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Determination of urinary catecholamines with capillary electrophoresis after solid-phase extraction

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Abstract

The stabilities of 3,4-dihydroxybenzylamine (DHBA), dopamine, 3-methoxytyramine, normetanephrine and metanephrine standards under acid, base and enzymatic hydrolysis conditions were studied. Basic incubation media were not suitable for 3,4-dihydroxy compounds, but acid and enzymatic hydrolysis conditions were applicable to all the compounds. The results of acid and enzymatic hydrolysis were comparable and the enzymatic hydrolysis was applied to a urine matrix. A method including solid-phase extraction (SPE) with a copolymer sorbent was developed for purification of the urine samples. Due to poor recovery of DHBA, the most frequently used internal standard in catecholamine analysis, this compound was replaced with the 3-*O*-methoxy structure. The recoveries of the compounds in spiked urine samples in SPE were between 96.4 and 124.4%. The repeatability of the combination of enzymatic hydrolysis and SPE pretreatment was good for all the compounds, except for dopamine and 3-methoxytyramine due to some matrix compounds still interfering with the separation. The analyses were performed with capillary electrophoresis in an ammonium acetate buffer with UV detection. The validation data for the compounds including limit of detection, limit of quantification, linearity and repeatability of the method are presented.

Keywords: Hydrolysis; Catecholamines

1. Introduction

Catecholamines, e.g., dopamine, adrenaline and noradrenaline, are neurotransmitters in the central and peripheral nervous systems. The measurement of catecholamines and their metabolites in biological samples is useful for clinical diagnosis of pheochromocytoma and neuroblastoma, of Parkinson's disease and in the investigation of stress systems [1].

The catecholamines are metabolized in organisms by oxidation–reduction reactions and by methylation (phase I). In phase II metabolization, glucose and sulfate conjugates are formed. Analysis of the total concentration of catecholamines and their phase I metabolites requires hydrolysis of the conjugate forms. The hydrolysis has commonly been performed with acid [2], base [3] or enzymes [4].

Sample preparation for the analysis of catecholamines in urine matrix has been performed with cation-exchange [5], with alumina [6] and with solid-phase extraction (SPE) with C_{18} sorbent [7]. Specific borate complexation with diphenyl borate has been utilized in liquid–liquid extraction with an ion pair reagent [8] as well as with SPE with PLRP-S, PRP-1 and different C_{18} sorbents [9]. In some investigations the separate isolation step has totally been avoided by directly injecting the urine sample into the

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analysis equipment. Examples include analyses with coupled-column high-performance liquid chromatography [10] or capillary electrophoresis (CE) with a high ionic strength electrolyte solution [11].

CE is an efficient analysis technique that is increasingly being adopted in clinical analysis at the expense of liquid chromatography. The advantage of CE is its ability to separate a wide range of molecules with different molecular sizes [12]. The small required sample volumes of microliters and minor volumes of waste are elements with ever-increasing importance [13]. However, the small sample volumes set requirements for the sensitivity of the detector in the case of low concentrations of the analytes in the sample. UV detection is widely used in CE due to its simplicity and applicability to routine analyses, although it is less sensitive than electrochemical and fluorescence detection [14]. Sample preconcentration can conveniently be performed with SPE. As a pretreatment method SPE has a number of distinct advantages over liquid–liquid extraction in combination with CE [12]. The development of new polymeric phase materials has increased the applicability of the SPE method for the purification of more polar compounds over a broad pH range [15].

The aim of this work was to compare different hydrolysis methods used in the hydrolysis of the glucose and sulfate conjugate forms of the catecholamines and to develop an efficient extraction method for their analysis in urine samples based on SPE with a polymeric sorbent material. The sorbent is water-wettable and therefore enhances the user-friendliness of the method. All the analyses were performed with CE using UV detection.

2. Experimental

2.1. Chemicals and materials

The catecholamines (CAs) dopamine (3-hydroxytyramine hydrochloride, DA), DL-normetanephrine (3-methoxybenzenemethanol hydrochloride, NMN), DL-metanephrine (DL-*m*-*O*-methylepinephrine hydrochloride, MN), 3-methoxytyramine (3-methoxy-4-hydroxyphenethylamine hydrochloride, 3MT), 3,4-dihydroxybenzylamine hydrobromide (DHBA) and 4-hydroxy-3-methoxybenzylamine hydrochloride (HMBA) were obtained from Sigma–Aldrich (Steinheim, Germany). Ammonium acetate, sodium acetate, disodium hydrogenphosphate and triethanolamine were obtained from Merck (Darmstadt, Germany), glacial acetic acid, sodium hydroxide Dilut-It and sodium dihydrogenphosphate from J.T. Baker (Deventer, The Netherlands), diisopropylamine, dibutylamine and triethylamine from Fluka (Buchs, Switzerland), concentrated hydrochloric acid from Riedel-de Haën (Seelze, Germany) and methanol from Rathburn (Walkerburn, UK). All the reagents and solvents used were of analytical purity. The deionized water used was purified with a Milli-Q Plus system (Millipore, Bedford, MA, USA).

