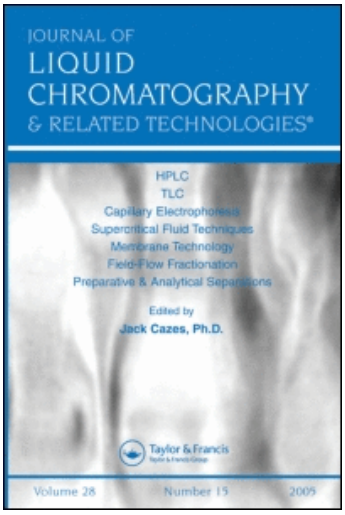

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Simultaneous Determination of Free Polyamines, Catecholamines and Metanephrines in Plasma and Urine

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Abstract: We describe a new, rapid, and sensitive HPLC method with fluorescent detection for simultaneous analysis of plasma and urine free catecholamines, metanephrines, and polyamines without prior treatment procedures via precolumn derivatization with Fmoc-Cl. The separation gradient was 40 minutes. The results showed good linearity across the calibration range 0.05–20 μM and linearity coefficient (r^2) higher than 0.990, except for epinephrine, $r^2 = 0.985$. The LOQ was in range 680–1300 fmol and LOD 204–390 fmol of these analytes per injection 20 microlitres. The reproducibility of the method for the analyzed samples, expressed as R.S.D. was from 1.2 to 4.5%. Isoproterenol was used as internal standard.

Keywords: Catecholamines, Fmoc-Cl, Metanephrines, Plasma, Polyamines, Urine.

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INTRODUCTION

Catecholamines (CA)-dopamine (D), norepinephrine (NE) and epinephrine (E), and polyamines (PA)-spermin (Spm), spermidin (Spd), diamines putrescin (Put) and cadaverin (Cad), are biologically active substances that have various regulatory functions in cells. Catecholamines and polyamines are synthesized in cells from respective amino acid precursors. For a long time, the monitoring of polyamine and catecholamine, their conjugates and metabolites, has been used as clinical biochemical markers for different diseases. Till now there are no data for direct relation between catecholamines and polyamines levels in cells, although increased levels of both types of substances are found in a number of diseases.

A broad spectrum of analytic methods for determining concentrations of different classes of substances is used in analytical and clinical chemistry. Diagnostic significance of concentration levels of these substances in plasma, urine and tissue samples has been undoubtedly demonstrated.[1,2]

Norepinephrine and epinephrine are neurotransmitters, closely related with the etiology of different physiologic and neurodegenerative diseases of the central nervous system. Diseases in which CA and MN are significantly elevated include Pheochromocytoma, Parkinson's Disease, Alzheimer, Neuroblastoma, Ganglioneuroblastoma, Von Hippel Lindau disease (VHL), Baroreflex Failure, Dopamine-β-hydroxylase deficiency (DBH), Chemodectina (Nonchromaffin paraganglioma), Familial Paraganglioma Sindrome, Genetic Deficiencies of Monoamine Oxidase (MAO Deficiencies), and Multiple endocrine neoplasia (MEN).[2,3] This gives rise to the significance of determining the levels of catecholamines and their metabolites, e.g., normetaepinephrine (NME) and metaepinephrine (ME), in tissue samples, serum, and urine, which can serve as biochemical markers for a number of diseases (Pheochromocytoma, Parkinson's Disease, Alzheimer, and others).[4–6]

It was found that measurement of plasma and urine free metanephrines could provide information about phenotype, size, and location of the pheochromocytoma. Furthermore, increases in plasma free MN can be useful in guiding further diagnostic decision making.[7]

The Pheochromocytoma (tumour that affects the suprarenal gland) is another example where pathologically increased levels of CA and metanephrines (MN), accompanied with higher blood pressure, are observed. Early laboratory diagnosis of this disease is of current interest due to autopsy studies revealing that the disease is not detected during a lifetime in 25–75% of those afflicted.[8]

Polyamines participate in eukaryotic and prokaryotic cells and have various functions in them. It is found that polyamines and

their metabolites take part in the processes of cell multiplication and regulation. Intensively growing tissues usually have increased polyamine levels, which have a stimulating effect on DNA, RNA, and the protein synthesis in cells. A reverse effect also is found; significant reduction in polyamine levels is associated with delay in cellular growth. Data exist that polyamines perform functions of growth factors, antioxidants, metabolite regulators, and membrane stabilizators, DNA, RNA, and protein structures.

