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Liquid chromatography method for simultaneous analysis of amino acids and biogenic amines in biological fluids with simultaneous gradient of pH and acetonitrile

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Abstract

A liquid chromatography method for simultaneous analysis of amino acids, polyamines, catecholeamines and metanephrines in human body fluids after derivatization with 9-fluorenylmethyloxycarbonyl chloride was developed. The chromatographic behavior of analytes at different pH of mobile phase was studied. Successful baseline resolution of all analyzed compounds was achieved using simultaneous gradient of pH and organic modifier in reverse phase mode of HPLC within 36 min. The repeatability of the proposed procedure in respect of retention time and peak area, expressed as RSD, ranges from 0.06 to 1.64% and 0.4 to 7.6%, respectively. The method linearity in the range of 1–200 μM for amino acids and in the range of 0.1–20 μM for polyamines, catecholeamines and metanephrines was found to be with correlation coefficients higher than 0.994. The limit of quantification (LOQ) was assessed to be in the range of 2.6–10 pmol for amino acids and 2–4 pmol for polyamines, catecholeamines and metanephrines.

Keywords: Liquid chromatography; Amino acids; Polyamines; Catecholeamines; Metanephrines; Fmoc

1. Introduction

Quantification of amino acids (AA), polyamines (PA) and catecholamines (CA) in biological fluids such as plasma, urine and cerebrospinal fluid has an important diagnostic implication in medicine.

The amino acid content of plasma and urine is expected to reflect the nutritional and metabolic status; the individual amino acid profile of biological fluid is of importance in confirming or other wise to rule out the suspected amino acidopathies [1].

Polyamines and catecholamines are members of a broad group of the so-called biogenic amines.

Polyamines – putrescine (Put), spermidine (Spd) and spermine (Spm) are polycationic compounds ubiquitous for all living organisms. They are essential for cell growth and differentiation and their intracellular concentrations increase during periods of rapid cell proliferation [2]. PAs are involved in the control of cell cycle progress and apoptosis [3]. Moreover, several lines of evidence suggest that PAs are also involved in cancer cells growth [4].

Catecholamines – epinephrine (E), norepinephrine (NE) and dopamine (D) containing an amino function on a side chain together with the catechol function (i.e. hydroxyl groups at 3- and 4-positions on a benzene ring) play an important role in the nervous system [5]. Catecholamines act via dopaminergic and adrenergic receptors, and are involved in the regulation of the response to stress, psychomotor activity, emotional processes, learning, sleep, and memory [6,7].

Metanephrines – metanephrine (MN) and normetaneprine (NMN) are *O*-methylated metabolites of catecholamines. NMN and MN have proved to be particularly important in the diagnosis of pheochromocytoma [8].

The most common analytical techniques used for determination of amino acids and biogenic amines in biological samples

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are HPLC and capillary electrophoresis (CE), combined with different detection techniques – spectrometric, fluorometric, electrochemical and mass spectrometric. During the last few years several reviews of methods for analysis of AA and biogenic amines were published [9,10,5]. There are several methods for simultaneous analysis of AA and PA [11–15]. During our literature survey we found only one published method for simultaneous detection of polyamines and catecholamines in cell extracts by CE [16]. However, according to our knowledge simultaneous determination of amino acids, polyamines and catecholamines in biological samples has never been demonstrated up to date.

In view of the diagnostic significance of all these compounds, a simultaneous method for their determination and quantification is of practical interest.

9-Fluorenylmethyloxycarbonyl chloride (Fmoc-Cl) is widely used as derivatizing agent for amino acid and polyamine analysis [17–20]. Fmoc-Cl was also successfully used for the determination of CA and MN in human urine samples [21,22]. The Fmoc-derivatives are stable at room temperature in acidic conditions [12,21], not susceptible to major matrix interference reactions, and able to be detected in low amounts.