Helix pomatia juice was obtained from BioSepra (Cergy-Saint-Christophe, France). The solution contained 100 000 Fishman Units (FU) of β-glucuronidase and 1 000 000 Roy Units (RU) of sulfatase per ml. The enzyme activity was not separately checked but the amount declared by the supplier was used as a basis for dosage.

The SPE was performed with Oasis HLB cartridges (30 mg) (Waters, Taunton, MA, USA). The solutions were filtered with 0.45-μm filters of Acrodisc 13 CR PTFE (Gelman Sciences, Ann Arbor, MI, USA) or HATF (Millipore). Incubations of the hydrolysis reactions were performed in a Multi-block dry bath (Lab-line Instruments, Melrose Park, IL, USA) and the SPE was performed in an IST Isolute vacuum manifold (International Sorbent Technology, Mid Glamorgan, UK). The pH values of the buffers were adjusted using a Denver Model 20 pH meter with a combination electrode (Denver Instrument Co., Denver, CO, USA) calibrated with pH 4.00, 7.00 and 10.00 commercial buffers (Reagecon, Co. Clare, Ireland).

2.2. Solutions

The 10 m*M* stock solutions of different CAs were prepared in glacial acetic acid–water solution (10:90, v/v). The 500 μ*M* standards in the hydrolysis studies were diluted from the stocks with 10% acetic acid solution. A 50 μ*M* CA mixture was used in the optimization of the SPE of the urine matrix. All the solutions were stored at −20°C.

The urine for the method development was collected from healthy volunteers in the laboratory. The

pooled urine was not filtered and no preservatives were added to the urine, which was stored in aliquots at −20°C.

The pH of the 0.15 *M* sodium acetate buffer used in enzymatic hydrolysis of the urine samples was adjusted with 10% acetic acid to 5.0. A 0.5 *M* phosphate buffer (pH 7.0) was mixed from 0.5 *M* solutions of disodium hydrogenphosphate and sodium dihydrogenphosphate and was used in the SPE. The electrolyte solutions for the CE analyses were made of 25 m*M* ammonium acetate or 50 m*M* ammonium acetate with 40 m*M* diisopropylamine. The pH (4.0) was adjusted with 10% acetic acid. All the solutions were stored at 4°C and filtered (0.45 μm) before use.

2.3. Capillary electrophoretic analysis

The analyses were performed with P/ACE MDQ CE equipment (Beckman Instruments, CA, USA) with a Beckman UV detector and temperature control units for the capillary and samples. The capillary (50 μm I.D.×375 μm O.D.) was purchased from Composite Metal Services (Werchster, UK). Hydrodynamic injections of 10 s were performed in triplicate with a pressure of 0.5 p.s.i. (1 p.s.i.= 6894.76 Pa). The voltage applied in separation was +20 kV. The temperatures of the capillary and the samples were maintained at 25°C and 18°C, respectively. New capillaries were conditioned by flushing at 20 p.s.i. pressure sequentially with 0.1 *M* NaOH, water and buffer solution for 15 min each. In the case of electrolyte solution containing diisopropylamine an additional flush at 20 p.s.i. pressure together with a voltage of +20 kV was applied for 15 min. Between analyses the capillary was flushed with buffer solution for 3–5 min.

2.4. Optimization of the CE separations

After the hydrolysis, the individual standard compounds were analyzed with a fast and high-throughput technique with the electrolyte buffer consisting of 25 m*M* ammonium acetate (pH 4.0). In the acid and base hydrolyses, the capillary length to the detector (L_{det}) and the total length (L_{tot}) were 10 and 60 cm, respectively. However, in the enzymatic hydrolyses the migration window of the capillary was 50 cm (L_{tot} 60 cm) due to interfering peaks originating from the enzyme solution. The detection wavelength was 214 nm.

Resolution between the analytes and the matrix compounds in urine was enhanced by increasing the ionic strength of the solution and by modifying the 50 m*M* ammonium acetate buffer with 40 m*M* diisopropylamine (pH 4.0). In addition, the migration window was further increased to 70 cm (L_{tot} 80 cm). The detection wavelength was changed to 200 nm, which increased the absorbance responses of the analytes by a factor of four.

2.5. Hydrolysis reactions of CA standards

In acid hydrolysis reactions, amounts of 10, 20, 50 and 100 μl of 0.5, 1 or 5 *M* hydrochloric acid solution were added to a solution of each CA in water. The total volumes of the samples were 1000 μl, corresponding to a CA concentration of 5 μ*M* and acid concentrations of 5–500 m*M*. The mixtures were incubated in different temperatures [room temperature (RT), 30, 38 or 50°C] for 30 min, after which the reaction was halted by adding sodium hydroxide solution to a final concentration of 5–500 m*M*, respectively. The samples were prepared and analyzed in triplicate by CE during 1 working day.

