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HPLC-FL/ED in the Analysis of Biogenic Amines and their Metabolites in Urine

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Abstract: The analysis of biogenic amine neurotransmitters (adrenaline, noradrenaline, dopamine, serotonin) and their metabolites (metanephrine, normetanephrine, 3,4-dihydroxyphenylacetic acid, vanillylmandelic acid, homovanillic acid, 5-hydroxyindoleacetic acid, 3,4-dihydroxyphenylglycol, 3-methoxy-4-hydroxyphenylglycol, 3-methoxytyramine) in body fluids may provide valuable information important in the diagnosis of the sympathoadrenomedullary system disturbances. An HPLC-FL/ED system using two Chromolith RP-18e columns and a mixture of acetate buffer (pH = 4.66) and methanol (97 : 3%, v/v) as the mobile phase was developed for the separation and determination of these compounds. Adrenaline, noradrenaline, dopamine and their synthetic analogs: dobutamine and isoproterenol, used in the treatment of clinical shock, were analyzed in urine of post-operative cardiosurgical patients and in urine of healthy controls. The extraction and pre-concentration step was performed on aluminium oxide SPE cartridges. Both detection techniques

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agreed well in final concentrations. Within-day coefficients of variation were below 2.3% and between-day coefficients of variation did not exceed 5%. The developed HPLC method is easy and reliable and could have a positive effect on the safety of severely ill patients treated with catecholamines and their synthetic analogues.

Keywords: Biogenic amine neurotransmitters, HPLC-FL/ED, SPE, urine

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INTRODUCTION

Biogenic amines (adrenaline, noradrenaline, dopamine, serotonin) regulate the sympathoadrenomedullary system. Catecholamines: adrenaline, noradrenaline, dopamine and their synthetic analogs: dobutamine and isoproterenol are usually used in the treatment of clinical shock (1). As large differences in metabolism rates are observed between individuals, monitoring of levels of these compounds in body fluids can aid in the treatment of the patients. The compounds of interest and their metabolites are usually analysed with the use of RP-HPLC methods either with fluorimetric (2–6) or electrochemical detection (7–11). Two detector systems have already been applied in the analysis of catecholamines but the analysis of these compounds together with their metabolites has not been widely investigated in such systems (12–14). Besides, most of the published one-detector methods are focused on the analysis of monoamines, their acidic metabolites and metanephrines (15, 16). Only Cao et al. (17) described a HPLC-ED method for simultaneous determination of these compounds together with their glycol metabolites. The above procedure required preparation and use of three mobile phases and analysis time of 80 minutes.

Previously we elaborated gradient HPLC-FL systems for the simultaneous determination of biogenic amine neurotransmitters, their acidic, glycol metabolites and metanephrines (18). A Chromolith RP-18e stationary phase and mixtures of buffers (of different pH) with methanol as the mobile phase were used. The Chromolith RP-18e column proved to be very efficient for the separation of these compounds in urine and enabled working with lower pressure than these observed in the systems using standard particulate columns.

In this work we have developed an isocratic, simple, and a relatively short method for the analysis of the compounds of interest, using two Chromolith RP-18e columns and fluorescence and electrochemical detection. Biogenic amine neurotransmitters and their metabolites may be analysed in this system in less than 50 minutes. Urine samples of cardiosurgical patients (children) were analysed for therapeutic drug monitoring in post-operative treatment. Urine of healthy children was analysed for comparison purposes.

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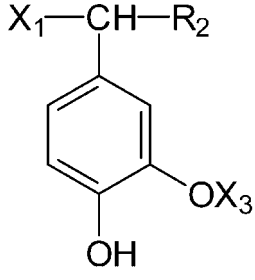
EXPERIMENTAL

Materials and Methods

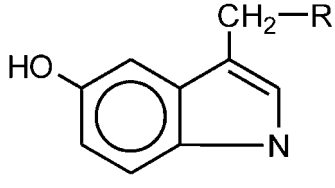
Standard solutions (1 mg/ml) in 0.1 M HCl were prepared of neurotransmitters (dopamine (DA), epinephrine (E), norepinephrine (NE), and serotonin (5HT)) and their metabolites: metanephrine (MN), normetanephrine (NMN), 3,4-dihydroxyphenylacetic acid (DOPAC), vanillylmandelic acid (VMA), homovanillic acid (HVA), 5-hydroxyindoleacetic acid (5HIAA), 3,4-dihydroxyphenylglycol (DHPG), 3-methoxy-4-hydroxyphenylglycol (MHPG), 3-methoxytyramine (3MT) (Sigma Chem. Co.)). The compounds of interest are presented in Fig. 1.