The aim of the presented study was to find chromatographic conditions allowing quantification of Fmoc – derivatives of diagnostically important compounds in human body fluids. All analyzed components exhibit different properties: (i) strongly ionic AA – His, Glu, Asn, Arg, Ser, Asp and Glu; (ii) uncharged AA as Ala, Val, Phe, Ile and Leu; (iii) Di-Fmoc substituted relatively hydrophobic – Lys, Orn, Tyr; (iv) very hydrophobic di- and three-substituted derivatives of PA and CA, respectively with different chromatographic behavior. Thus, the selection of the mobile phase properties was crucial in order to create a useful and simple method for their separation. In this paper we present a method based on the simultaneous increase of organic modifier and pH during linear gradient elution for successful separation and quantification of the compounds of interest. As eluents, acidic (A with pH = 2.1) and basic (B with pH = 8.4) ammonium acetate buffers containing different amounts of dibutylamine were used. The gradual increase of pH of mobile phase was achieved with perchloric acid that also acts as an ion-pairing agent.

The linearity, reproducibility and recovery of the method were studied and were found to be satisfactory.

The method was examined for its application to human plasma and urine samples.

2. Experimental

2.1. Materials

Standard analytical kit, containing 21 amino acids, 2-aminoheptanoic acid and Fmoc-Cl were obtained from Fluka (Buchs, Switzerland). Putrescine, cadaverine, spermine, spermidine, epinephrine, dopamine, norepinephrine, metanephrine and normetanephrine were purchased from Sigma (Germany). The acetonitrile of gradient grade was obtained from Sigma (Germany). Dibutylamine and perchloric acid were purchased from Merck (Germany). The water used for buffer preparations was purified on Purelab UHQ II system (ELGA, Vivendi Water Systems, Netherlands). All other reagents were of the highest purity available.

2.2. Standards and samples

0.1 mM stock standard solutions of AA, PA, CA and MN were prepared in 0.1 N HCl and stored at 4 °C. Working standard solutions at different concentrations were prepared from the stock solution by appropriate mixing and diluting with 0.1 N HCl.

Samples (human plasma and urine) used during these study were disposed from Clinical laboratory of Department of Urology at Medical University of Sofia.

2.3. Sample preparation

2.3.1. Extraction and protein precipitation

A 100 μl volume of sample (plasma, urine or standard solution) was mixed with 30 μl 2.0 M $HClO_4$ and an aliquot of internal standard (IS) solution (generally we used 2.5 μl of 2.0 mM 2-aminoheptanoic acid) in a 500 μl well-capped centrifuge type tube. The mixture was thoroughly vortexed for few seconds and incubated for 10 min at room temperature and then centrifuged at 13,000 × *g* for 12 min. An aliquot of 50 μl of the supernatant was used for derivatization.

2.3.2. Derivatization

An aliquot of 50 μl of the supernatant was added to 80 μl of 0.5 M sodium hydrogen carbonate/sodium carbonate buffer with pH = 10.2 in 500 μl well-capped reaction vessels. Then 150 μl of 5 mM Fmoc-Cl solution in acetone was added and allowed to react at 40 °C for 10 min (The resulting pH of the reaction mixture was in the range of 8–8.5 depending on the sample's nature). The reaction was quenched by addition of 10 μl conc. HCl. The acidified samples were kept at 4 °C and 20 μl aliquots were injected for analysis.

2.4. HPLC analysis

2.4.1. Equipment

The analyses were carried out on HPLC system consisting of P2000 binary gradient pump (ThermoSystem, USA), HP 4926A fluorescent detector (Hewlett Packart, Germany) and 20 μl fixed loop manual injector model 7125NS (Rheodyne, USA). The fluorescence of Fmoc derivatives was measured using 262 nm for execution and 615 nm of emission wavelengths. Data acquisition and presiding was achieved with CW 1.7 chromatographic software (DataApex, Czech Republic).