The base hydrolysis reactions were carried out analogously to the acid reactions but with sodium hydroxide as the hydrolyzing agent and hydrochloric acid as the stopping agent.

In the enzyme hydrolyses, amounts of 3, 5, 7, 10 or 12 μl of *Helix pomatia* juice were added to a solution of each CA in water. The total volumes of the samples were 1000 μl, corresponding to a CA concentration of 5 μ*M* and enzyme activities of 300–1200 FU and 3000–12 000 RU. The mixtures were incubated at different temperatures (room temperature 38°C at intervals of 3°C) for 20 min. No reagent was used to halt the reaction but the samples were analyzed immediately by CE. The samples were prepared in triplicate.

2.6. Enzymatic hydrolysis in the urine matrix

To 1 ml of urine were added 0.5 ml of 0.15 *M* sodium acetate buffer (pH 5.0) and *Helix pomatia* juice corresponding to enzyme activities of 1000 FU

and 10 000 RU. The mixture was incubated at 37°C for 3 h. After cooling, the samples were immediately purified by SPE. The enzymatic hydrolysis studies were performed with the pooled urine spiked with standards and also with a blank sample of the pooled urine.

2.7. Solid-phase extraction of the urine matrix

To the enzyme-hydrolyzed urine sample was added 0.5 ml of 0.5 *M* phosphate buffer (pH 7.0) and the sample was loaded onto a conditioned SPE cartridge. The conditioning was performed with 1 ml of methanol and 1 ml of 0.5 *M* phosphate buffer (pH 7.0). After adsorption of the sample the column was washed with 2 ml of water and dried for 1 min at 350 mbar vacuum (1 bar=10^5 Pa). The CAs were eluted with 1 ml of methanol. The methanol was dried under a stream of nitrogen at 37°C and the residue was dissolved in 200 μl of water. Thus the sample was concentrated by a factor of five. The samples were analyzed by CE. The extraction studies were optimized with pure solutions and with pooled urine samples, all of them spiked with the CAs. In addition, a blank urine sample was analyzed in order to determine the basal levels of the endogenous CAs in the pooled sample.

2.8. Calculation of pK_a values

The ionizations of the different functional groups of the CAs were estimated with the aid of the pK_a values. The pK_a values of the functionalities were based on predictions of the Pallas 1.2 program (CompuDrug Chemistry, Budapest, Hungary).

3. Results and discussion

3.1. CE analysis

The background electrolyte solution was selected to keep the CAs positively charged at their primary and secondary amine functionalities during the analyses. In ammonium acetate buffer the resolution between the CAs increased with decreasing pH (in the range of 4–6) and with increasing ionic strength (in the range of 25–50 m*M*). After validation the 50 m*M* ammonium acetate electrolyte solution (pH 4.0) was selected for the analysis of the CA mixture.

The migration order of the CAs in CE follows the order of increasing molecular mass (M_r) (Fig. 1). The differences in M_r values are small (14 or 16 g/mol), corresponding to structures of $-CH_2-$ and –O–, respectively (Fig. 2). Although the M_r values of HMBA and DA are equal, the resolution between these analytes is more substantial than between DA and 3MT, with a difference in M_r of 14 g/mol. It is apparent that the methylation of the 3-hydroxy group affects the polarity and hence the electrophoretic mobility of the molecule more than the elongation of the side chain by one carbon.

After pretreatment of the urine samples there were some matrix compounds left which emerged in the electropherograms with the same migration velocities as DA and 3MT. With the aim of increased resolution between these analytes and the matrix components, 10% of 2-propanol was added to the buffer in order to obtain a solution with higher viscosity. This resulted in band broadening and thus in a decrease in resolution. Therefore, the selectivity of the buffer had to be increased by other means. For this purpose, additives such as triethylamine, diisopropylamine, dibutylamine and triethanolamine were used in 50 m*M* ammonium acetate buffer (pH 4.0) in order to form dynamic coatings on the capillary wall [16]. The results obtained with the modified buffers showed only minor differences between the separation efficiencies of these systems, and finally the 40 m*M* diisopropylamine was selected. The differences in the results might have been more substantial with a more basic electrolyte solution, as the addition of a cationic modifier to the buffer would have a much greater effect on the electroosmotic flow in basic media than in a more acidic medium, in which the silanol groups would not be as fully dissociated [17]. Compared to the analyses with the pure ammonium acetate buffer, this resulted in partial resolution between the matrix compounds and DA and 3MT together with a baseline resolution between NMN and an unknown matrix compound which was not detected after CA standard spiking of the sample (Fig. 1).