It is found that the concentration of polyamines (PA) and their metabolites in biological fluids (urine, plasma) and tissues is significantly increased in patients with cancer diseases. On the other hand, decreasing of that concentration directly reflects the effectiveness of the applied therapy. In other words, polyamine levels in biosamples can be used as a marker for the presence of cancer diseases and for the effectiveness of the applied therapy.[1–9]

Various methods have been described for determination of catecholamines, metanephrines, and polyamines, namely high performance liquid chromatography (HPLC) with electrochemical or fluorescent detection.[10–12] and capillary electrophoresis.[1] Of these methods, HPLC with electrochemical detection has been most used for quantization of catecholamines.[13,14] HPLC with fluorescent detection is widely applied for determination of polyamines levels.

Since polyamines, catecholamines, and metanephrines have no appropriate chromophore groups for direct registration, they are derivatized via appropriate chromophores for analysis at low concentrations.

Orthophthal aldehyde, benzoil chloride, danzil chloride, 9-fluoreniloxycarbonile chloride, benzilamine, 1,2-diphenilethilendiamine, and others are used as derivatization agents.[10,15–18]

9-Fluoreniloxycarbonile chloride reacts intensively with primary and secondary amino groups, as well as with hydroxyl groups. Moreover, derivatives that are stable acidic and neutral medium are formed. The stability of Fmoc-derivatized catecholamines, metanephrines, and polyamines were described.[18,19]

This paper describes a simple HPLC fluorescent assay for the simultaneous determination of free polyamines, catecholamines, and metanephrines in plasma and urine after pre-column derivatization with Fmoc-Cl. The separation of the analyzed components was done within the gradient of 40 minutes by means of three column system.

Till now, there is no developed method for simultaneous HPLC separation of CA, PA, and MN with consequent fluorescence detection and, till now, there is no method for HPLC separation of CA, PA, and MN in urine and plasma samples without a prior treatment procedure.

The method that we propose for simultaneous analysis of CA, PA, and MN is especially appropriate for early diagnostics of diseases, where changes in concentrations of these substances are expected to occur.

EXPERIMENTAL

Materials

Catecholamines and metanephrines, necessary for the analysis, were purchased from Fluka (Switzerland). Polyamines were purchased from Sigma (Germany). The internal standard 1-[3′, 4′-Dihydroxyphenyl]-2-isopropylethanol (Isoproterenol) and Fmoc-Cl were obtained from Sigma (Germany). The acetonitril for liquid chromatography (super gradient) was purchased from Sigma (Germany) and DBA (dibutylamine) from Merck (Germany). We purified water used for preparing buffers with the Purelab UHQ II system (ELGA Vivendi Water System, Netherland).

Urine and plasma samples were obtained from patients at the University Hospital for active treatment of endocrinology "Acad. Ivan Penchev"after their whitten informed consent was obtained. The study was approved by the Ethics committee of the Medical University-Sofia.

Standards

Standard solutions of polyamines and catecholamines with a concentration of 1.0 mM were prepared in 0.1 M HCI. Prepared solutions were kept at a temperature of 4°C. All calibration and working solutions were prepared from the initial standard solutions via dilution with 0.1 M HCI.

Sample Preparation

Urine and plasma samples were kept at −70°C and were unfrozen immediately prior to the process of derivatization.