2.4.2. Preparation of elution buffers

Buffer A was a mixture of 20 mM ammonium acetate and acetonitrile (95/5%, v/v) containing 1 mM dibutylamine. The buffer pH was adjusted to 2.1 with 35% perchloric acid on pH meter (Schott, Germany) equipped with SenTix 41 electrode (WTW, Germany).

Table 1
Chromatographic gradient condition.

Time (min)	Eluent A (%)	Eluent B (%)	pH[a]
0	74	26	2.4
5	69	31	2.5
15	63	37	3.6
16	52	48	4.3
21	44	56	4.8
25	32	68	5.4
27	14	86	5.8
30	0	100	6.2
36	0	100	8.4
38	74	26	2.4
43	74	26	2.4

[a] Reported pH values were measured on output of column in100 μl aliquot using pH-meter equipped with microelectrode (Blue Line 16, WTW, Weilheim, Germany).

Buffer B was a mixture of 20 mM ammonium acetate and acetonitrile (90/10%, v/v) containing 5 mM dibutylamine. The resulting pH of the buffer was 8.4.

Both buffers were filtered on nylon 0.45 μm membrane and degassed in sonic bath under vacuum before use.

2.4.3. Chromatographic conditions

The separation of Fmoc-derivatives of AA, PA, MN and CA was achieved on Nucleodur 100-5 C_{18} ec (125 × 4) column (Masharey-Nagel, Germany) at flow rate of 1.5 ml min^{-1} using binary gradient elution. The gradients profile is shown in Table 1.

3. Result and discussion

3.1. Sample preparation

3.1.1. Extraction and protein precipitation

The quantitative analysis of amino acids and biogenic amines in complex matrices such as plasma or urine needs a special attention on the sample preparation and cleaning up procedure in order to obtain complete extraction of all analyzed components and removal of proteins and other sample components. The organic solvent or acid precipitation and solid-phase extractions or ultra filtration are often methods used for these purposes [23,24] All of them have some advantages and drawbacks. The acidic precipitation/extraction procedure seems to be the most promising method for complete recovery of low molecule weight compounds from complex matrices. In this study perchloric acid was used as precipitating agent. Series of experiments were carried out with different perchloric acid concentrations (from 0.5 M to 5 M), time for incubation (5–40 min.) and temperature (0–50 °C) to find the optimal sample preparation conditions (data not shown). The following conditions were selected as apparently best: 2.0 M perchloric acid at room temperature for 10 min.

3.1.2. Derivatization

The derivatization was carried out according to our previously reported procedure [3] with some modifications. As derivatization reagent Fmoc-Cl was used, because it reacts not only with amino groups but also with phenolic groups of catecholamines and methanephrines.

In order to achieve complete derivatization of CA and PA, different reaction time and temperatures were studied. The reaction was carried out at room and elevated (up to 60 °C) temperature for different periods of time. Temperatures over 50 °C caused evaporation of the acetone from the reaction mixture resulting in irreproducible sample preconcentration and respectively incorrect results. At room temperature incomplete derivatization of CA and Spm was observed even for 60 min. In addition, His and Tyr were represented with more than one peak on the chromatograms (data not shown). The reaction with Fmoc-Cl for 10 min at 40 °C was selected because of the complete derivatization of all components. We observed full hydrolysis of the excess derivatizing reagent. In these conditions single peaks for problematic amino acids His and Tyr were also presented on the chromatograms.

3.2. Chromatographic analysis. Design and optimization of the mobile phase

The Fmoc derivatives of PA and CA are hydrophobic and strongly retained compounds. We used DBA as mobile phase modifier to reduce their retention times and to achieve shorter analysis. The influence of different DBA concentration in the mobile phase has been studied. Series of experiments varying only the DBA concentration (from 1 mM to 10 mM, in step of 0.5 mM) was carried out. Concentrations above 5 mM DBA added to both eluents had favorable effect on the elution and separation of hydrophobic compounds but resulted in more rapid elution and peaks overlapping of more hydrophilic AA. The lower DBA concentration turned out to be insufficient for the elution of PA and CA. To overcome this problem, we utilized 1 mM DBA concentration in solvent A and 5 mM in solvent B so that during the mobile phase gradient all AA were separated and hydrophobic components were eluted at appropriate retention times without increasing the analysis time.