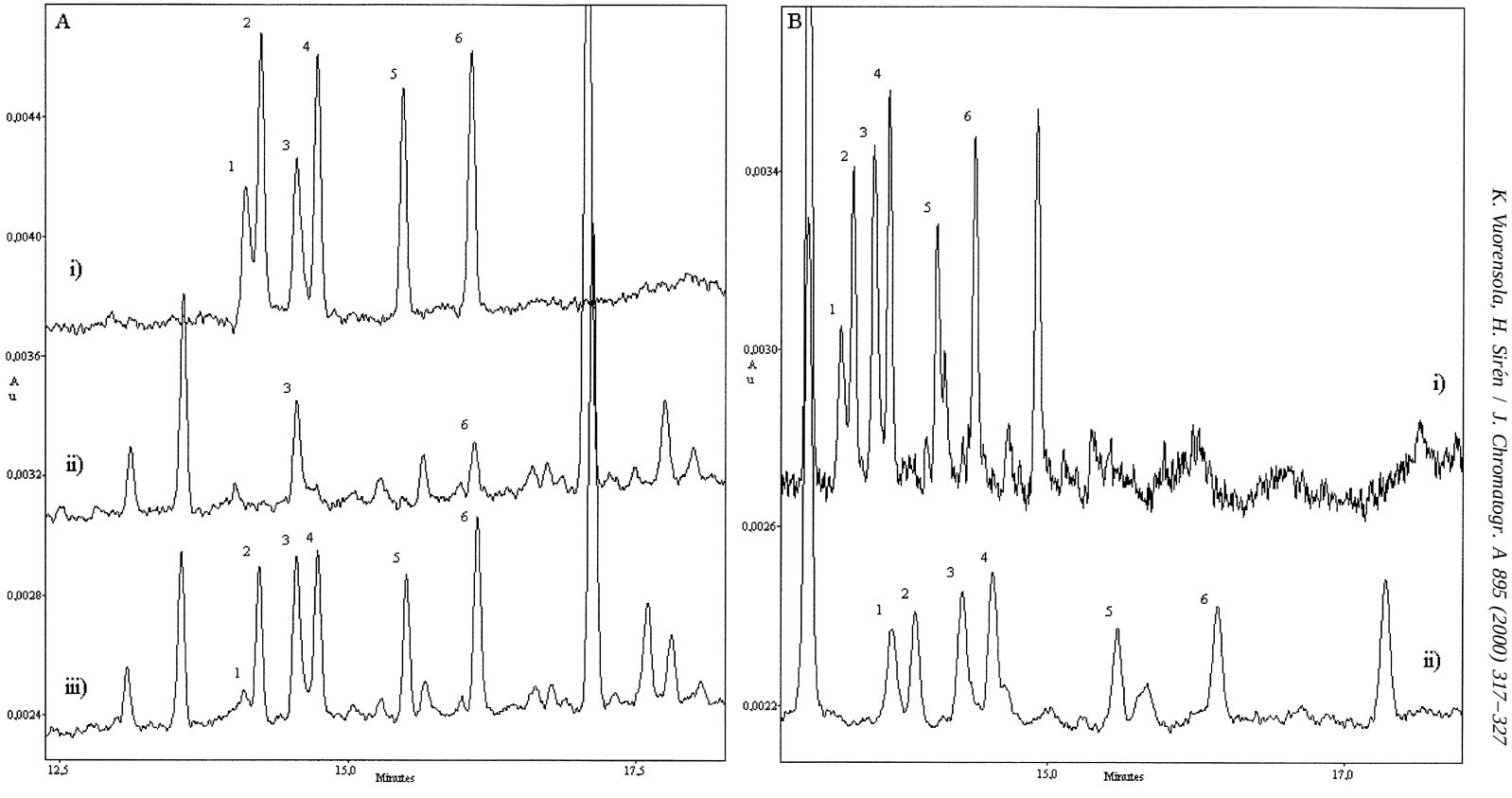

Fig. 1. (A) Separation of the catecholamines. (i) Catecholamine standards, (ii) blank urine sample (pooled), (iii) pooled urine sample spiked with standards. Peak identification: 1=DHBA, 2=HMBA, 3=DA, 4=3MT, 5=NMN, 6=MN. Conditions: buffer 50 m*M* ammonium acetate–40 m*M* diisopropylamine (pH 4.0), capillary 70/80 cm (L_{det}/L_{tot}), +20 kV, 37 μA, 25°C, 200 nm, injection 10 s at 0.5 p.s.i. (B) Effect of dynamic coating on the capillary on separation between the analytes and matrix compounds. Aligned electropherograms (i) buffer 50 m*M* ammonium acetate (pH 4.0), (ii) buffer 50 m*M* ammonium acetate–40 m*M* diisopropylamine (pH 4.0). Peaks and other conditions as in (A) except current in (i) 27 μA.