R_2 = CH_2-NH-X_2 (METHOXY)CATECHOLAMINES
X_1=H, X_2=H, X_3=H - DOPAMINE (DA)
X_1=OH, X_2=CH_3, X_3=H - ADRENALINE (E)
X_1=OH, X_2=H, X_3=H - NORADRENALINE (NE)
X_1=H, X_2=H, X_3=CH_3 - 3-METHOXYTYRAMINE (3MT)
X_1=OH, X_2=CH_3, X_3=CH_3 - METANEPHRINE (MN)
X_1=OH, X_2=H, X_3=CH_3 - NORMETANEPHRINE (NMN)

R_2 = COOH CARBOXYLIC ACIDS
X_1=OH, X_3=CH_3 - VANILLYLMANDELIC ACID (VMA)
X_1=H, X_3=CH_3 - HOMOVANILLIC ACID (HVA)
X_1=H,X_3=H - 3,4-DIHYDROXYPHENYLACETIC ACID (DOPAC)

R_2 = CH_2-OH GLYCOLS
X_1=OH, X_3=H - 3,4-DIHYDROXYPHENYLETHYLENEGLYCOL (DHPG)
X_1=OH, X_3=CH_3 - 3-METHOXY-4-HYDROXYPHENYLETHYLENE GLYCOL (MHPG)



R = CH_2NH_2 - SEROTONIN (5HT)
R = COOH - 5-HYDROXYINDOLEACETIC ACID (5HIAA)

Figure 1. Biogenic amine neurotransmitters and their metabolites.

High-Performance Liquid Chromatography (HPLC)

A Merck-Hitachi L4500 A chromatograph equipped with two Chromolith RP-18e, 100-4.6 columns (Merck, Darmstadt, Germany), an FL detector (excitation and emission wavelengths: $\lambda_{ex} = 285$ nm, $\lambda_{em} = 315$ nm) and an ED detector (10 nA, +0.6 V and +0.8 V), was applied to the HPLC investigations. Acetate (pH = 4.66) buffer solution with methanol (Merck, Darmstadt, Germany) were used as the components of the mobile phase. The measurements were performed at column temperature of 14°C.

Sample Collection and Preparation for HPLC

During the clinical phase of the study the catecholamines and their metabolites concentrations were measured in 24 h urine samples (250–2000 ml) collected from Cardiosurgical Postoperative Intensive Care Unit patients (children between 2 months and 11 years). Thirteen patients were treated with single agents (patients 1–9, 13) and the combinations (patients 10–12) of: three endogenous catecholamines: dopamine, epinephrine, and norepinephrine and two synthetic compounds: dobutamine and isoproterenol. Nine patients were treated with dopamine because of systemic hypotension and low cardiac output state after cardiac surgery (patient 1–9). One patient was on isoproterenol due to bradycardia (patient 13). Three critically ill ones were on combinations of dopamine and dobutamine (patient 10), epinephrine (patient 11) or norepinephrine (patient 12) when septic shock or cardiogenic shock were of concern. Three patients were not treated with catecholamines and their concentrations in urine reflected their endogenous source (patients 14–16). pH of fresh urine samples was adjusted to 8.6 with 2 M ammonium hydroxide. BAKERBOND spe Alumina (Al_2O_3) columns (3 ml/500 mg) were conditioned with 2 × 3 ml methanol, followed by 2 × 3 ml borate buffer, pH 9. After adding 2 ml of the urine sample, the columns were washed with 2 × 3 ml HPLC grade water and aired dry under vacuum for 2 minutes.

Biogenic amine neurotransmitters and their metabolites were eluted with 2 × 0.5 ml 1 M acetic acid, vortexed to mix and 20 μl injected into HPLC for analysis.

Urine samples spiked with standards were treated with the same procedure. BAKERBOND spe-12G system (J.T. Baker Inc.) and Refrigerated Universal Centrifuge Z 323 K (Hermle Labortechnik GmbH, Germany) were employed during the sample preparation.

Method Validation

Within-run precision and accuracy was calculated from repeated analysis (n = 3) of urine spiked with standards during one working day.

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Between-day precision and accuracy was calculated from repeated analysis of urine spiked with standards on five consecutive working days.

The efficiency of the SPE procedures was evaluated by comparing peak area ratios of the compounds with and without extraction.

Calibration curves as peak area [AU] vs. standard concentrations [ng/ml] were obtained with the use of least-squares linear regression method.

The limit of detection (LOD) was based on signal-to-noise ratio of 3 : 1 at the baseline.

