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Study of catecholamines in patient urine samples by capillary electrophoresis

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Abstract

Capillary zone electrophoresis with photodiode array detection at 220 nm was used for analysis of catechol compounds in human urine. The method was optimized with reference compounds 3,4-dihydroxybenzylamine, adrenaline, noradrenaline, normetanephrine, dopamine, dopac (homogensitic acid), methanephrine, vanillyl-mandelic acid, 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid and 3-methoxytyramic acid at pH 4.0 and 8.0 for their electrophoretic separation. The UV spectra of the catechols were detected at a concentration of 20 μM. Repeatability of the method calculated using the absolute migration times of the catechols was below 1.5% and using the peak areas below 5%. The patient samples were hydrolyzed by 0.5 *M* acid or base solutions. In the studies, a few patient samples were analyzed using 3,4-dihydroxybenzylamine as an internal standard. In the hydrolysis steps needed for their detection in urine, all the other catecholamines, except 5-HIAA, did not decompose to detectable species at 220 or 254 nm. The concentrations of the catecholamines observed in real samples were at n*M* levels.

Keywords: Catecholamines

1. Introduction

Biogenic amines such as serotonin, catecholamines and their metabolites have effects on nerve systems. The name catecholamines is usually equated with to dopamine, noradrenaline and adrenaline. In particular, dopamine is an important neurotransmitter in the brain. It is also a precursor for the two other catecholamines. Their determination from blood, urine and other body fluids has an essential role in the diagnostics of diseases related to them [1,2]. In particular, the analysis of catecholamines and metabolites of serotonin is applied in tumor diagnostics [3]. Usually, in the routine analysis of vanillylmandelic acid (VMA), homovanillic acid (HVA) and its acidic metabolite serotonin (5-hydroxy-3-indoleacetic acid, 5-HIAA) are determined with high-resolution gas chromatography (HRGC) [4–7], high-performance liquid chromatography (HPLC) [8–11] and capillary electrophoresis (CE) [12–18]. In addition, the basic metabolites of the catecholamines 3-methoxytyramine (3-MTA), normetamephrine (NM) and metanephrine (M) have been detected from body fluids. Noradrenaline (NA), adrenaline (A) and dopamine (DA) have been analyzed even from nerve cells [13,19,20], but also from plasma and serum [21,22], urine [23], saliva [24] and brain samples [23,25].

In HPLC screening of patient samples special detectors, such as electrochemical and fluorescence detectors, are needed to measure catecholamines.

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Because the catechols do not have native fluorescence, they have been derivatized in pre-column or post-column methods using e.g., *o*-phthalic aldehyde (OPA) [26,27], benzylamine [28] or 2-cyanicacetamide [29]. Interest in testing the capability of capillary electrophoresis in screening and determining the catechols has also increased recently. Especially, micellar electrokinetic chromatography (MECC) has demonstrated its potential in pharmaceutical and biomedical fields.

Physiological concentrations of biogenic amines are related to the time of day, the degree of disease, drug abuse and mental condition of a patient. Furthermore, the body fluid matrix has an effect on the concentrations through excretion of the biogenic amine: the amount of dopamine in urine is ten-times higher than the amount of noradrenaline, whereas concentrations of free catecholamines in plasma are ten-times higher than that of noradrenaline [30]. Catecholamines are easily oxidized, for which reason antioxidants (ascorbinic acid, EDTA) are added to the sample before analysis: 6 *M* HCl has been an addition solution [31–33] when cold storing of the patient samples is needed.

The aim of this work was to obtain more information about the electrophoretic conditions needed in screening of catecholamines and to validate a separation method for their fast determination in patient samples. The hydrolysis and concentration of patient samples were also tested. The advantages of capillary electrophoresis in the analysis of catechols are briefly compared with those obtained with HPLC–electrochemical detection (ED) studies made at the laboratory of the Helsinki University Hospital [31–33].

2. Experimental

2.1. Materials

All chemicals used were of analytical grade. Sodium acetate, ammonium acetate, glacial acetic acid, disodium hydrogenphosphate and sodium tetraborate decahydrate were from Merck (Darmstadt, Germany). 3-Hydroxytyramine (dopamine, DOPAM), (±)-arterenol (noradrenaline, NA), (−)-epinephine (adrenaline, A), 2,5-dihydroxyphenylacetic acid (homogentisic acid, DOPAC), DL-normetanephrine (NM), 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid, HVA), 3-methoxy-4-hydroxyphenylethylamine (3-methoxytyramine, 3-MTA), DL-4-hydroxy-3-methoxymandelic acid (vanillylmandelic acid, VMA), 5-hydroxyindoleacetic acid (5-HIAA) and 3,4-dihydroxybenzylamine (DHBA) were purchased from Sigma (Sigma–Aldrich, Deisenhofen, Germany).

