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Determination of dopamine and methoxycatecholamines in patient urine by liquid chromatography with electrochemical detection and by capillary electrophoresis coupled with spectrophotometry and mass spectrometry

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Abstract

The applicability of capillary electrophoresis (CE) with UV and mass spectrometric (MS) detection for the determination of dopamine and methoxycatecholamines in urine was evaluated in comparison with the liquid chromatography–electrochemical detection (LC–EC) method widely used in catecholamine analysis. The catecholamines in urine were deconjugated with acid or enzyme hydrolysis, purified by cation exchange (CEX) or solid-phase extraction (SPE) with a copolymer of *N*-divinylpyrrolidone and divinylbenzene and analyzed by LC–EC, CE–UV, and CE–MS. Acid hydrolysis was more effective in the deconjugation than enzymatic hydrolysis with *Helix pomatia*. However, the recoveries of HMBA, DA and NMN from spiked samples were less than 30% after acid hydrolysis and SPE purification. The CEX purification was more efficient than SPE in removing matrix compounds from the urine samples. The limits of detection were lower in LC–EC analysis than in CE–UV or CE–MS. Many factors in the analytical procedure caused deviations in the concentrations measured for urinary dopamine and methoxycatecholamines. The recovery of HMBA, which was used as the internal standard, was poor after acid hydrolysis and SPE purification. The purification methods were validated in conjunction with the analytical methods and therefore cross analysis was unsuccessful. The LC–EC method was the most sensitive, but CE–UV and CE–MS were sensitive enough for the determination of dopamine and methoxycatecholamines even in healthy patient urine. The EC and MS detections were superior to the UV detection in specificity since, after acid hydrolysis, some matrix compounds were migrating close to I.S., DA and 3MT.

Keywords: Dopamine; Methoxycatecholamines

1. Introduction

Catecholamines dopamine (DA), noradrenaline, and adrenaline as well as their metabolites, the

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methoxycatecholamines 3-methoxytyramine (3MT), normetanephrine (NMN), and metanephrine (MN) are neurotransmitters, which are determined in urine for the diagnosis of pheochromocytoma [1]. NMN and MN have proved to be particularly important in the diagnosis [2]. In addition, catecholamines and their metabolites are involved in a variety of regulatory systems such as stress and learning as well as in the control of many processes of metabolism and the immune system [3].

The analytical methods along with many sample preparation, separation, and detection methods used for the determination of catecholamines and their metabolites in biological samples have recently been reviewed [3,4]. Sample preparation for catecholamine and methoxycatecholamine analysis most often includes cation exchange (CEX) purification [5]. Also a two-step purification including a CEX extraction for catecholamines followed by an anion exchange extraction for methoxycatecholamines has been developed [6]. Both enzymatic and acid hydrolyses for the deconjugation of catecholamines and methoxycatecholamines have been carried out [5,7,8]. The analysis by liquid chromatography (LC) with electrochemical detection (EC) is the most widely used technique for catecholamine and methoxycatecholamine determination [6,9]. Catecholamines and their metabolites have also been separated by capillary electrophoresis (CE) using UV [10], fluorescence [11], and electrochemical detections. The dual-electrode amperometric detection of catecholamines in diluted urine samples without purification resulted in very sensitive analyses [12].

Several publications have compared the use of LC and CE as separation techniques with UV detection. Sample matrices included urine, plasma, and other biological matrices [13–17]; pharmaceutical formulation [18]; and soil [19]. In general, the results obtained by LC and CE correlated well. CE provides two to seven times faster analysis with high column efficiency, whereas the LC method provides at least two to three times better sensitivity. The possibility to totally avoid sample preparation with CE separation reduces the total analysis time considerably [15]. The sample purification needed for LC analysis, in turn, often leads to cleaner chromatograms and lower limits of detection (LOD) [17].

The mass spectrometer (MS) is a highly specific detector, which allows peak identification even in complex sample matrices. It also gives structural information about the analytes, which increases the reliability of the identification of analytes. The MS analyses of catecholamines and methoxycatecholamines with atmospheric pressure ionization techniques include both direct infusion of samples into MS and analyses with LC or CE on-line coupled with MS. Catecholamines have been identified in the immune system by direct infusion of the extract of human peripheral blood mononuclear cells into MS [20]. The use of sonic spray ionization in order to achieve lower detection levels compared to electrospray ionization has been demonstrated in the analysis of catecholamines [21]. Furthermore, catecholamine metabolites and glucuronide and sulfate conjugates have been identified by MS [22]. The use of negative ion electrospray ionization for catecholamines has been reported to yield more informative MS–MS spectra than using positive ion mode [23].

A sensitive LC–MS–MS analytical technique has been developed for urinary catecholamines after a specific sample purification step [24]. MS detection with atmospheric pressure chemical ionization has been found to be superior to EC detection in terms of selectivity in the analysis of urinary catecholamines and methoxycatecholamines [25]. Atmospheric pressure chemical ionization was also used in the LC–MS analysis of 9-fluorenylmethyloxycarbonyl derivatized catecholamines in urine [26]. The derivatization allowed fluorometric detection of the analytes prior to MS detection. Catecholamine and methoxycatecholamine standards have been analysed by CE–MS in aqueous and non-aqueous conditions with sheath liquid coupling [27,28] as well as with sheathless nanospray coupling [29]. In the latter study, the determination of catecholamines and methoxycatecholamines was also demonstrated, with non-aqueous separation conditions and nanospray coupling, in urine samples.