3.2. Effect of hydrolyses on the stability of catecholamines

The CAs and their phase I metabolites form glucose and sulfate conjugates in cellular metabolism. With no commercial reference compounds available for these conjugates, the biological samples must be hydrolyzed prior to the analysis in order to quantify the total concentration of the CAs in the sample. Release of the analytes can be performed by acid, base or enzymatic hydrolysis. All the techniques have their advantages and disadvantages [18]. However, during the release of the conjugates the concentrations of the free CAs must not alter. With this aspect in mind, the stabilities of the standard compounds were tested in the conditions of the different hydrolysis formats.

In acid hydrolysis the CAs were degraded completely in 50–500 m*M* hydrochloric acid at room temperature. The degradation was up to 70% in 20–25 m*M* acid and up to 35% in 5–10 m*M* acid at room temperature. The effect of temperature was tested with 5–10 m*M* acid up to 50°C. The degradation did not increase markedly up to a temperature of 38°C. The degradation results for the catecholamines in 10 m*M* HCl at 30°C are shown in Table 1.

The results indicate that MN would be least affected by the acidic conditions. This result is contradictory to the result of Hay and Mormède [19], who reported that MN was most affected by the acid hydrolysis. In acidic conditions the primary or secondary amine is positively charged. The 4-hydroxy group may be protonated, forming a cation which is resonance-stabilized in the aromatic nucleus. Via highly metastable intermediates with two positive charges the CAs form quinone structures by losing a proton or a water molecule as in the case of NMN and MN [20].

In this study DHBA and DA with the 3,4-dihydroxy structure were totally hydrolysed under basic conditions irrespective of the base concentration. The 3-*O*-methylated CAs 3MT, NMN and MN were also totally degraded in 50–500 m*M* sodium hydroxide concentrations. At room temperature the extent of degradation was 10–20% in 5–20 m*M* solutions. At elevated temperatures the degree of hydrolysis was greater, but the quantitation was not reliable due to the poor resolution between the analytes and the degradation products. The hydrolysis results for the CAs in 5 m*M* NaOH at 30°C are shown in Table 1.

Under basic conditions the ionization of the catechol structure is preferred at the 3-hydroxy group rather than at the 4-hydroxy group, as it leads to a more resonance-stabilized intermediate. In the 3-*O*-methylated compounds this ionization is prevented,

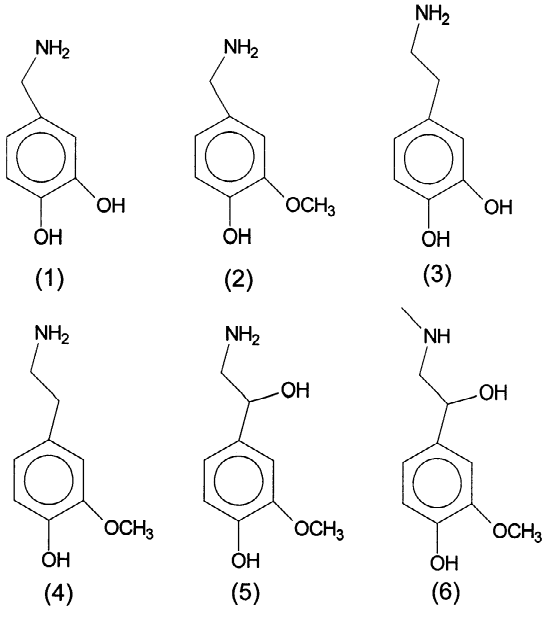

Fig. 2. Structures of the catecholamines. (1) DHBA, (2) HMBA, (3) DA, (4) 3MT, (5) NMN, (6) MN.

Table 1
Degradation of the catecholamine standards in acid, base and enzymatic hydrolyses[a]

Hydrolysis	Degradation (%)					
	DHBA	HMBA	DA	3MT	NMN	MN
Acid	32	–	27	13	16	7
Base	100	–	100	14	10	9
Enzymatic	–	25	11	35	0.3	11

[a] Conditions: 10 m*M* HCl, 30°C, 30 min; 5 m*M* NaOH, 30°C, 30 min; enzyme activities of 300 FU and 3000 RU from *Helix pomatia*, 32°C, 20 min ($n=3$).

indicating greater stability of 3MT, NMN and MN in basic conditions than of DHBA and DA. The degradation of the 3-*O*-methylated compounds starts only in stronger conditions including higher base concentration and temperature.

In enzymatic hydrolysis, degradation of the compounds was observed in the electropherograms with increasing peak area compared to non-hydrolyzed compounds. By increasing the enzyme activity at constant temperature the degradation of the CAs was linear up to 80%. The decrease in degradation with increasing temperature up to 50°C may result from decrease in the enzyme activity due to thermal denaturation. Minor degradation was obtained at 32°C with enzyme activities of 300 FU and 3000 RU (Table 1).