RESULTS AND DISCUSSION

In this work we developed an isocratic HPLC-FL-ED system for the analysis of biogenic amines, their acidic and glycol metabolites and metanephrines. The method is simple (uses one mobile phase: acetate buffer (pH = 4.66) and methanol (97 : 3%, v/v), flow rate 0.8 ml/min, 14°C) and relatively rapid (50 min vs. earlier 80 min (17)). The use of two Chromolith RP-18e columns enables efficient separation of the compounds of interest at lower working pressures than while using microparticulate columns. Application of two detector systems: fluorescence and electrochemical enables the evaluation of peak purity and possible interferences. The chromatogram of the compounds in the developed system is presented in Fig. 2.

Urine samples of post-operative cardiosurgical patients were analysed for therapeutic drug monitoring in post-operative treatment. Urine of healthy children was analysed for comparison purposes. As the aim of this analysis was the determination of catecholamine (adrenaline, noradrenaline, dopamine) levels, extraction and pre-concentration of the compounds of interest from urine was performed on aluminium oxide SPE cartridges.

The presence of DA, MHPG, NMN, E, VMA, DHPG, NE, and DOPAC in the urine has been confirmed through the analysis of urine spiked with standards. Spiking random urine samples with standards of 2 μg/ml gave recovery of 90.2; 75.3; 76.1; 90.0; 71.3; 88.4, 91.3, and 92.4 for DA, MHPG, NMN, E, VMA, DHPG, NE, and DOPAC, respectively. Within-day coefficients of variation were below 2.3% and between-day coefficients of variation did not exceed 5%. The slope and intercept of the regression equations, linearity range, and limits of detection obtained for both detectors are presented in Table 1. As expected, the electrochemical detector provided significantly lower detection limits for the compounds of interest. Application of two detectors enabled identity assurance for the analysed compounds. Both techniques agreed well in final concentrations. An exemplary chromatogram of the urine sample is presented in Fig. 3.

The concentrations of the catecholamines and their metabolites determined in the urine samples of Cardiosurgical Postoperative Intensive Care Unit patients are presented in Table 2.

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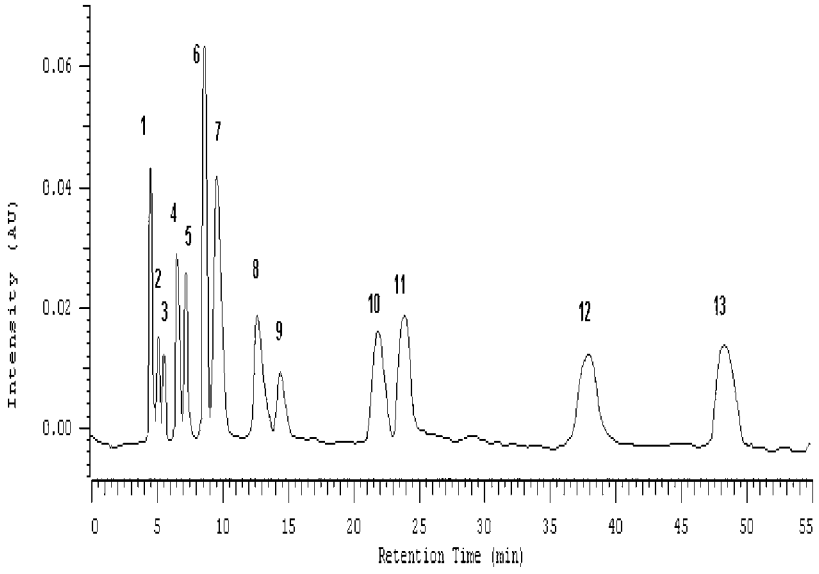

Figure 2. Chromatogram of biogenic amines and their metabolites in the isocratic HPLC system described in the text. Under these conditions the retention times of the compounds of interest are [min]: **1**–NE (FL = 4.55, ED = −), **2**–VMA (FL = 5.09, ED = 5.25), **3**–E (FL = 5.48, ED = 5.63), **4**–DHPG (FL = 6.54, ED = 6.70), **5**–NMN (FL = 7.25, ED = 7.40), **6**–DA (FL = 8.65, ED = 8.89), **7**–MN (FL = 9.56, ED = 9.82), **8**–MHPG (FL = 12.63, ED = 12.87), **9**–DOPAC (FL = 14.30, ED = 14.51), **10**–5HT (FL = 21.67, ED = 21.95), **11**–3MT (FL = 23.96, ED = 24.17), **12**–5HIAA (FL = 37.99, ED = 38.17), **13**–VA (FL = 48.14, ED = 48.40).