Next point of our investigations was the examination of the effect of mobile phase pH on the resolution. Firstly, we tried to find an acid that could be used for pH correction in broad interval without jumps. A number of acids were examined. Phosphoric and trifluoracetic acids were found to be unsuitable because slight concentration changes are accompanied with wide variations in pH. They could be used in limited pH intervals 1.8–2.6 and 3.8–4.6. The next studied perchloric acid turned out to be the most proper for our purposes. In buffers (ammonium acetate or citric acid) containing DBA, it allowed gradual correction of pH in broad pH interval from 2.0 to 8.0. Moreover, as it was reported recently perchloric ion performs superior properties as ion-pairing agent in comparison with other studied acids [25].

Secondly, the effect of pH on the separation was studied. We carried out series of analyses using the same gradient of acetonitrile and buffer composition varying only perchloric acid concentration, respectively pH of the mobile phase (A – 20 mM ammonium acetate containing 5% acetonitrile (v/v)

and 3 mM DBA with different pH adjusted with 35% $HClO_4$; B – A/acetotitrile 10/90%, v/v). The investigated range of pH was from 2.1 to 8.5. As expected the analysis of charged small molecules is conducted at around pH 4.0 of elution buffer; we investigated the performance of our analysis in additional narrow pH interval in 0.1 steps ranging from 3.8 to 4.6. The results indicated that these pH values of the mobile phase enabled good separation of the hydrophobic AA and relatively complete resolution of PA and CA but caused peak overlapping of the first-eluting charged amino acids. (Fig. 1b) Insignificant differences on the performance in this narrow pH interval were observed. Carrying out the chromatographic process at basic pH of the mobile phase, the separation of all amino acids was unsuccessful. The retention of hydrophobic PA and CA was not obviously different from that obtained at pH 4.0 (Fig. 1c). Baseline resolution and well-defined peaks for early-eluting hydrophilic amino acids were achieved at pH in range 2.1–2.6. However, this low pH of the elution solvent in comparison with more basic conditions markedly deteriorated the separation of strongly retained hydrophobic AA, especially of Phe, Ile and Leu. Under these acidic mobile phase conditions poor separation and overlapping peaks were also observed for PA and CA. Co-elution of NME and Cad as well as NE and Spd was found in the chromatogram (Fig. 1a).

On the basis of above described studies, we tried to provide the analysis varying pH of the mobile phase during gradient elution. For this purpose buffers with different pH were designed (A – pH 2.11, B – pH 8.4). To verify that during the organic solvent gradient, gradient increase of pH was also formed; we measured the pH of the mobile phase on the every step of the gradient program at the column outlet and inlet. This elution program allowed the pH of the elution solvent to be increased simultaneously with the gradient of the organic content. In this way the hydrophilic AA were eluted at low pH with well-defined and separated peaks. When their elution was completed, the pH of the eluent started to increase so that the hydrophobic AA, PA and CA were also well resolved because of the basic pH of the mobile phase. Moreover, the peaks of these compounds were more intensive at this pH because their Fmoc-derivatives according to our observation demonstrated higher quantum yield of fluorescence in basic media in comparison with this in acidic media. This is an important advantage of the method because PA and CA contents in biological matrices are negligible (Fig. 2).