The aim of this study was to determine dopamine and methoxycatecholamines in patient urine samples by CE–UV and CE–MS [10,27] and compare the results to those obtained by LC with EC detection. Also, the effects of acid and enzymatic hydrolyses and of purification methods based on CEX and solid-phase extraction (SPE) were investigated on a

copolymer of *N*-divinylpyrrolidone and divinylbenzene.

2. Experimental

2.1. Materials

4-Hydroxy-3-methoxybenzylamine hydrochloride (HMBA, used as internal standard, I.S.), dopamine (3-hydroxytyramine hydrochloride, DA), 3-methoxytyramine (3-methoxy-4-hydroxyphenethylamine hydrochloride, 3MT), DL-normetanephrine (3-methoxybenzenemethanol hydrochloride, NMN), and DL-metanephrine (DL-*m*-*O*-methylepinephrine hydrochloride, MN) (98%), were obtained from Sigma–Aldrich (Steinheim, Germany).

Ammonium acetate was from Sigma–Aldrich; glacial acetic acid and methanol for LC analyses were from Rathburn (Walkerburn, Scotland, UK); and methanol for CE analyses, ammonium hydroxide, and sodium hydroxide solutions were from J.T. Baker (Deventer, The Netherlands). Sodium acetate, sodium dihydrogen phosphate, disodium hydrogen phosphate, trichloroacetic acid (TCA), hydrochloric acid (HCl) (37%), and ammonia (25%) were from Merck (Darmstadt, Germany), and ethylenediamine tetraacetic acid (EDTA) (IDRANAL III) was from Riedel de Haën (Seelze, Germany). All reagents were of analytical purity unless otherwise stated. *Helix pomatia* juice was from BioSepra (Cergy-Saint-Christophe, France) and contained 100 000 Fishman Units (FU) of β-glucuronidase and 1 000 000 Roy Units (RU) of sulfatase per ml. The deionized water was purified with a Milli-Q Plus system (Millipore, Bedford, MA, USA).

2.2. Instruments

The LC instrument consisted of the following modules: an HP 1050 autosampler (Agilent Technologies, Palo Alto, CA, USA), an LKB 2150 HPLC pump (LKB, Bromma, Sweden), an HPLC column heater (Bio-Rad Laboratories, Hercules, CA, USA), and an amperometric electrochemical detector (LC-4B, Bioanalytical Systems, West Lafayette, IN, USA). The detector used a dual glassy carbon working electrode (MF-1000) and a RE-5B reference electrode (MF-2052). The cell thickness was 0.005 inch. The system was used in oxidation mode at +800 V. The LC column was a 15-cm-long Nucleosil 10C18 column (I.D. 4.6 mm; HPLC Technology, Welvyn Garden City, Hertfordshire, UK) thermostated at 40 °C. The LC runs were isocratic with the flow-rate adjusted to 0.9–1.2 ml/min to obtain suitable retention times for NMN, MN, and I.S. (3.5, 5.0 and 6.4 min, respectively). The total run time was 20 min. A 10-μl volume of the purified sample was injected into the column.

The CE equipment used in CE–UV analyses was a P/ACE 2200 series instrument (Beckman-Coulter Instruments, Fullerton, CA, USA). The capillary was 77 cm long (effective length 70 cm, 50 μm I.D.) and was obtained from Composite Metal Services Ltd. (The Chase, Hallow, Worcestershire, UK). The capillary was thermostated at 25 °C. Triplicate injections were carried out hydrodynamically with a pressure of 3.45 kPa for 20 s. Separation voltage was +20 kV corresponding to a field strength of 260 V/cm. UV detection was carried out at 200 nm.

The CE instrument used in CE–MS analyses was from Prince Technologies (TC Emmen, The Netherlands). The capillary was 80 cm long (50 μm I.D.). Injections were carried out with a pressure of 3.50 kPa for 30 s in triplicate. Separation of the catecholamines was carried out with +20 kV together with a 0.7 kPa pressure from the capillary inlet. The sample tray and capillary were maintained at 20 and 25 °C, respectively.

The MS instrument was an Esquire ion trap mass spectrometer from Bruker Daltonic (Bremen, Germany). Temperature of the ion source was 60 °C; nebulizing gas pressure and drying gas flow-rate were 34.47 kPa and 8 l/min, respectively. Capillary voltage was 3500 V, end-plate offset −680 V, and trap drive value 35. Capillary voltage was activated 30 s after activation of the separation voltage from CE. Sheath liquid flow-rate was 6 μl/min, and the sheath liquid was introduced by a syringe pump from Cole Parmer Instrument Company (Vernon Hills, IL, USA). Mass range m/z 125–210 was scanned for the catecholamines. The ions used in quantitation were m/z 137, 154, 168, 166, and 180 for I.S., DA, 3MT, NMN, and MN, respectively.

2.3. LC column and CE capillary conditioning

Methanol, water and the mobile phase were bubbled with helium for 15 min before use in LC. The column was flushed with methanol for 10–15 min, with water for 30 min, and finally with the mobile phase for 1 h with a flow-rate of 1.3 ml/min. During conditioning with the mobile phase, the potential of the EC detector was slowly adjusted to +800 V.