Distilled water was further purified with a Water-I System through ion-exchange and carbon black sorbents (Gelman Sciences, Ann Arbor, MI, USA) and filtered through 0.45-μm membranes (Waters, Molsheim, France).

2.2. CE instrument

A Hewlett-Packard ^{3D}CE system (Hewlett-Packard, Waldbronn, Germany) with a diode array detector was used for determination of catechols in patient urine samples. The wavelengths used in their detection were 220 and 254 nm. The total length of a fused-silica capillary (50 μm I.D., Composite Metal Services, The Chase, Hallow, UK) was 68.5 cm (length to the detector 60 cm). Injection was made hydrodynamically by pressure (50 mbar$=5\cdot10^3$ Pa) for 5–40 s.

In addition, a Beckman instrument P/ACE MDQ with a PDA detector (Beckman Instruments, Fullerton, CA, USA) was used for testing the chemical parameters in the separation procedure in acidic media. The wavelength used in their detection was 220 nm. The outlet length of a fused-silica capillary (50 μm I.D., Composite Metal Services) to the detector was 10 cm (total length 64.2 cm). Injection was made hydrodynamically by pressure (0.50 p.s.i.$=3.5\cdot10^3$ Pa) for 5 s.

Before use, the capillaries were conditioned with 0.1 *M* NaOH for 5 min, with purified water for 5 min, and then with the electrolyte solution for 10 min. The injection was performed hydrodynamically with a pressure of 50 mbar for 5 s (1 bar$=10^5$ Pa). The applied voltage used was +20 kV. Temperatures were +23°C and +20°C in capillary and in the autosampler unit (HP instrument), respectively. UV spectra (190–450 nm) were collected from every peak for their identification.

2.3. Standards

The reference mixtures of the catecholamines, DOPAM, NA, A, DOPAC, NM, methanephrine (M), HVA, 3-MTA, VMA, 5-HIAA and DHBA for internal standard were prepared from their stock solutions, which were made as 450 μ*M* in methanol. The mixtures used in method validation contained (a) 1 μ*M* of the analytes and (b) 750 n*M* VMA, 1.5 μ*M* 5-HIAA, 1.5 μ*M* HVA, 1 μ*M* NA, 1 μ*M* A and 1 μ*M* DOPAC prepared in 450 μl of a water–methanol solution (50:50, v/v).

2.4. Sample pretreatment

The patient urine samples were obtained from the University Hospital in Helsinki. Samples containing antioxidants (EDTA) (1 ml) were pretreated by hydrolysis with acid (0.5 *M* HCl, 100°C, 45 min) or base (0.5 *M* NaOH, 100°C, 45 min) and centrifuged for 5 min at 2000 rpm. Before injection 6 *M* HCl was added to the samples to prevent oxidation [33]. The samples were filtered through 0.45-μm PTFE membranes, after which they were analyzed by CE. A stock solution containing all the catechols in methanol at a concentration of 50 μ*M* was used to spike the blank urine samples.

2.5. Electrolyte solutions

The 10, 25, 30, 40, 48.8 and 50 m*M* electrolyte solutions were prepared from sodium or ammonium acetate and the pH was adjusted to 4.0 with acetic acid. In the basic region different concentrations of disodium hydrogenphosphate and sodium tetraborate were mixed to buffers (10–50 m*M*) used at neutral and basic pH regions.

3. Results and discussion

The concentrations of catecholamines were very low in real samples, therefore optimization and enhancing of their detection were needed. The tests also showed that selective hydrolysis (optimized with temperature and amount of reagent) for basic and acidic compounds was important to prevent their decomposition. It was noticed when acidic metabolites were studied in urine, that hydrolysis step is not necessary in that case.

The separation of the analytes was optimized by using standard mixtures. The effects of pH and concentrations of the sodium and ammonium acetate in the buffers used as the electrolyte medium for the migration of the catechols were tested. The Figs. 1 and 2 show that both the effects have a very high influence on the migration of cationic catechols having positively charged amine in their molecule. However, the acidic catechols were analyzed faster in phosphate–tetraborate buffer at pH 8.0, which can be used when the anionic metabolites are measured.