Depending on the clinical condition of the patients, various doses of catecholamines were involved and the clinical improvement varied significantly too. Generally speaking, the concentrations measured in urine were concordant with drug doses (patients 1–3, 5, 7, 9, 11–16). In some cases however, neither expected clinical improvement nor concentrations measured in urine samples correlated strongly with the doses of pharmacological agents used (patients 4, 6, 8, 10). A possible explanation of the above observation may be the fact that the patients are simultaneously on a great endogenic catecholamine drive and the effects of the catecholamine drugs must be added to the effects of the endogenic ones. Another problem is that antecedent congestive heart failure and sustained use of catecholamines by these patients can lead to a down regulation of their receptors in human body and therefore decrease the efficacy of the treatment. In these situations the possibility of measuring the concentrations of catecholamines and their metabolites in urine seems to be of a paramount importance. A physician that cares for the patient, when no expected response to the drug is

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Table 1. Data on regression equations for determination of biogenic amines and their metabolites

	Slope	Intercept	r	Concentration range [ng/ml]	LOD [ng/ml]
a) Fluorescence detector					
NE	2680.2 ± 23.7	1083.0 ± 59.5	0.999	25–2000	8
E	4513.1 ± 230.7	−4218.0 ± 1485.8	0.999	25–2000	10
DA	3260.0 ± 53.1	−876.2 ± 175.9	0.999	25–4000	8
NMN	7055.6 ± 230.8	−6972.3 ± 867.4	0.999	25–2000	10
DHPG	2560.6 ± 79.4	3291.2 ± 633.9	0.999	25–2000	8
MHPG	3605.9 ± 151.6	−2450.7 ± 569.5	0.999	25–2000	10
VMA	449.2 ± 8.4	506.9 ± 65.2	0.999	25–2000	15
DOPAC	1033.7 ± 38.9	4949.0 ± 1784.3	0.998	25–2000	10
b) Electrochemical detector					
NE	61430.9 ± 2981.1	−33070.4 ± 11202.1	0.999	1–2000	0.2
E	50005.5 ± 3394.1	−119741.4 ± 21863.0	0.998	1–2000	0.5
DA	56365.7 ± 3748.3	−25.2 ± 14085.1	0.998	1–2000	0.1
NMN	48450.5 ± 1949.8	−41314.3 ± 7326.9	0.999	1–2000	0.2
DHPG	23654.4 ± 860.0	63417.0 ± 5539.6	0.999	1–2000	0.1
MHPG	33689.9 ± 2161.2	−19914.3 ± 8121.2	0.998	1–2000	0.2
VMA	5377.1 ± 231.0	−16986.1 ± 1989.2	0.999	1–2000	0.5
DOPAC	44005.1 ± 856.2	37276.7 ± 1608.7	0.999	1–2000	0.1

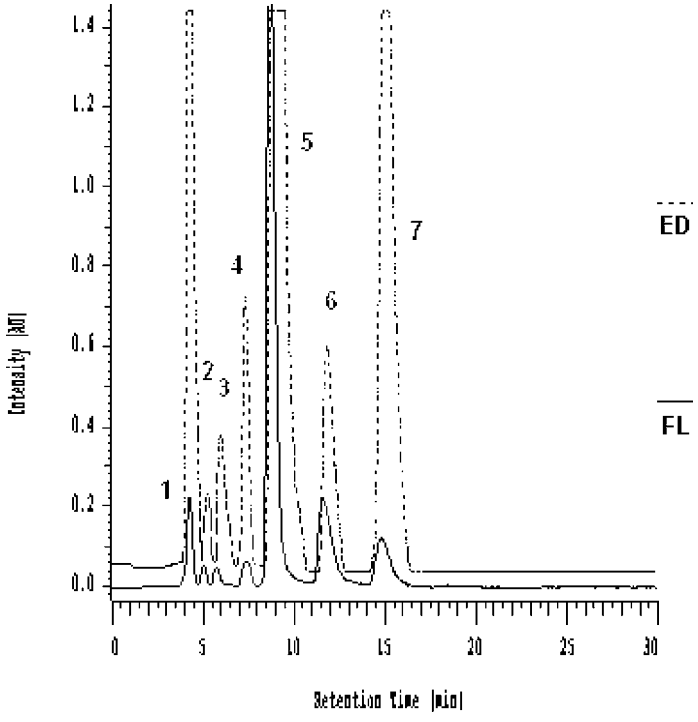

Figure 3. Chromatogram of urine sample in the isocratic system described in the text. Retention times of the determined compounds [min]: **1**–NE (FL = 4.29, ED = −), **2**–VMA (FL = 5.04, ED = 5.24), **3**–E (FL = 5.63, ED = 5.88), **4**–NMN (FL = 7.19, ED = 7.34), **5**–DA (FL = 8.71, ED = 8.86), **6**–MHPG (FL = 11.57, ED = 11.83), **7**–DOPAC (FL = 14.79, ED = 14.93).

achieved, tends to increase the drug dose. It may have negative consequences when severe adverse effects of the drugs appear. With the access to the information that high levels of drugs in urine were achieved the physician is expected to choose another alternative drug rather, than face the risk of dose related adverse effects.