In CE–UV analyses, new capillaries were conditioned by flushing at 138 kPa pressure sequentially with 0.1 mol/l sodium hydroxide, water and electrolyte solution for 15 min each. Between analyses, the capillary was flushed with 0.1 mol/l sodium hydroxide and water for 1 min and with electrolyte solution for 3 min. A new CE–MS capillary was conditioned by flushing at 140 kPa pressure with 0.1 mol/l ammonium hydroxide, water and electrolyte solution for 15 min each. Between analyses, the capillary was flushed with electrolyte solution for 1 min. After three injections, the capillary was reconditioned with 0.1 mol/l ammonium hydroxide and water for 3 min each and with the electrolyte solution for 6 min.

2.4. Solutions

The buffer solution of the mobile phase for LC–EC was sodium acetate containing 0.1 mol/l TCA and 0.1 m*M* EDTA. The pH of the buffer was adjusted to 3 using solid anhydrous sodium acetate, and the solution was filtered through a 0.45-μm membrane (HAWP04700; Millipore, Bedford, MA, USA). To obtain the final mobile phase, 222.6 ml of methanol was added to 2000 ml of the TCA–EDTA buffer solution. The elution solution used with CEX columns was ammonium–methanol; 1.5 ml of ammonia solution (25%) was added to 1000 ml of methanol. Acidic methanol was prepared by adding 1.8 ml of HCl (37%) to 100 ml of methanol.

For sample preparation, 0.15 mol/l sodium acetate (pH 5.0) and 0.5 mol/l phosphate buffer (pH 7.0) were used by enzymatic hydrolysis and SPE, respectively. The phosphate buffer was a mixture of 0.5 mol/l solutions of disodium hydrogenphosphate and sodium dihydrogenphosphate. The electrolyte solution in CE–UV analyses was 50 m*M* ammonium acetate–40 m*M* diisopropylamine (pH 4.0). The electrolyte solution in CE–MS analyses was 50 m*M* ammonium acetate (pH 4.0). The pH was adjusted with acetic acid using an inoLab pH meter and a combination electrode (WTW, Weilheim, Germany) calibrated with commercial aqueous buffers of pH 4 and 7 (Merck, Darmstadt, Germany). The electrolyte solutions were filtered through 0.45-μm nylon membranes (Tracer). Sheath liquid in CE–MS analyses was methanol–water (80:20, v/v) with 0.5% (v/v) glacial acetic acid added.

The 5 m*M* stock solutions of I.S., DA, 3MT, NMN and MN were prepared in 0.5% (v/v) acetic acid in water. The final concentrations of I.S. in the purified samples were 16.3 μmol/l and 5.0 μmol/l in LC and CE analyses, respectively. For spiked samples, a mixture of 50 μmol/l of each DA, 3MT, NMN and MN was used.

2.5. Samples

Six urine samples (Table 1) having a wide range of excreted amounts of NMN and MN were selected from the daily routine of a hospital laboratory. Daily excretion of NMN and MN was approximately normal in two of the samples, slightly elevated in two, and very high in two. The 24-h urine was collected into a vessel containing 5 ml of 6 mol/l HCl, and a second 5-ml portion of 6 mol/l HCl was added. The samples were stored at −20 °C and filtered before use through a 0.22-μm Millex-GS filter (Millipore). The samples were pretreated and analyzed simultaneously at two laboratories, one using LC and the other CE.

Table 1
Properties of patient urine samples analyzed in this study

Sample	V_{tot} (24 h) (ml)	Female/male	Age (years)
1	1160	M	35
2	2000	M	39
3	2400	F	38
4	1300	M	48
5	2150	F	43
6	3500	F	45

2.6. Sample pretreatment

2.6.1. Hydrolysis

In acid hydrolysis, 50 μl of 6 mol/l HCl solution was added to 1 ml urine and the mixture was incubated for 20 min at 100 °C. In enzymatic hydrolysis, 0.5 ml of 0.15 mol/l sodium acetate (pH 5.0) and 10 μl of *H. pomatia* juice were added to 1 ml urine and incubation was carried out for 3 h at 37 °C.

2.6.2. Purification

2.6.2.1. Cation exchange extraction. Before LC–EC analysis, urine samples were purified on columns of strong CEX resin; 160 g of resin (AG MP-50, 100–200 mesh, Bio-Rad Laboratories) was suspended in 2 l of water. After the suspension had settled, it was decanted to remove small particles. The resin was washed with 2 l of 500 m*M* sodium hydroxide and with the same volume of water. For the last step, the resin was washed with 2 l of 500 m*M* sodium bicarbonate and twice with the same volume of water. The CEX columns were prepared daily using a suspension of resin in an equal volume of water. Disks (Quik-Sep®, Isolab, Akron, OH, USA) were placed at the bottom of the plastic columns (Quik-Snap Columns, Isolab) and 3 ml of resin suspension was added. The pH of the hydrolyzed urine samples was made alkaline with 50 m*M* sodium phosphate, with Thymol Blue as the indicator, and the samples were quantitatively transferred to the columns. The columns were washed with 4.5 ml each of water, 50% methanol and 100% methanol and the methoxycatecholamines were eluted with 3.5 ml of ammonium–methanol solution. The eluent was collected into tubes containing 100 μl of acidic methanol. The eluent was evaporated and 200 μl of TCA–EDTA buffer was added to each tube.