3.1. Electropherograms

Catecholamines and their metabolites could be efficiently separated from each other which was useful in the analyses of patient urine samples. By using 30 m*M* acetic acid buffer at pH 4.0 the analytes could be selectively separated from each other: primary and secondary amines migrated before electroosmosis, followed by the carboxylic groups containing catecholamines (Fig. 3). It was calculated that under the acidic conditions adrenaline had the same electrophoretic mobility as the acetate ion.

At pH<7 the amine groups of the basic catecholamines are fully protonated and the difference in mobility was shown to be based on their molecular size and/or shape. On the other hand, at pH>7 the acidic catecholamines were either negatively charged or if they contained only an aliphatic hydroxyl group they may not dissociate quantitatively enough at pH<12.0. In 30 m*M* acetic acid buffer the theoretical electrophoretic mobilities of adrenaline, noradrenaline, NM, dopam, dopac, M, 5-HIAA, HVA and 3-MTA were $1.03\cdot10^{-2}$, $1.05\cdot10^{-2}$, $1.34\cdot10^{-2}$, $1.44\cdot10^{-2}$, $2.57\cdot10^{-2}$, $3.14\cdot10^{-2}$, $3.60\cdot10^{-2}$, $7.66\cdot10^{-2}$ and $4.07\cdot10^{-2}$ V^{-1} m^2 s^{-1} and their relative mobilities ($RRT=t_R/t_{DHBA}$) were 1.04, 1.06, 1.27, 1.48, 2.58, 3.19. 3.65, 3.67 and 4.13 in acetic acid buffer made from sodium acetate (pH 4.0 adjusted with acetic acid).

Under basic conditions (phosphate–tetraborate buffer, pH 8.0) tested for fast separation of anionic catecholamines, all the analytes migrated very slowly, partly due to their complexation with tetraborate present in the buffer solution, since only the disso-

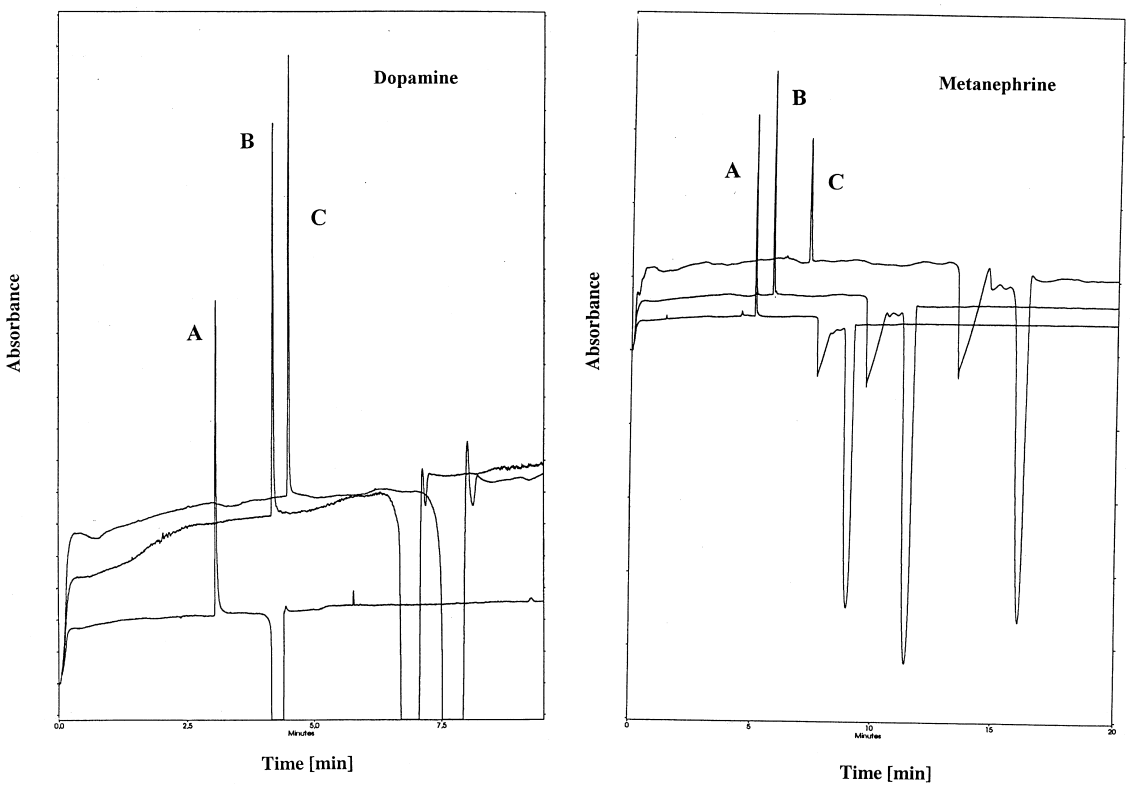

Fig. 1. Effect of concentration of ammonium acetate in electrolyte solution. (I) pH not adjusted and (II) pH adjusted. (A) 30 m*M*, (B) 40 m*M* and (C) 50 m*M*. Sample amount, 1 μ*M*. Injection, 5 s. Equipment, Beckman MDQ.