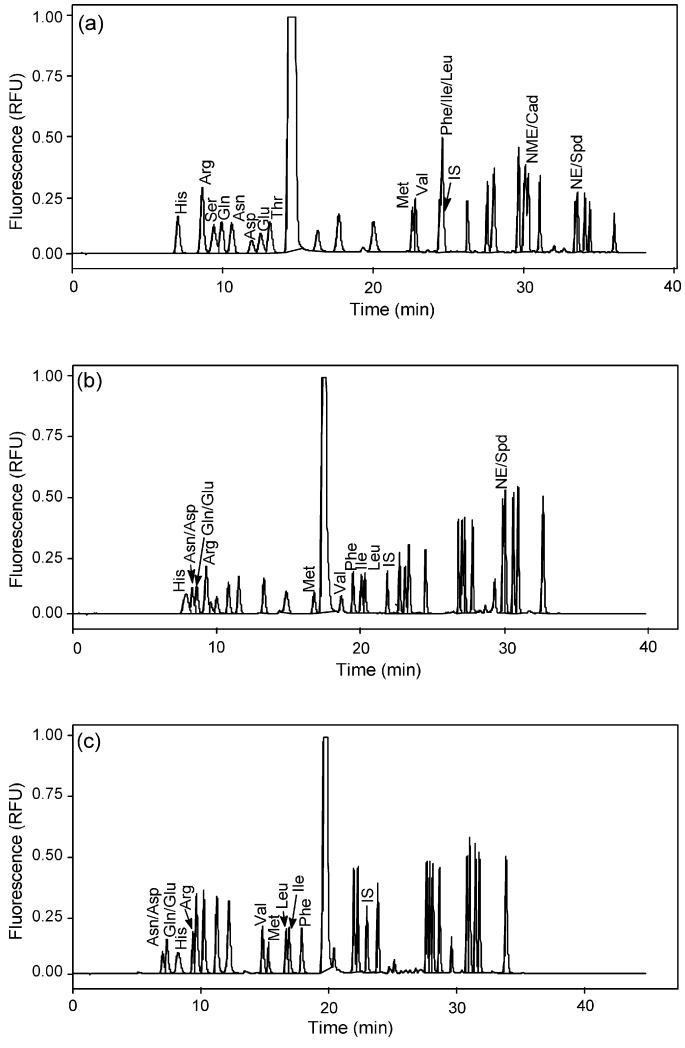

Fig. 1. Selected chromatograms obtained at different pH of mobile phase: (a) pH 2.8; (b) pH 4.6; (c) pH 7.4. Analyzed standard sample contains 65 pmol of each AA, PA, CA and MN per 20 μl injection. Eluent A – 20 mM ammonium acetate containing 5% acetonitrile (v/v) and 3 mM DBA, the pH value was adjusted with 35% $HClO_4$; B– A/acetotitrile 10/90%, v/v). Only selected derivatives are labeled for easy apprehending.

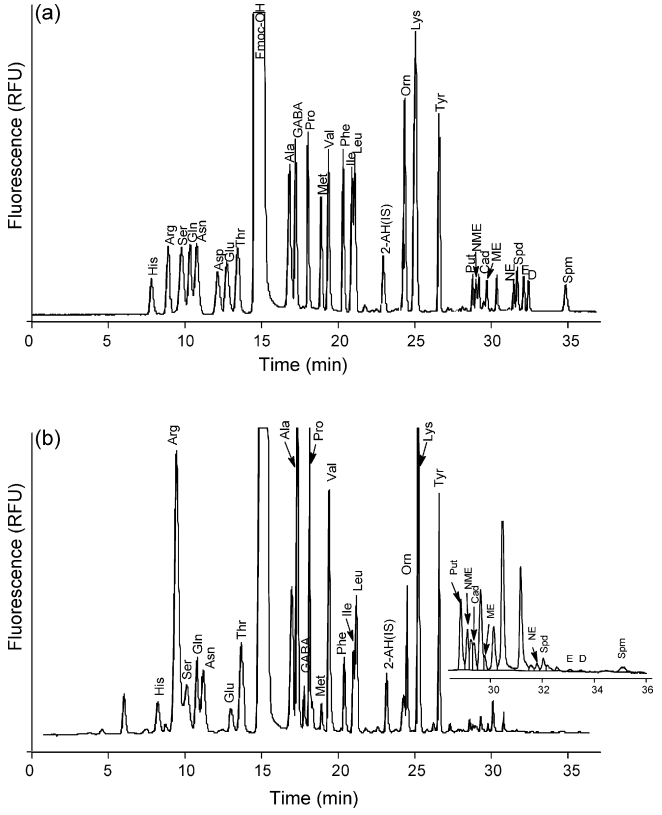

Fig. 2. Chromatograms of (a) standard sample contains 130 pmol of each AA and 5 pmol of each PA, CA and MN per 20 μl injection and real (b, plasma) samples under optimized gradient conditions (for details see text).