Derivatization

To a 50 μL sample was added 5 μL internal standard solution (containing 0.1 mM Isoproterenol) in an "ependorf" tube with a volume of 1.5 mL. After that were added 150 μL derivatization buffer (0.5 M $NaHCO_3/Na_2CO_3$ with $pH = 10.2$) and 300 μL Fmoc-Cl dissolved

in acetone. Fmoc-Cl, 5 mM, was used for derivatization of standard solutions, 35 mM Fmoc-Cl–for urine samples, and 75 mM Fmoc-Cl–for plasma samples. The mixture was vortexed well and then was incubated at room temperature for 30 min on a test rotator (Snijders, Tilburg, the Netherlands). The process of derivatization was interrupted by addition of 50 μL concentrated HCI. Derivatized samples were centrifuged for 5 min, at 13000 rpm. From the supernatant of each sample, 20 μL was used for analysis. The derivatized samples were kept at 4°C.

HPLC Analysis

Equipment

All the experiments were carried out on a chromatographic system using a pump from Thermo Finingan (model P2000), with Rheodyne (Germany) injector loupe 20 μL and a fluorescent detector (Hewlett-Packard, model 1046A), with extinction and emission wavelength 262 nm and 615 nm, respectively. Data were collected and processed by chromatographic software-CSW 1.7 (Data Apex, Chech Republic).

Chromatographic Conditions

The process of polyamines and catecholamines separation took place via a system of three consecutive bound short columns with the following characteristics: Nucleodur C_{18}, 50 mm × 4.6 mm, 5 μm size of the filling particles, and 100 Å size of the pores (Macherey-Nagel, Germany).

The flow rate during separation of the Fmoc-Cl derivatives of polyamine, metanephrine, and catecholamine, was 1.5 ml min^{-1}. Eluent A consisted of 10 mM ammonium acetate, 3 mM dibutylamine, 5% acetonitril, with pH 4.20, prepared by adjusting with 50% solution of trifluoroacetic acid. Eluent B was a mixture of 95/5% (v/v) acetonitril and phase A. Both phases were filtrated via a 0.45 μm nylon membrane (Hewlett-Packard) and degassed in a vacuum ultrasonic bath. The gradient of the mobile phase is shown in Table 1. The analyses were carried out at room temperature.

RESULTS AND DISCUSSION

Derivatization

The purpose of our assay was based on development of simultaneous determination of free catecholamine, metanephrines, and polyamines in plasma and urine samples without prior treatment procedures.

Table 1. Chromatographic gradient conditions for analysis of polyamine, catecholamine and metanephrine

Time (min)	Eluent A (%)	Eluent B (%)
0	44	56
2	44	56
8	29	71
11	29	71
14	23	77
17	21	79
18	18	82
35	0	100
38	0	100
40	44	56

When choosing a derivatization reagent, we explored derivatization via 9-fluorenylmethoxycarbonyl chloride (Fmoc-Cl). It was confirmed that by use of Fmoc-Cl, metanephrines are di- and catecholamine are tri-Fmoc derivatives, which have a higher fluorescent signal.[18] Therefore, we ceased our choice of derivatization reagent for Fmoc-Cl, although more side effects were likely to take place with it.

In urine and plasma samples, there is the presence of a lot of compounds, such as metabolites of catecholamine, metanephrines and polyamines, amino acids and peptides, which may also react with Fmoc-Cl.

Those were the reasons which necessitated the selection of optimum conditions for the process of derivatization.

To optimize the sample condition for preparation, series of experiments with 5 mM Fmoc-Cl were carried out with varying times for incubation (5–60 min). By this concentration of Fmoc-Cl, derivatization was not fully completed even after 60 min. The reaction was carried out at room and elevated (up to 50°C) temperatures for different durations of time and in an ultrasonic bath. Use of ultrasonic baths leads to more side products. The process of derivatization did not proceed efficiently. Therefore, it was necessary to optimize the concentration of Fmoc-C1.

