
|---|---|---|
| *N* | Mean ± *SD*, μg $l^{-1}$ | *CV*, % |
| 10 | 23.3 ± 1.5 | 6.4 |
| 10 | 45.2 ± 2.1 | 4.6 |
| (B) Recovery | | |
| Added, μg $l^{-1}$ | Observed, μg $l^{-1}$ [a)] | Recovery, % |
| 21.0 | 16.8 ± 1.4 | 80.0 |
| 42.0 | 34.6 ± 2.7 | 82.4 |
| 84.0 | 69.7 ± 5.0 | 83.0 |
| 126.0 | 114.7 ± 6.9 | 91.0 |
| 168.0 | 156.2 ± 8.0 | 93.0 |
| - | Mean | 85.9 ± 5.7 |
| - | *CV* | 6.7 |

[a]Mean of triplicate assays is recorded

for a routine analysis) was linear in the whole range tested: 2-200 μg $l^{-1}$ for E, 5-500 μg $l^{-1}$ for NE and 20-2000 μg $l^{-1}$ for D (Figs 4-6). The lowest concentration that can be quantified with acceptable accuracy and precision was: 2 μg $l^{-1}$ (100 pg) for E, 5 μg $l^{-1}$ (250 pg) for NE and 20 μg $l^{-1}$ (1000 pg) for D. Furthermore, limits of detection for E, NE and D, defined as signal-to-noise (*S/N*) ratio of 3:1, were: 0.6 μg $l^{-1}$ (30 pg), 1.5 μg $l^{-1}$ (75 pg) and 6 μg $l^{-1}$ (300 pg), respectively.

## The Determination of Epinephrine, Norepinephrine and Dopamine in Human Urine

The levels of E, NE and D in a group of donors were 8.1 ± 6.2 μg $d^{-1}$ (0.88 ± 0.67 mg $mol^{-1}$ of creatinine), 47.2 ± 28.1 μg $d^{-1}$ (5.13 ± 3.05 mg $mol^{-1}$ of creatinine) and 198 ± 74 μg $d^{-1}$ (21.5 ± 8.04 mg $mol^{-1}$ of creatinine), respectively. The normal values for catecholamine differ between laboratories [38-40]. We detected practically no levels of E in some donors; levels of NE and D are similar to those published previously.

Table IV Precision and recovery of dopamine determination in urine

| (A) Precision (within-day) | | |
|---|---|---|
| *N* | Mean ± *SD*, μg l$^{-1}$ | *CV*, % |
| 10 | 92.5 ± 5.3 | 5.7 |
| 10 | 220.1 ± 8.6 | 3.9 |
| (B) Recovery | | |
| Added, μg l$^{-1}$ | Observed, μg l$^{-1}$ [a)] | Recovery, % |
| 5.6 | 54.0 ± 3.2 | 96.4 |
| 112.0 | 108.6 ± 5.5 | 97.0 |
| 224.0 | 226.2 ± 11.1 | 101.0 |
| 336.0 | 349.4 ± 14.3 | 104.0 |
| 448.0 | 480.3 ± 18.2 | 107.2 |
| - | Mean | 101.1 ± 4.6 |
| - | *CV* | 5.4 |

[a]Mean of triplicate assays is recorded

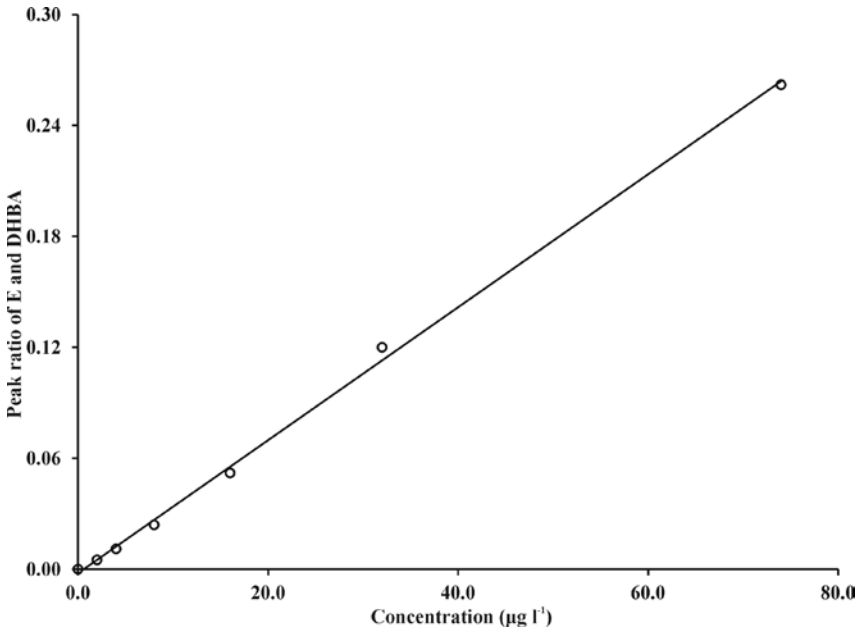


Fig. 4 Typical standard curve for HPLC quantification of epinephrine. Regression equation: $y = 0.0036x - 0.0022$; $R = 0.9993$