ciated acid compounds were observed with resolution. However, with optimization of the instrumental parameters faster analysis could be performed.

3.2. Identification from the UV spectrum

The UV spectra of the catecholamines in acetic acid were similar. Therefore, their identification according to absorption was not possible. However, their identity was tested using BHDA as the internal standard by calculating their relative migration times, especially, in patient samples. In addition, spiking was used to secure their migration order.

3.3. Precision and accuracy

The precision was examined by analyzing nine times the standard mixture. Their concentrations were 1 μg/ml. The relative standard deviations (RSDs) of the absolute migration times varied from 0.8 to 1.4%. The precision in analyses of spiked urine samples were between 2 and 6% of the repeated screening of the absolute migration times. Peak areas showed RSD values of 5% for standard mixtures.

3.4. Detection limit and limit of quantification

Limits of detection (LODs) were calculated from the electropherograms. The LOD concentrations for the analytes in methanol (acidified with 4% of 5 *M* HCl) was 5 n*M* ($S/N=2$). The limits of quantification (LOQs) were 10 n*M* at $S/N=2$ for normethanephrine, dopac, metanephrine, 5-HIAA, HVA and 3-MTA and $S/N=5$ for the others (Fig. 2).

3.5. Quantification

The linearity range of the analyses was studied by analyzing five blank urine samples containing differ-