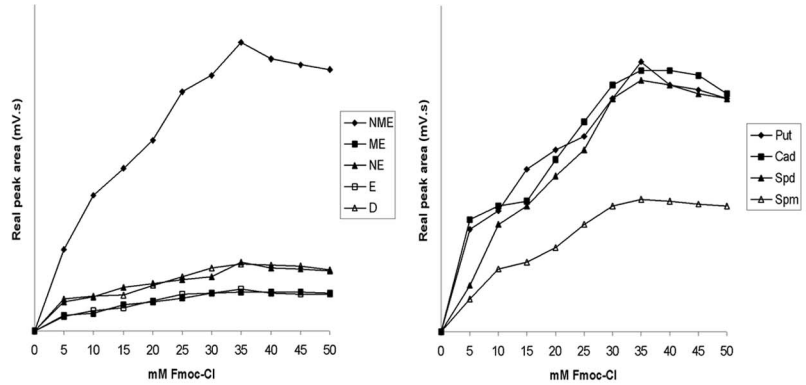
Urine Samples

In order to optimize the derivatization reagent concentration during the analysis of urine samples, we explored the process of derivatization at concentrations from 5 mM to 50 mM Fmoc-Cl (in steps of 5). The mixture was vortexed well and then was incubated at room

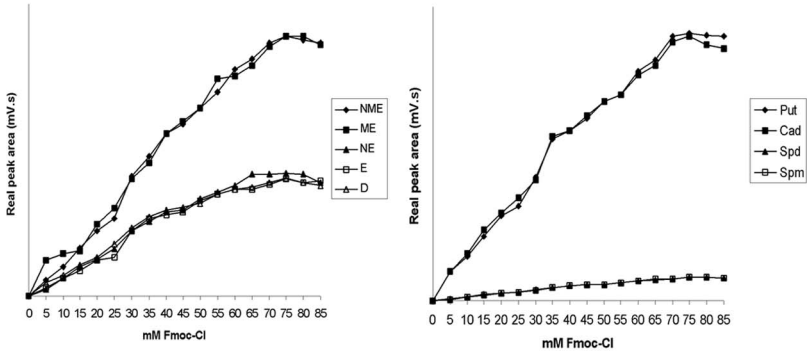
temperature for 30 min on a test rotator. The process of derivatization was interrupted by adding 50 μL concentrated HCI. Derivatized samples were centrifuged for 5 min, at 13000 rpm. From the supernatant of each sample, 20 μL was used for analysis. Maximum peak heights were attained at 35 mM Fmoc-Cl. Results are shown in Figure 1a.

Plasma Samples

To optimize derivatization reagent concentration during the analysis of plasma samples, we explored the process of derivatization at concentrations from 5 mM to 85 mM Fmoc-Cl (in steps of 5).



(a)



(b)

Figure 1. Effect of Fmoc Cl concentration on derivatization of NE, E, D, NME, ME, Put, Cad, Spd, Spm, and Isoproterenol (IS) in urine and plasma samples.

The mixture was vortexed well and then was incubated at room temperature for 30 min on a test rotator. The process of derivatization was interrupted by adding 50 μL concentrated HCI. Derivatized samples were centrifuged for 5 min, at 13000 rpm. From the supernatant of each sample, 20 μL was used for analysis. It was found that 75 mM Fmoc-Cl resulted in the highest signal intensities of Fmoc-derivatives. Results are shown in Figure 1b.

At lower concentrations, enough derivatization reagent was used to allow the explored components to react fully. Also, at lower concentrations, the derivatization process ran at a lower rate, and catecholamines and metanephrines that were unstable in alkaline medium would have to be submitted to the longer impact of the derivatization buffer (pH = 10.2).

Validation Characteristics

We explored the reproducibility of developed methods via 8 different derivatizations of one and the same sample. We explored derivatization of standard polyamine, catecholamine, and metanephrine solutions at concentrations of 1 μM and of one urine sample and one plasma sample. In Tables 2 and 3 we show data for the relative standard deviations, linearity, and sensibility of Fmoc-derivatives. Relative standard deviations showed good reproducibility of the derivatization process.