2.6.2.2. Solid-phase extraction. Before CE–UV and CE–MS analysis, urine samples were purified with a SPE method developed earlier [10]. Briefly, 0.5 or 2 ml of phosphate buffer (0.5 mol/l, pH 7.0) was added to a urine sample after enzymatic or acid hydrolysis, respectively. The sample was introduced to the Oasis HLB copolymer SPE sorbent (Waters, Taunton, MA, USA) after conditioning of the sorbent with methanol and phosphate buffer. The sorbent was washed with water and the analytes were eluted with methanol. The final dissolution after evaporation was made in water (200 μl). The purified samples were stored at −20 °C if not analyzed directly.

The hydrolysis, purification and analysis method combinations (Methods 1–7) used in the study are listed in Table 2.

3. Results and discussion

Method 1 has been developed in our laboratory for the analysis of methoxycatecholamines 3MT, NMN and MN, of which NMN and MN are routinely

Table 2
Combinations of pretreatment and analysis methods used in this study

Method no.	Hydrolysis		Purification		Analysis			Quantitation	
	Acid	Enzyme	CEX	SPE	LC–EC	CE–UV	CE–MS	I.S.	E.S.
1	×		×		×			×	
2		×		×		×		×	
3	×			×		×		×	
4	×			×		×			×
5		×		×			×	×	
6	×			×			×	×	
7	×			×			×		×

I.S., internal standard; E.S., external standard.

analyzed. Methods 2–7 are intended for the analysis of DA, in addition to the methoxycatecholamines. The methods listed in Table 2 are compared below in terms of hydrolysis, purification and analysis steps, including separation and detection. The results of patient urine analyses (Table 3) by Methods 1–7 (Table 2) are evaluated and discussed.

3.1. Hydrolysis methods

The hydrolysis of the glucuronide and sulfate conjugates of dopamine and the methoxycatecholamines was carried out chemically with mineral acid and enzymatically with *H. pomatia* juice. The chemical hydrolysis was considerably faster than enzymatic hydrolysis. Preliminary studies gave lower concentrations for metanephrines after enzymatic hydrolysis than after acid hydrolysis and the enzyme activity in the reaction was therefore increased from 1000 FU and 10 000 RU up to 20 000 FU and 200 000 RU, respectively, and incubations of 4 h at 37 °C and 1 h at 55 °C were carried out. However, no increase in the amounts of free metanephrines was noticed. On the contrary, the I.S. signal degraded with increasing enzyme activity.

Enzymatic hydrolysis with *H. pomatia* for the deconjugation of analytes is reported in the literature, also in comparison with acid hydrolysis. Hydrolysis with *H. pomatia* gave higher efficiencies for both nortestosterone and diethylstilboestrol glucuronides than methanolysis, the efficiency of the deconjugation was 43–52% [30]. In addition, incubation for 2 h at 55 °C gave higher hydrolysis efficiency than overnight incubation at 37 °C. The comparison of acid and enzymatic hydrolyses with *H. pomatia* and *Patella vulgata* showed most efficient cleavage of the conjugates with *H. pomatia* [31]. However, incomplete hydrolysis of anabolic steroid conjugates using *H. pomatia* has been reported even under optimum conditions [32]. The enzymatic hydrolysis of urinary and plasma catecholamine and methoxycatecholamine sulfates has often been carried out

Table 3
Results for NMN, MN, 3MT and DA (μmol/l) in patient urine samples with different methods

Sample		Method						
		1	2	3	4	5	6	7
1	NMN	1.21	0.27	1.75	0.54	<0.20	0.93	0.54
	MN	0.78	0.51	3.68	1.09	nd	2.44	1.41
	3MT	na	0.66	2.93	0.88	0.96	2.02	1.20
	DA	na	2.71	5.70	1.68	2.72	3.13	1.69
2	NMN	0.95	0.70	1.15	0.83	0.24	0.72	0.43
	MN	0.50	<0.19	1.13	0.81	0.62	1.66	0.95
	3MT	na	0.72	0.63	0.46	0.67	0.80	0.49
	DA	na	1.37	1.60	1.14	1.20	2.40	1.34
3	NMN	0.54	nd	nd	nd	nd	nd	nd
	MN	0.29	1.03	31.47	1.87	nd	nd	nd
	3MT	na	0.90	1.98	0.15	0.71	nd	nd
	DA	na	1.01	5.13	0.34	nd	nd	nd
4	NMN	1.38	0.41	1.04	0.77	0.22	0.82	0.85
	MN	0.62	0.43	1.15	0.84	nd	1.33	1.35
	3MT	na	0.57	0.77	0.58	0.63	0.79	0.84
	DA	na	2.55	3.59	2.57	2.67	5.07	5.09
5	NMN	2.79	0.38	1.00	0.82	0.73	2.92	1.11
	MN	2.09	0.90	3.44	2.73	1.24	10.66	4.28
	3MT	na	1.06	1.06	0.87	1.15	2.96	1.11
	DA	na	1.03	0.55	0.48	0.80	2.53	0.71
6	NMN	1.66	0.32	1.90	0.41	nd	0.75	<0.20
	MN	0.71	0.71	2.20	0.45	nd	1.60	0.35
	3MT	na	0.77	1.91	0.40	0.57	0.79	0.21
	DA	na	2.72	3.13	0.63	3.26	2.21	0.42

nd, not detected; na, not analysed.

with sulfatase from *Aerobacter aerogenes*, which leads to complete deconjugation in 60 min or less [8,33–35]. However, enzymatic hydrolysis of urine with glusulase preparation containing both β-glucuronidase and sulfatase activity yielded lower values of free catecholamines than the acid lyophilization method [7]. On the other hand, equal recovery of free methoxycatecholamines has been reported with sulfatase or acid [36].