No reagent was used to inactivate the enzyme and halt the hydrolysis reaction and therefore the results may be affected by the time elapsing between the reaction time of 20 min and the time of analysis. The temperature of the sample track in the CE instrument was kept slightly below room temperature (18°C) in order to diminish the enzyme activity during the time between the end of the reaction and introduction of the sample to the CE instrument.

3.3. Enzymatic hydrolysis of urine

Hydrolysis in standard solutions may not proceed at the same rate as in biological samples [18]. Therefore, overall control of the analysis is necessary. Addition of the enzyme into acid-buffered intermediate did not lead to precipitation of the enzyme juice, in contrast to CA tests made with uncontrolled media. However, if the media in CA tests had been performed in buffered solution, the buffer would have caused deterioration in the quality of the capillary electrophoretic analyses by peak broadening of the CAs.

Hydrolysis reactions using β-glucuronidase or sulfatase from *Helix pomatia* have been performed in 0.12–0.2 *M* sodium acetate buffer (pH 5.0–5.4) [4,21,22]. The capacity of a 0.15 *M* sodium acetate buffer to compensate the pH of the urine (pH 6) was found to be adequate, and therefore 0.15 *M* sodium acetate (pH 5.0) was selected for the enzymatic hydrolysis reactions in urine matrix, although 1.0 *M* sodium acetate with pH 4.8±0.2 was suggested by the supplier.

Taylor et al. [23] reported that morphine-6-β-D-glucuronide was not fully hydrolysed by *Helix pomatia* extract within the 1.5 h reaction time. Furthermore, Lanz et al. [21] observed that conjugates of 4-hydroxy-3-methoxymethamphetamine, structurally related to MN, were only 50% hydrolysed during 4 h incubation at 37°C with an activity of 2000 U per ml urine. In their studies the most effective hydrolysis was obtained with an activity of 10 000 U per ml and an incubation time of 16 h at 37°C. On the basis of their studies, in our tests the reaction time was increased from 20 min to 3 h in order to complete the reaction. The final reaction temperature was optimized to the physiological temperature (37°C). The optimized urine hydrolysis were performed with 1000 FU of β-glucuronidase and 10 000 RU of sulfatase in 1 ml urine. An ice bath has been used to halt the enzymatic hydrolysis reaction [4]. In our work the samples were subjected directly to SPE treatment and therefore a method to halt the reaction was not considered necessary.

3.4. Solid-phase extraction of the urine matrix

On the basis of our own results and those of Pastoris et al. [9], the silica-based packings in SPE yield poorer recoveries of the CAs than polymer-based resins. We moved on to test the applicability of the Waters Oasis HLB copolymer sorbent of *N*-vinylpyrrolidone and divinylbenzene in the purification of CAs in the urine matrix. The copolymer is non-ionizable at any pH, but the ionization and thus the stability of the CAs is strongly dependent on the pH. The analytes are positively charged at the amine functionality at pH below 12 and are negatively charged at the phenol group at pH above 7, showing a double charge in the pH range of 7–12. In acidic solution the CAs were not retained by the sorbent and in basic solutions the 3,4-dihydroxy compounds DHBA and DA were degraded. Usually the decomposition of the CAs, due to oxidation, begins already at about pH 7 [24].

Phosphate buffer at pH 7 gave better extraction recoveries than borate or mixed phosphate–borate buffers. The recoveries of the analytes were in-

creased with increasing buffer concentration in the range of 0.03 to 0.5 *M* due to the higher buffer capacities of the higher ionic strength buffers. The capacity of the 0.5 *M* phosphate buffer to compensate for the pH of the sodium acetate buffer (pH 5.0) used in enzymatic hydrolysis of the urine was found to be adequate. Increase of the buffer concentration above 0.5 *M* decreased the recoveries of the CAs.