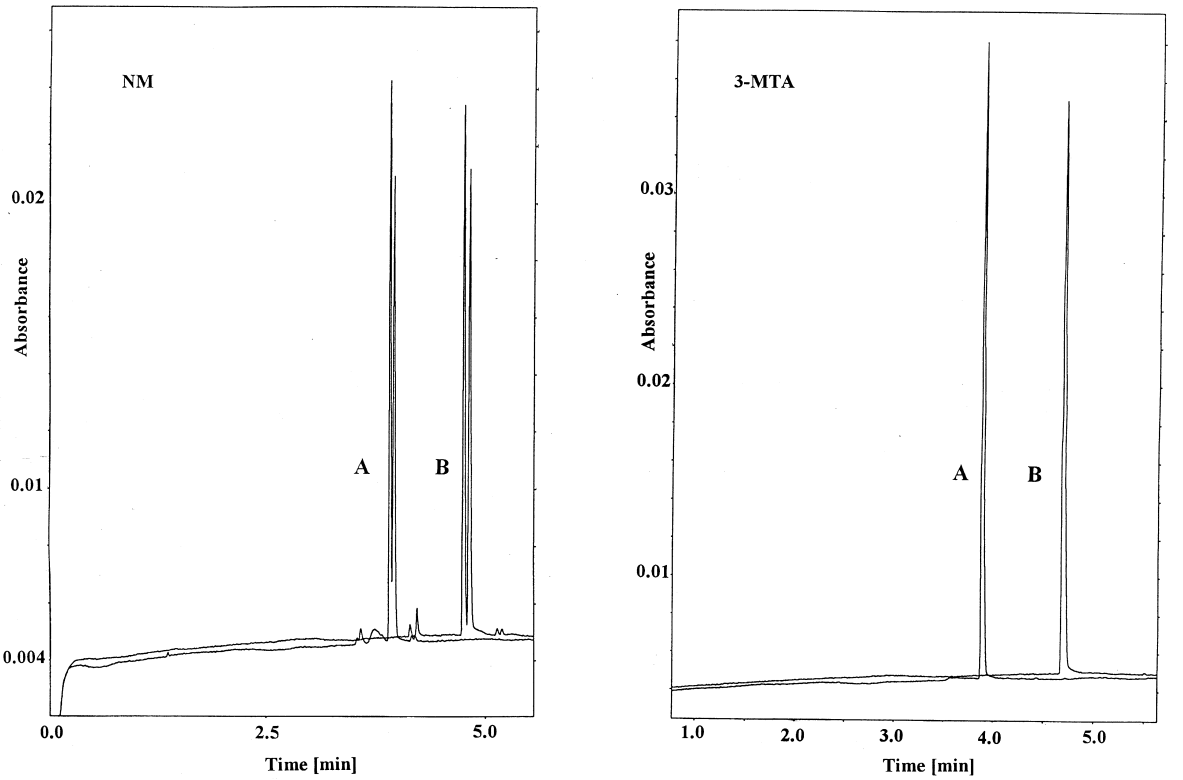

Fig. 2. Effect of pH on the migration of normetanephrine and 3-MTA in 30 m*M* ammonium acetate. (A) pH adjusted to 4.0 and (B) pH not adjusted. Sample amount, 1 μ*M*. Injection, 5 s. Equipment, Beckman MDQ.

ent concentrations of the catechols studied. There was linear correlation between the area and the absolute concentration of the analyte from 10 n*M* to 200 μ*M* for most of the analytes. The statistical concentrations of catecholamines and their metabolites in human urines of Finnish healthy people are

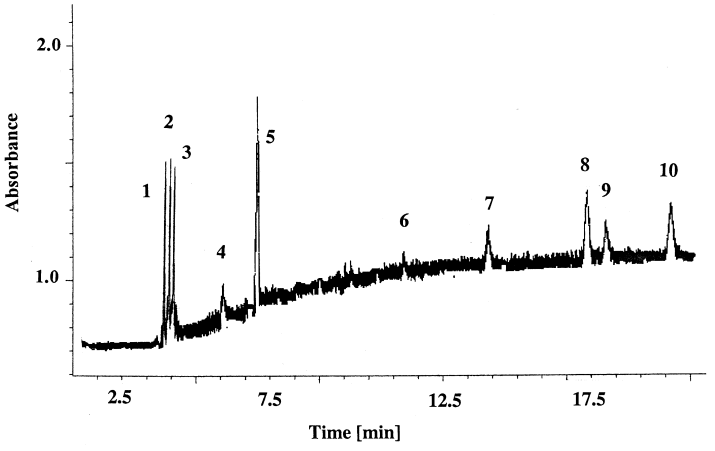

Fig. 3. Electrophoretic separation of catecholamine standards in basic electrolyte solution. (1) DHBA, (2) adrenaline, (3) noradrenaline, (4) NM, (5) dopam, (6) dopac, (7) M, (8) 5-HIAA, (9) HVA and (10) 3-MTA. Concentrations: 5 μ*M*. Equipment: HP ^{3D}CE.

7–30 μ*M*, 10–40 μ*M*, 5–40 μ*M*, below 4.0 μ*M*, below 1.7 μ*M* and below 1.2 μ*M* for VMA, HVA, 5-HIAA, NMN, NM and 3-MTA, respectively. Relative shares of VMA, metanephrine, adrenaline, 5-HIAA, noradrenaline and normetanephrine found in human urine are 65%, 1%, 0.5%, 30%, 2% and 1.5. In this study, concentrations of the analytes in 1.0 ml samples ranged between 5 n*M* and 200 μ*M*.

3.6. Patient samples

After hydrolysis, the standard deviations of the peak areas were 10%. The studies of the patient samples showed that the screening profile of catecholamines in urine varied from patient to patient. Further optimization was needed, since the best separation for the catechols and the compounds from the matrix in patient samples were obtained in 48.8 m*M* acetic acid solution (Fig. 4). When compared to available HPLC studies, the advantages of the CE techniques used here were high selectivity and resolution enhancement. The patient urine samples contained VMA, noradrenaline, NM, DOPAM, DOPAC, M, 5-HIAA, HVA and 3-MTA [32]. The concentrations of six patient samples containing 5-HIAA varied from below 12 to 475 μ*M*. VMA was measured from four samples and its concentration was between 14–27 μ*M*. When the samples were rich in one of catecholamines it was diluted to get the concentration of the analyte to linear capacity range of the system.

In particular, the amounts of the acidic metabolites were in some samples extremely low and therefore these analytes were below the LOQ limits in CE.