Acid hydrolysis at 100 °C for 120 min has yielded complete hydrolysis of catecholamine sulfate conjugates determined with sulfate standards [37]. The hydrolysis did not result in degradation of the free catecholamines. Even hydrolysis time of 15 min has yielded quantitative deconjugation with acid [38]. Acid hydrolysis of plasma dopamine has been reported to be complete in 40 min and the recovery of the added dopamine standard was 103% [39]. However, at pH 1 at 100 °C, degradation of the free methoxycatecholamines was noticed to begin after 20 min hydrolysis [36] and degradation of free standard and sample dopamine has been noticed even after hydrolysis of just 10 min [34]. However, under conditions of optimal acid strength, the sulfate conjugates are hydrolyzed without damage to the free amine [40]. Sodium metabisulfite and dithiothreitol have been added as antioxidants in acid hydrolyses of catecholamines to preserve the free forms from oxidation [38,41].

The urinary dopamine and methoxycatecholamines are 42–67% and 49–87% sulfoconjugated, respectively [8,37,42], glucuronide conjugates comprise only a few percent [43]. As enzyme activity depends on the electrolyte content of the medium, the degree of hydrolysis may vary from one biological sample to another [7]. The sulfatase is inhibited by the salts in urine matrix, but desalting has been reported to abolish the inhibition [36]. Acid hydrolysis leads to incomplete glucuronide hydrolysis [34,36] but is efficient in hydrolysing the sulfate ester bond [44,45]. Therefore, in addition to enzymatic hydrolysis, we evaluated acid hydrolysis as a means of obtaining high deconjugation.

The concentrations of free metanephrines in the samples were higher after acid hydrolysis than enzymatic hydrolysis indicating more efficient release of the metanephrines from the conjugates. The recoveries of the analytes in spiked urine samples after acid hydrolysis and SPE purification were 30, 13, 99, 26 and 79% for I.S., DA, 3MT, NMN, and MN, respectively. The reason for the poor recoveries of I.S., DA, and NMN could be degradation in acid hydrolysis. Kema [44] has reported poor acid stability for both NMN and MN in spiked urine samples. However, DA has been reported to be stable in acid [45]. The acid stability of 3MT is reported to be good, but Hay [6] has reported poor recovery for MN after acid hydrolysis. Another reason could be poor retention of I.S., DA, and NMN in the SPE phase after acid hydrolysis. The volume of phosphate buffer added to the acid-hydrolyzed sample was increased relative to the volume used after enzymatic hydrolysis to obtain neutral pH and to provide retention of the catecholamines in the SPE phase. However, it was observed earlier that the ionic strength of the sample has a major effect on the retention of the analytes in SPE [10], and therefore poor recovery for I.S., DA and NMN could also be due to poor retention in SPE due to increased ionic strength of the sample.

3.2. Purification methods

The sample purification methods that we investigated were CEX and SPE. The SPE sorbent was a copolymer of *N*-divinylpyrrolidone and divinylbenzene. The recoveries of dopamine and the methoxycatecholamines with the SPE method are in the range of 96–124% [10]. The repeatability of the purification of duplicate samples was improved in this study by optimizing the dissolution time and stirring speed after the drying of the eluent from the SPE column. The optimized conditions yielded good reproducibility, with RSD% of 5.0, 5.3, 4.7, 5.4, and 3.7 for I.S., DA, 3MT, NMN, and MN, respectively.

A cross-checking analysis was carried out to evaluate the efficiency of the purification methods. Three samples were prepared and analysed as follows: (1) acid hydrolysis followed by CEX extraction and analysis by CE–UV and (2) enzymatic hydrolysis followed by SPE and analysis by LC–EC. The results showed that the purification methods were not suitable for analytical techniques they were not originally developed for. The matrix compounds still present in the sample after SPE purification interfered drastically with the LC–EC analysis and

methoxycatecholamines could not be identified in the chromatogram. Similarly, the high salt concentration in the sample after CEX purification interfered with the CE separation, leading to very wide peaks and shifts in the migration times of the methoxycatecholamines. The profiles of the patient urine samples were very clean, however, with only a few matrix compounds left after the CEX clean-up.

3.3. Analytical methods

The LC and CE analyses were compared in terms of limits of detection (LOD), repeatability, analysis time, and resolution between analytes and matrix compounds. LODs for DA, 3MT, NMN, and MN were determined as signal-to-noise ratio of 3 ($S/N=3$) and are presented in Table 4. Considerably lower LODs for methoxycatecholamines were obtained with LC–EC due to the larger injection volume and better sensitivity of the detector than for CE–UV and CE–MS techniques. The LODs for CE–MS analyses were lower with the ion trap analyzer in scan mode than with the triple quadrupole analyzer in single ion monitoring mode as used earlier [27], but still higher than the LODs in CE–UV analysis. The pretreatment of the urine sample increased the concentration of the analytes by a factor of five, and thus the sensitivities of the CE–UV and CE–MS methods are high enough for the determination of dopamine and methoxycatecholamines even in healthy patient urine [1,5,44].