It can be observed in Table 2 that some variation in the excreted metabolites between patients given the same agent—dopamine is present. It may be partly explained by the fact that the expected effects of catecholamines are dose related, although it should be emphasized that the actual response of an individual patient will largely depend on his/her clinical state at the time the drug is administered. The renal, liver, neurological, and haemodynamic condition of the patients may differ significantly. Since dopamine is metabolized in the liver, the kidney and plasma by monoamine oxidase and catechol-O-methyltranspherase, to the inactive compounds, and about 1/4 of the dose is taken up into specialized neurosecretory vesicles, where it is

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Table 2. Concentrations of catecholamines and their metabolites found in the analyzed urine samples related to the children's disorders and age

Medicine dosage sample [μg/kg/min]	Disorder child's age	Compound concentration in urine [μmol/24 h]							
		DA	MHPG	NMN	E	VMA	DHPG	NE	DOPAC
(1) 3 DA	Complete correction of ventricular septal defect 6 months	10.1	1.7	—	—	—	1.2	—	—
(2) 3.5 DA	Pulmonary artery banding in ventricular septal defect 2 months	7.0	1.0	2.4	0.7	—	—	—	3.4
(3) 5 DA	Pulmonary artery debanding and complete correction of ventricular septal defect 13 months	24.5	3.1	—	—	9.2	—	—	2.1
(4) 5 DA	Complete correction of ventricular septal defect 6 months	0.4	1.4	—	—	—	—	—	—
(5) 5 DA	Complete correction of atrioventricular septal defect 5 months	23.7	3.1	1.1	0.6	—	2.5	0.8	4.7
(6) 5 DA	Complete correction of atrial septal defect sinus venosus and partially anomalous pulmonary venous connection 5 years	42.2	2.0	1.1	0.6	9.3	1.2	1.0	2.9
(7) 10 DA	Tetralogy of Fallot—complete correction 1 year	6.0	17.5	5.7	—	16.5	1.9	—	—

(*continued*)

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Table 2. Continued

Medicine dosage sample [μg/kg/min]	Disorder child's age	Compound concentration in urine [μmol/24 h]							
		DA	MHPG	NMN	E	VMA	DHPG	NE	DOPAC
(8) 10 DA	Complete correction of atrioventricular septal defect 4 months	3.3	1.1	1.3	0.4	—	5.6	1.0	4.1
(9) 10 DA	Tricuspid atresia-total cavopulmonary connection 11 years	12.3	1.0	1.5	—	18.1	3.2	1.0	1.9
(10) 10 DA; 10 DB	Cardiogenic shock in end stage cardiomyopathy 10 years	1.1	1.1	2.9	0.7	—	3.0	—	—
(11) 10 DA; 0.2 E	Cardiogenic shock after tetralogy of Fallot correction 10 months	50.2	5.5	0.9	1.1	19.4	—	5.2	11.3
(12) 10 DA; 10 DB; 0.1 NE	Septic shock after total cavopulmonary connection in hypoplastic left heart syndrome 2 years	6.2	1.8	1.0	0.5	—	—	1.1	3.3
(13) 0.05 iP	Complete heart block 2 years	—	1.5	—	0.1	—	—	—	—
(14)	Healthy control 7 years	3.3	1.3	—	—	7.3	—	—	—
(15)	Healthy control 11 years	1.9	1.2	1.0	—	—	—	—	—
(16)	Healthy control 10 years	1.4	1.1	—	—	—	—	—	—

hydroxylated to norepinephrine—the differences in the functions of the above mentioned organs may considerably influence the final urine concentrations of the analyzed compounds.

The above data suggests that an easy and reliable HPLC method on urine catecholamine levels could have a positive effect on the safety of treatment of severely ill patients like the ones treated in Cardiosurgical Postoperative Intensive Care Units.

The HPLC procedure, together with another sample preparation method, may also be applied for the analysis of other biogenic amines and their metabolites in body fluids.

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