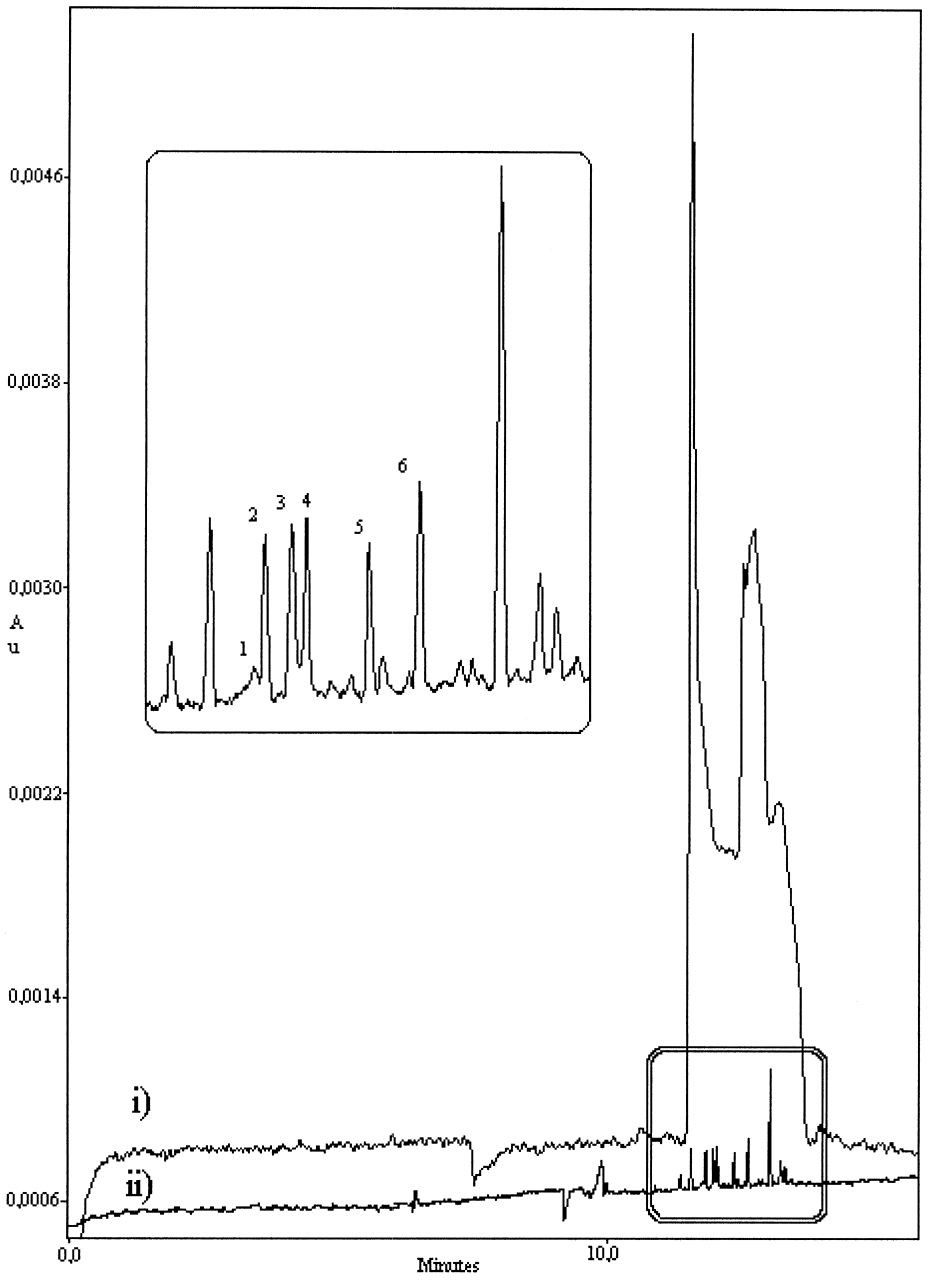

Fig. 3. Effect of solid-phase extraction on the analysis of catecholamines in urine. Spiked urine sample (i) with no pretreatment, (ii) after solid-phase extraction. Peaks and other conditions as in Fig. 1A.

More quantitative results of the analytes were obtained when the conditioning of the sorbent material was performed with the phosphate buffer rather than with water after the methanol treatment.

In SPE the recovery of DHBA, commonly used as an internal standard in CA analyses [1,3–5,8,9], was observed to be strongly dependent on the concentration of the phosphate buffer. In the range of 0.03 to 0.1 *M* phosphate buffer its recovery was increased from 49 to 89%. However, at 0.2 *M* and above its recovery was only 20%. After enzymatic hydrolysis of the urine sample the recovery of DHBA was diminished to zero. Our studies showed that the compound was not suitable as the internal standard in the present method. Therefore DHBA was replaced with HMBA, the 3-*O*-methylated form of DHBA, which showed good recovery in SPE. The low recovery of DHBA is possibly related to the differences in the polarities of the molecules. The recovery of DA, structurally similar to DHBA and differing only by one carbon in the side chain, was not affected by the phosphate concentration. DHBA is more polar than DA and HMBA. The polarity of DA is lower due to the increase of the length of the side chain. The polarity of HMBA is lower due to *O*-methylation [25]. The 3,4-dihydroxy compounds can form intrahydrogen bonds between the two phenolic hydroxy groups, thus diminishing the extent of solvation and leading to a more hydrophobic character. Moreover, the amine group in DA may form an intrahydrogen bond with the phenolic hydroxy group, thus further increasing the hydrophobicity [26]. In DHBA this kind of intramolecular bonding may be restricted by steric effects due to the shorter side chain. In the Oasis HLB SPE copolymer sorbent composed of *N*-divinylpyrrolidone and divinylbenzene, the interactions between the sorbent and the analyte are based on hydrophobic Van der Waals forces [27]. Thus a more polar analyte is less strongly attached to the sorbent. An increase in the ionic strength of the phosphate buffer further decreases the attachment of a more polar analyte to the sorbent material.