The repeatabilities of peak heights and retention times were good in LC–EC analysis: RSD% 5.6–9.4 and RSD% 0.8–1.2, respectively. Also, the repeatability of peak areas between injections was good with CE–UV, with RSD% 0.3–10 for analytes present in high concentration. High RSD% values (up to 40%) originated from analytes with very low signals and interference of a matrix peak with the integrated electropherogram peak. The repeatabilities of absolute migration times of dopamine and methoxycatecholamines in CE–UV using the dynamic coating agent in the electrolyte solution were very good (RSD% 0.30–0.38). Owing to the matrix effect, the migration times in CE–MS analyses fluctuated, leading to higher RSD% values of 2.8–3.0. However, the relative migration times were highly repeatable with RSD% 0.18–0.25. In addition, the specificity of the MS detection allowed reliable identification of the analytes in the samples. Because of variation in the ionization process the repeatability of peak areas between injections with MS detection was low (RSD% 10–30).

Table 4
Limits of detection (μmol/l) for DA, 3MT, NMN and MN, with different analytical methods

	DA	3MT	NMN	MN
LC–EC	nd	0.2	0.1	0.1
CE–UV	0.7	0.5	0.5	0.8
CE–MS	1.2	0.9	0.7	1.4

nd, not determined.

The analysis time for determining the metanephrines and 3MT in isocratic LC–EC run is 20 min. Some matrix compounds eluting after the analytes must be eluted from the column before the injection of the next sample, which increases the total analysis time beyond the retention times of the analytes. In CE analysis, DA and the methoxycatecholamines are separated within 18 min, but the between-run conditioning of the CE capillary increases the total analysis to 23 min. In CE–MS analyses, the separation time is 13 min plus 5 min conditioning time per sample.

A baseline separation between dopamine, the methoxycatecholamines, and I.S. was obtained with all the methods (Figs. 1 and 2). More matrix compounds were detected with UV detection than with the specific EC and MS detections. After acid hydrolysis, the sample contains a compound that migrates very close to the I.S., interfering with its quantitation. In addition, a larger matrix compound is detected between DA and 3MT after acid hydrolysis, interfering with the quantitation of both DA and 3MT (Fig. 1B). With MS detection, matrix interference was not observed in the extracted ion electropherograms (Fig. 2B).

3.4. Results from patient urine samples

3.4.1. NMN and MN

The results for NMN and MN in the patient urine samples are presented in Table 3. As can be seen, notably lower concentrations for NMN were obtained with Method 2 than with Method 1, and the concentrations measured for MN were also lower