Linear calibration was acquired for polyamines, catecholamines, and metanephrines solutions with known concentrations, from 0.05 to

Table 2. Relative standard deviation (%) of peak area for Fmoc derivatives of polyamine, catecholamine and metanephrine

	1 μM ($n = 8$)	Urine ($n = 8$)	Plasma ($n = 8$)
Put	1.3	1.4	1.5
NME	1.5	1.7	2.0
Cad	1.4	1.4	1.8
ME	2.4	3.0	3.5
NE	2.0	1.9	1.9
Spd	2.0	2.0	2.1
E	3.5	3.0	3.9
D	2.4	4.0	4.1
Spm	4.5	3.0	4.0

Table 3. Linearity and sensibility of Fmoc- derivatives of polyamine, catecholamine and metanephrine

	Linearity		Sensibility	
	$Y = ax$	R^2	LOQ fmol	LOD fmol
Put	$Y = 0.229x$	0.9972	680	204
NME	$Y = 0.168x$	0.9984	920	276
Cad	$Y = 0.233x$	0.9968	740	222
ME	$Y = 0.168x$	0.9962	960	288
NE	$Y = 0.210x$	0.9974	860	258
Spd	$Y = 0.318x$	0.9970	800	240
E	$Y = 0.197x$	0.9944	1100	330
D	$Y = 0.212x$	0.9990	960	288
Spm	$Y = 0.340x$	0.9974	1300	390

20 μM (0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20). The linearity coefficient (R^2) was higher than 0.994 in the range 0.05–20 μM.

As internal standard, we chose isoproterenol for several reasons. On the one hand, it has analogous structure to catecholamines and metanephrines, and therefore, it would be derivatized in a similar way. On the other hand, it has high correlation ratio $R^2 = 0.9970$ (commensurable with that of the explored components) within the concentration interval from 0.05 to 20 μM.

In Figures 2 and 3, we have shown the application of the described methods for analysis of polyamines, catecholamines, and metanephrines with standard solutions, urine, and plasma samples.

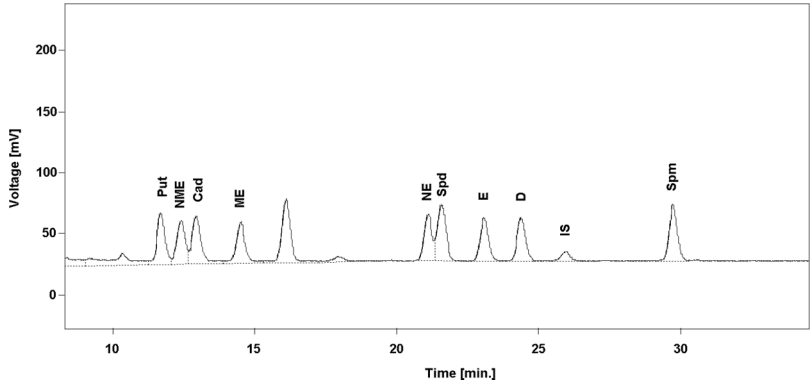


Figure 2. Chromatogram of 5 μM standart solution of CA, MN, and PA with internal standard (IS).