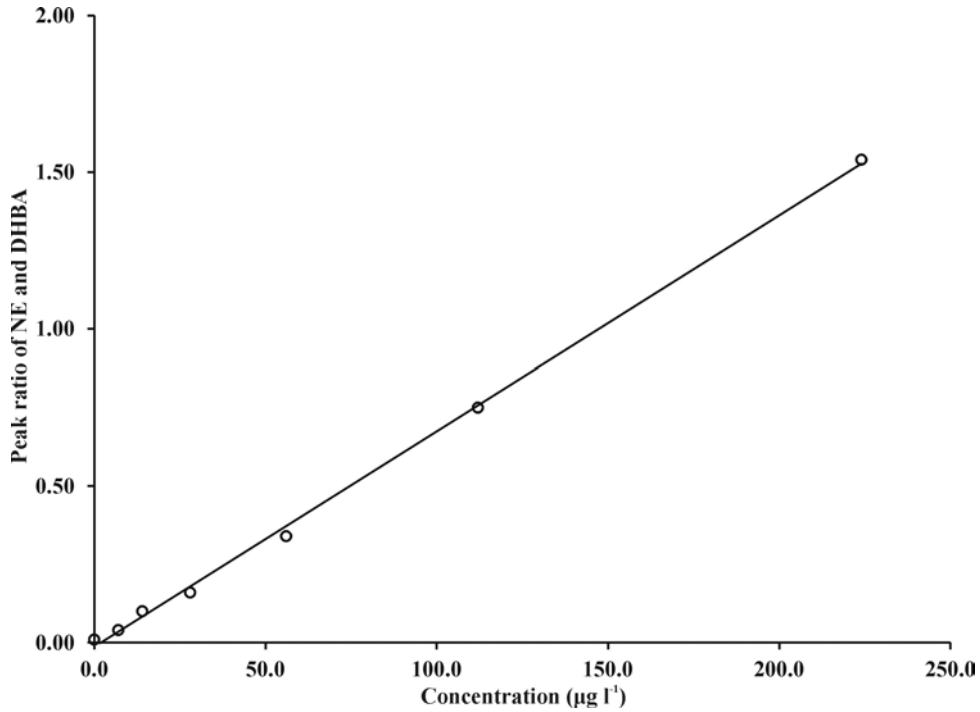


Fig. 5 Typical standard curve for HPLC quantification of norepinephrine. Regression equation: $y = 0.0069x - 0.0133$; $R = 0.9993$

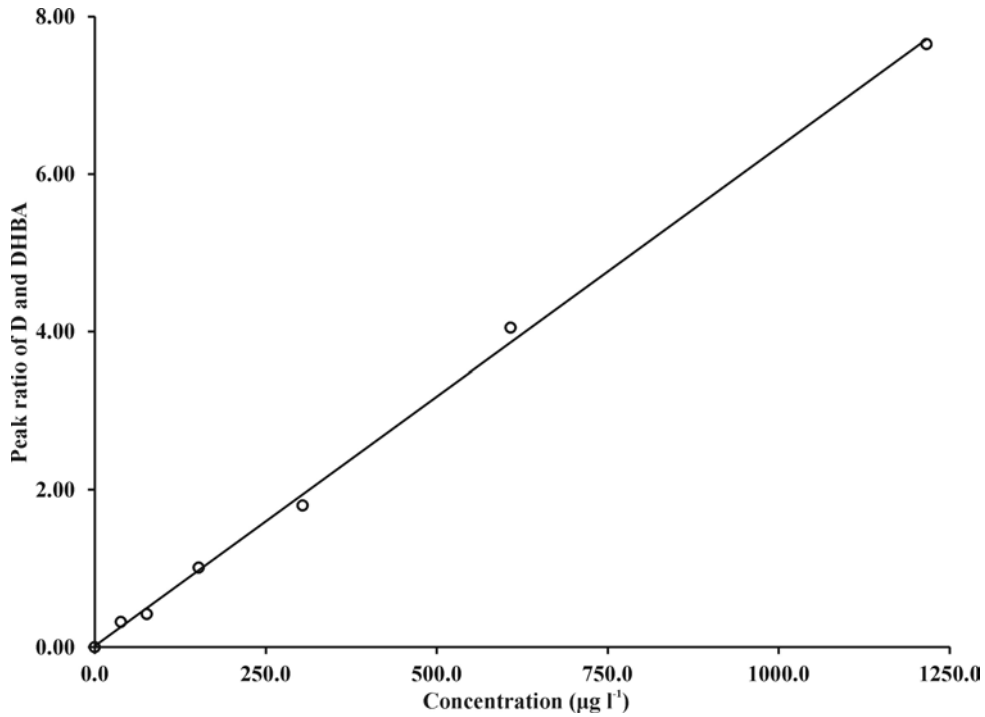


Fig. 6 Typical standard curve for HPLC quantification of dopamine. Regression equation: $y = 0.0063x + 0.0137$; $R = 0.9994$

## Conclusion

We have developed a relatively rapid, simple and very sensitive HPLC method with coulometric electrochemical detection for the simultaneous determination of E, NE and D in human urine. The described method is relatively inexpensive and suitable for clinical trials.


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