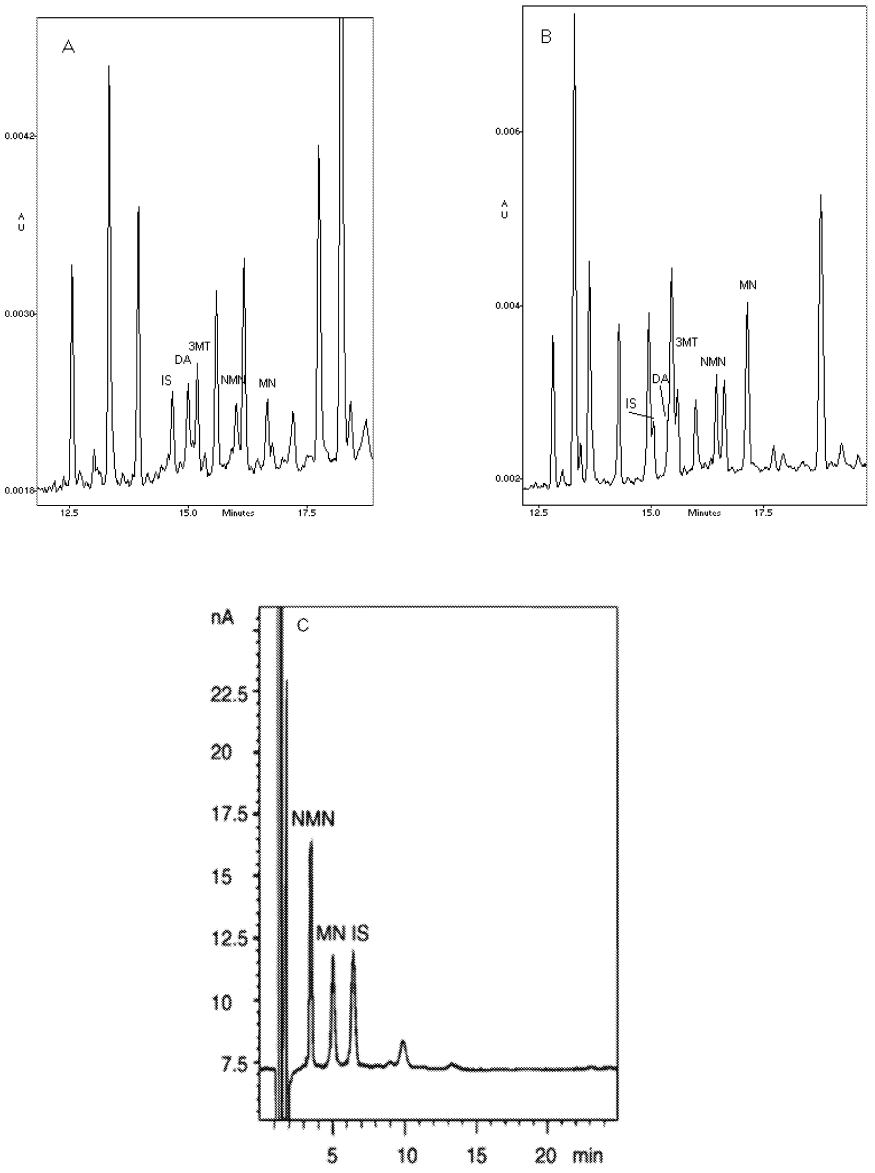

Fig. 1. Analysis of urine sample 5 by Method 2 (A), Method 3 (B) and Method 1 (C).

except for sample 3. Since good recoveries of NMN and MN were obtained in SPE method development [10], we directed our attention to the hydrolysis step and further to Method 3 where acid hydrolysis was combined with SPE purification and CE–UV analysis. Comparison of the results of Methods 2 (enzyme hydrolysis) and 3 (acid hydrolysis), both with SPE, showed that concentrations obtained for NMN and MN were clearly higher with Method 3 than with Method 2 indicating more efficient release of metanephrines from the conjugates with acid hydrolysis. In the case of NMN, the results obtained with Methods 1 and 3 were more similar than the results obtained with Methods 1 and 2. In the case of MN, however, the results with Method 3 were very high and the results were more similar with Methods 1 and 2. An explanation for the high values in Method 3 may be found in the recovery tests for the

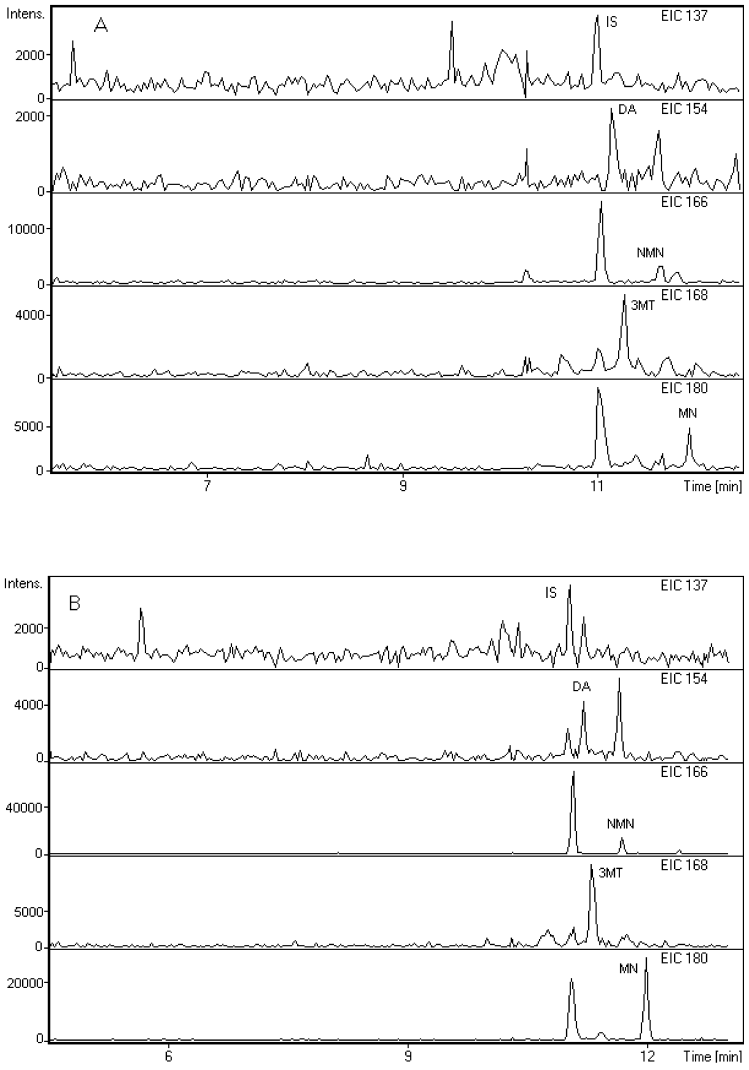

Fig. 2. Analysis of urine sample 5 by Method 5 (A) and by Method 6 (B).

methoxycatecholamines after acid hydrolysis in urine matrix, where the recovery of I.S. was 30% while the recovery of MN was almost 80%. The recoveries of NMN and I.S., in turn, were similarly low, and more similar results were thus obtained for NMN with Methods 3 and 1. When the quantitation was done instead with external standard (Method 4), the calculated concentrations of both NMN and MN were lower than with Method 3. However, a difference in the concentrations between Methods 1 and 4 persisted owing to the unequal recoveries in the two purification methods. Lower recoveries for methoxycatecholamines were obtained for spiked control samples in the sample sequence in this study (Table 5) than in earlier method development studies. In addition, the recoveries for I.S. were unrepeatable from sample to sample (Table 6).