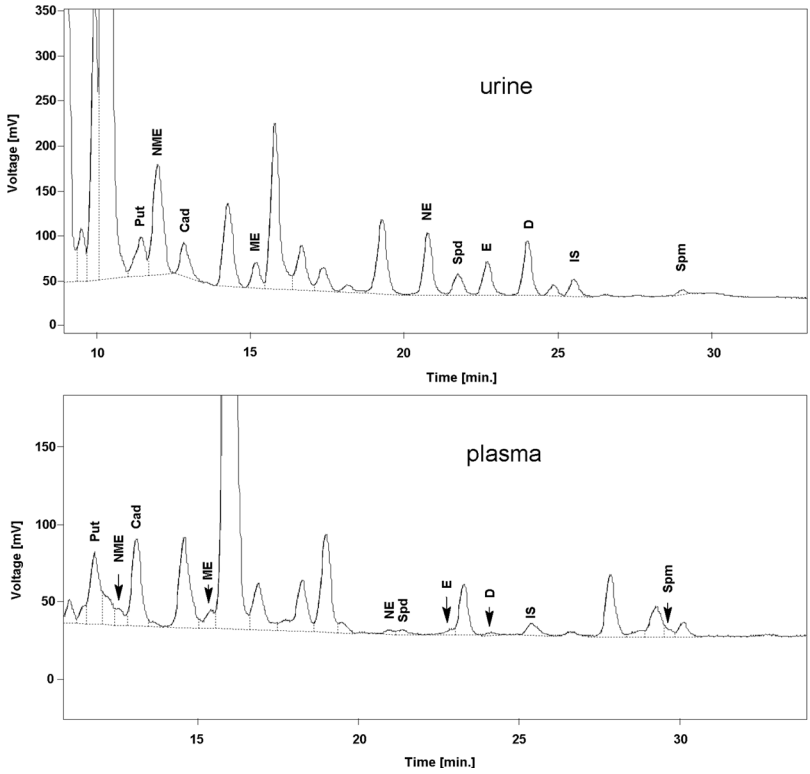


Figure 3. Chromatogram of real samples of urine and plasma with internal standard (IS).

Table 4. Concentrations of CA, MN and PA in plasma and urine samples ($nM.ml^{-1}$)

		Incidentaloma		Pheochomocytoma	
	Controls plasma ($n = 40$)	plasma ($n = 15$)	urine ($n = 7$)	plasma ($n = 15$)	urine ($n = 11$)
NME	0.02 – 5.02	4.03 – 8.42	3.01 – 9.35	7.67 – 11.98	14.03 – 28.81
ME	0.01 – 5.44	3.82 – 10.01	4.03 – 8.67	9.02 – 17.97	10.58 – 27.44
NE	0.07 – 3.06	0.10 – 2.52	0.08 – 1.82	0.15 – 7.02	2.21 – 7.77
E	0.07 – 1.23	0.09 – 3.03	1.68 – 2.41	0.17 – 3.97	4.01 – 7.62
D	0.05 – 0.27	0.03 – 1.42	1.10 – 1.57	0.06 – 3.81	1.38 – 4.46
Put	1.02 – 8.08	3.40 – 10.00	0.39 – 3.73	2.84 – 14.77	7.50 – 15.01
Cad	1.64 – 8.23	7.64 – 12.55	4.81 – 9.04	8.81 – 25.00	9.62 – 13.00
Spd	0.21 – 2.24	0.58 – 2.19	0.11 – 0.72	1.02 – 4.76	0.02 – 1.02
Spm	1.80 – 4.21	2.59 – 7.21	0.10 – 1.30	3.03 – 6.21	1.06 – 1.79

Application

This method was used in our laboratory for analysis of urine and plasma samples in 70 patients (40 healthy volunteers, 15 patients with incidentaloma-a tumor, found by coincidence without clinical symptoms and suspicion, and 15 cases of pheochromocytoma). Results are shown in Table 4.

The method that we have developed could provide information for early diagnostics of Pheochromocytoma, as well as for exploration of postoperative recovery of patients with that disease.

CONCLUSION

In conclusion, we have developed a HPLC method with fluorescent detection for determining of polyamines and catecholamines (epinephrine, norepinephrine) and their 3-O-methylized metabolites (metanephrine and normetanephrine) in plasma and urine. That method is characterized with high sensitivity, selectivity, and comparative simplicity, when measuring concentrations of catecholamines and metanephrines.

This method permits a selective, simple, and highly sensitive determination of urinary and plasma concentrations of free polyamines, catecholamines, and metanephrines. Moreover, the measuring of plasma NMN and MN also allows not only the identification of pheochromocytoma but make it possible to provide information about adrenomedullary hypofunction.[8]

This method could also be a reliable marker for the detection of catecholamine secreting tumours.

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