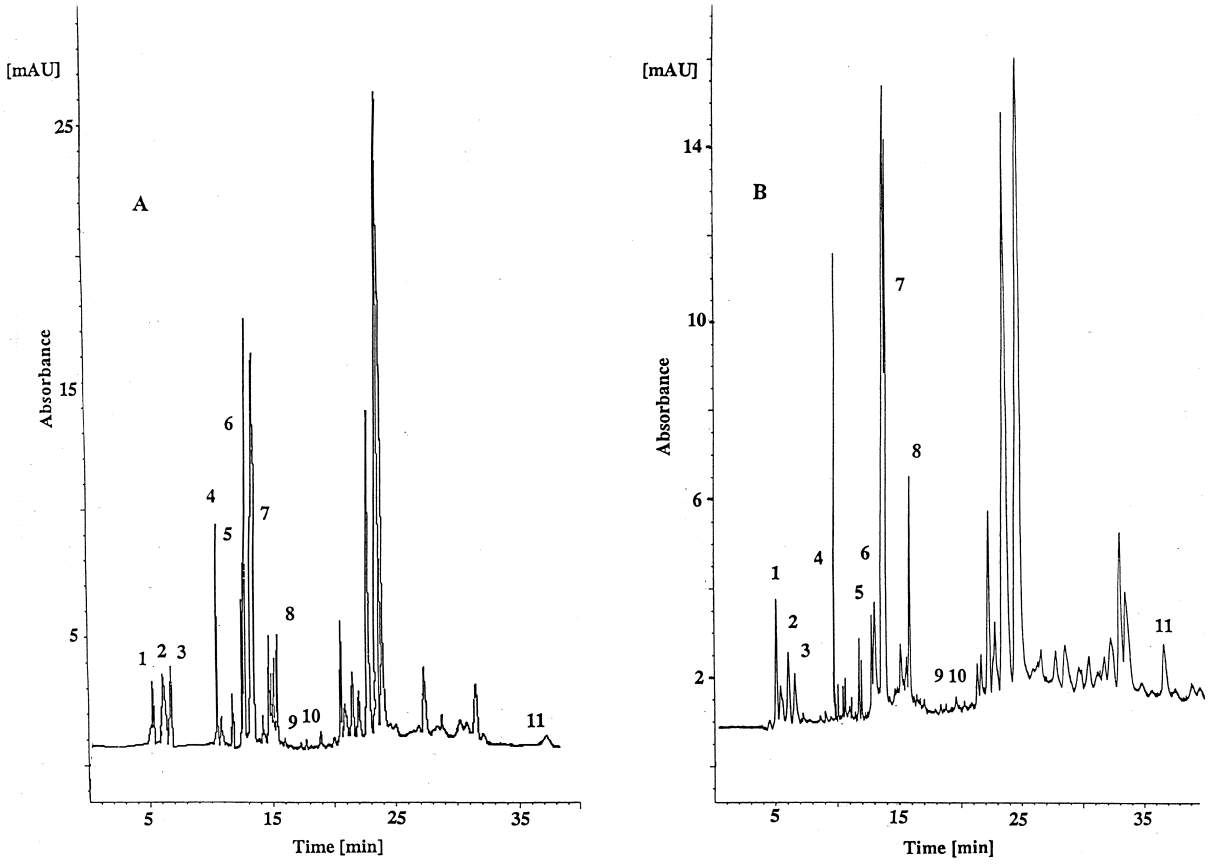

Fig. 4. Electrophoretic separation of a patient samples. Biogenic amines identified from the urine. Compounds as in Fig. 3 and peak number (11) VMA. (A) Female urine and (B) male urine. Injection time, 5 s. Equipment, HP ^{3D}CE.

However, when assayed with HPLC–ED they could be determined.

4. Conclusions

The studies showed that capillary electrophoresis technique has good separation efficiency for 3,4-hydroxybenzylamine, adrenaline, noradrenaline, normetanephrine, dopamine, dopac, metanephrine, vanillylmandelic acid, 5-hydroxyindoleacetic acid, homovanillic acid and 3-methoxytyraminic acid. Combined with off-line sample pretreatment, capillary electrophoresis with UV detection can be used in the analyses of catecholamines analyzed from patient urines. In acidic buffer solution the cationic metabolites containing amine groups can easily be analyzed within 15 min. However, when acidic metabolites are also screened, it is suggested that two separate analysis methods are used, including two hydrolysis procedures to get a fast screening method.

The concentrations of biogenic amines were low in real samples and therefore sensitivity was increased by off-line hydrolysis, off-line concentration and sample modification. The results showed that simultaneous analysis of acidic and basic catechols is possible. The advantages of CE over HPLC–ED are injection of non-extracted urine and high resolution of the separation procedure. When acetate buffer is used, there is a great possibility to adapt the CE method to on-line coupled electrospray mass spectrometry.

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