All the results obtained for NMN with MS detection (Methods 5–7) are smaller than the results obtained with UV detection (Methods 2–4) except for sample 5 (Table 3). The higher concentrations for sample 5 are due to lower I.S. recovery with MS than with UV from this sample (Table 6). The recovery of I.S. in acid-hydrolyzed sample 5 with

Table 5
Recoveries (%) of spiked samples analyzed by CE–UV with SPE purification

	Recovery %				
	I.S.	DA	3MT	NMN	MN
Enzymatic hydrolysis					
I.S., 1.0 μmol/l	65	61	129	83	134
I.S., 5.0 μmol/l	72	51	130	86	120
E.S., 1.0 μmol/l	65	31	69	46	74
E.S., 5.0 μmol/l	72	35	83	53	75
Acid hydrolysis					
I.S., 1.0 μmol/l	33	21	367	107	282
I.S., 5.0 μmol/l	38	47	295	93	233
E.S., 1.0 μmol/l	33	3	106	30	80
E.S., 5.0 μmol/l	38	20	100	33	80

I.S., quantitation based on internal standard; E.S., quantitation based on external standard.

MS detection can be interpreted as the more reliable quantitation owing to absence of the matrix compound in the extracted ion electropherogram of I.S. (Fig. 2B), which was interfering in the quantitation with UV detection (Fig. 1B). The higher LOD for MN with CE–MS than with CE–UV explains the large number of ''not detected'' results.

3.4.2. DA and 3MT

DA is a catecholamine, while the other analytes are methoxycatecholamines. The recoveries of DA (Table 5) from the spiked samples are lower than those reported earlier [10]. Therefore, the DA concentrations obtained with Method 2 were assumed to be too low; the results with Method 3 were assumed to be slightly too high because the recovery of I.S. after acid hydrolysis was higher than that of DA and the results with Method 4 too low as poor recovery was obtained for DA after acid hydrolysis (Table 3). In particular, after acid hydrolysis, the electropherograms show a large matrix peak interfering with the quantitation of both I.S. and DA (Fig. 1B). The results for DA with CE–MS analysis are comparable with those with CE–UV analysis after enzymatic hydrolysis (Methods 2 and 5), likewise, the results obtained with Methods 4 and 7 after acid hydrolysis with external standard are comparable.

Table 6
I.S. recoveries (%) in patient urine samples analyzed, after enzymatic and acid hydrolyses, by CE–UV and CE–MS

Sample	Recovery %			
	CE–UV		CE–MS	
	Enzymatic	Acid	Enzymatic	Acid
1	92	33	82	63
2	100	75	103	65
3	42	6	47	nd
4	88	77	90	106
5	76	83	54	41
6	78	31	97	34

nd, not detected.

The highest recovery of the methoxycatecholamines from acidic medium was for 3MT. The recoveries of 3MT in spiked urine samples with external standard method were higher from acid-hydrolysed samples than from enzymatically hydrolysed samples (Table 5). Recoveries were increased with the use of I.S. quantitation with acid hydrolysis in Method 3 (Table 3). The results obtained with UV and MS detection after enzymatic hydrolysis (Methods 2 and 5) were very similar. However, the results are different after acid hydrolysis (Methods 3 and 6 as well as Methods 4 and 7), where the matrix compounds interfered in the quantitation of both I.S. and 3MT with UV detection (Fig. 1B).

4. Conclusions

Methods were compared for the analysis of dopamine and methoxycatecholamines in the complex matrix of urine. Variables in the methods were the hydrolysis, purification, separation and detection steps and quantitation with internal or external

standard. The many different variables gave rise to wide deviations in the final concentrations measured for the analytes.

Acid hydrolysis was more rapid and efficient in the deconjugation of methoxycatecholamines from sulfate conjugates than was enzymatic hydrolysis with *H. pomatia*. However, the recoveries of HMBA, DA, and NMN were poor after acid hydrolysis and SPE purification: 30, 13 and 26%, respectively. Concentrations of 3MT and MN, whose recoveries were considerably better (99 and 79%, respectively) were thus elevated in methods where HMBA was used as internal standard. In cross-check analysis, the CEX purification was found to be more efficient than polymer-based SPE in removing matrix compounds from the urine sample. However, the results showed that the purification methods developed for LC–EC and CE–UV could not be interchanged with other methods.

The sensitivity of detection was considerably poorer for CE–UV than for LC–EC analysis, owing to the lower injection volume and poorer sensitivity of the detector. In turn, the CE–MS method was 30-40% less sensitive than the CE–UV method. The CE–UV and CE–MS methods are nevertheless both sensitive enough for the determination of dopamine and methoxycatecholamines in healthy patient urine. Repeatabilities of retention times and migration times were good with all methods, and peak height and peak area repeatabilities were good for LC–EC and CE–UV, respectively. Only the peak area repeatability for CE–MS was poorer owing to variation in the ionization process. The LC–EC, CE–UV, and CE–MS analyses were comparable in terms of total run time, with 20, 23 and 18 min, respectively. The greater specificity of the EC and MS detections over UV detection was clear from the urine sample profiles, where more matrix compounds were seen in the electropherograms with UV detection. Matrix compounds interfered with the quantitation of I.S., DA and 3MT after acid hydrolysis.

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