The SPE purification was found to be very effective. The broad and massive peak (Fig. 3) caused by different endogenous compounds in the urine matrix was eliminated by the pretreatment and a clear profile with only a few peaks of endogenous compounds was found in the migration window of the CAs. The results of the recoveries of the SPE treatment for the CAs are listed in Table 2.

3.5. Method validation

The limit of detection (LOD) for every CA was determined with standards as a signal-to-noise (S/N) ratio of 3. The results are given in Table 2. The linearity of the compounds was found to be good in the range tested. The limits of quantification (LOQs) for the analytes were determined in the pooled urine by spiking with low concentrations of CA standards and calculating the lowest limit corresponding to an S/N ratio of 3 after the SPE treatment. The LOQ studies were performed without enzymatic hydrolysis. The concentrations of the endogenous CAs in the blank urine were also estimated. The results showed that only the concentrations of DA and MN were above their LOQ values.

The reproducibility of the method including enzymatic hydrolysis was evaluated on two concen-

Table 2
Validation data of the catecholamines in urine

	HMBA	DA	3MT	NMN	MN
LOD (μM)	0.40	0.70	0.53	0.57	0.40
Linearity, r^2 (0.50–15.0 μM)	0.996	0.994	0.996	0.995	0.999
LOQ (μM)[b]	0.49	0.83	0.67	0.87	0.50
Recovery (%)[a]	96.4	109.2	112.5	106.1	104.6
Recovery (%)[a]	101.9	97.8	122.7	103.9	124.4

[a] $n=6$.

[b] Due to the concentration of the sample by a factor of five in the SPE treatment, concentrations of 1/5 of the LOQ values can be determined in urine samples (1 ml).

Table 3
Repeatability of the method including enzymatic hydrolysis

n	HMBA (μ*M*)		DA (μ*M*)		3MT (μ*M*)		NMN (μ*M*)		MN (μ*M*)	
	2.5	5.0	2.5	5.0	2.5	5.0	2.5	5.0	2.5	5.0
1	2.7	4.2	1.2	−5.4	5.7	3.3	2.3	4.0	2.5	3.9
2	2.9	4.9	1.2	3.5	5.3	7.9	2.3	4.8	2.8	4.8
3	2.9	5.2	1.8	2.0	6.2	6.9	2.6	5.3	2.9	5.0
4	3.0	5.2	0.0	4.5	3.9	9.5	2.6	5.4	3.0	5.4
5	2.6	4.8	1.4	−4.7	5.1	5.0	2.3	4.4	3.0	5.0
6	2.9	6.2	1.5	5.8	5.2	11.0	2.3	6.0	2.7	6.4
Mean	2.8	5.1	1.2	0.9	5.2	7.3	2.4	5.0	2.8	5.1
SD	0.1	0.7	0.6	4.8	0.8	2.8	0.1	0.7	0.2	0.8

tration levels by spiking the urine samples with the CA standards (Table 3). The repeatabilities of the injections of the samples were good, ranging from 1.8 to 9.1% (RSD) and from 1.0 to 5.3% at concentrations of 2.5 and 5.0 μ*M*, respectively. The repeatability of the recoveries in replicate samples was good in the case of HMBA, NMN and MN. However, in the case of DA and 3MT the results were not correlated within the replicates due to the extensive variations in the peak areas. Some matrix compounds with the same mass-to-charge ratio as DA and 3MT may induce interference in the method.

4. Conclusions

Of the hydrolysis reactions of the CA standards it can be concluded that the basic hydrolysis conditions seem not to be suitable for the CAs without the use of preservatives, due to total degradation of the 3,4-dihydroxy compounds. The results from acid and enzymatic hydrolysis are comparable. However, only the enzymatic hydrolysis cleaves both ether and ester bonds of the glucose and sulfate conjugates of the CAs. Therefore enzymatic hydrolysis was applied to the urine matrix. Total resolution between the CAs was achieved in CE with an ammonium acetate buffer. However, the direct injection of a urine sample to CE at acidic pH resulted in total loss of resolution between the analytes and the matrix components. Therefore a purification step based on SPE with a copolymer sorbent was developed, with good recoveries of the CAs. The resolution between the analytes and endogenous compounds was enhanced by using dynamically coated capillaries. The reproducibility of the method was good for all the analytes except for DA and 3MT. In the case of these two compounds, unknown peaks were still interfering their analysis in CE.

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