Table 2
Validation characteristic of analytical procedure

	Repeatability					Linearity[b]	Sensitivity	
	Retention time[a]			Peak area[a]		r^2	LOD (pmol)	LOQ (pmol)
	Time (min)	SD (min)	RSD (%)	Standard[c] RSD (%)	Plasma RSD (%)			
His	7.85	±0.13	1.64	4.62	7.46	0.974	6.92	14.10
Arg	8.94	±0.08	0.92	2.44	1.47	0.996	2.26	7.84
Ser	9.83	±0.13	1.29	2.16	1.38	0.998	1.90	6.26
Gln	10.36	±0.12	1.12	1.85	1.24	0.999	2.14	6.86
Asn	10.83	±0.07	0.63	2.46	2.06	0.999	2.16	6.62
Asp	12.19	±0.10	0.81	1.66	2.88	0.998	3.38	11.64
Glu	12.82	±0.08	0.66	3.28	1.01	0.998	2.23	8.20
Thr	13.49	±0.07	0.54	2.34	1.34	0.994	2.30	7.72
Ala	17.31	±0.04	0.22	1.34	1.06	0.999	1.66	5.76
Pro	18.13	±0.07	0.37	2.60	0.71	0.999	1.68	5.44
Met	18.95	±0.08	0.43	1.85	1.84	0.988	2.98	10.18
Val	19.47	±0.08	0.39	1.51	1.51	0.997	3.50	9.48
Phe	20.43	±0.08	0.40	2.49	2.01	0.999	1.76	5.78
Ile	20.97	±0.08	0.37	1.14	1.77	0.998	1.82	6.72
Leu	21.15	±0.08	0.38	3.12	0.40	0.998	1.44	5.24
Orn	24.42	±0.05	0.20	1.97	5.18	0.999	1.10	3.96
Lys	25.16	±0.15	0.58	2.74	0.78	0.994	0.56	2.46
Tyr	26.67	±0.17	0.62	3.58	4.62	0.998	2.40	5.10
Put	28.87	±0.04	0.15	3.44	3.54	0.999	0.96	3.16
NMN	29.13	±0.01	0.03	4.21	3.66	0.995	0.98	2.78
Cad	29.32	±0.04	0.13	4.39	3.44	0.996	0.84	2.72
MN	29.81	±0.05	0.16	2.60	5.52	0.997	1.08	3.48
NE	31.59	±0.03	0.09	4.10	7.49	0.999	1.26	4.12
Spd	31.81	±0.03	0.10	2.08	n.d.	0.999	0.86	2.64
E	32.22	±0.03	0.10	2.54	2.42	0.999	0.92	3.00
D	32.57	±0.03	0.09	3.34	4.96	0.999	1.28	3.60
Spm	34.99	±0.02	0.06	3.48	4.04	0.999	0.64	2.16

nd: not detected.
[a] Presented data are average values from five replicates.
[b] Based on the peak-area ratios between the components and IS.
[c] Standard sample was with concentration of 50 μM of each AA and 2 μM of each PA, CA and MN.

Furthermore, utilization of simultaneous gradient of pH and organic solvent allows Fmoc-OH peak to be tuned in position on chromatogram where it does not interfere with the resolution of any compounds of interest.

3.3. Method validation characteristics

All quantitative analyses were performed by internal standard procedure using 2-aminoheptanoic acid as internal standard.

The chromatographic conditions of our method include simultaneous gradient of organic solvent and pH; thus it is of particular interest to ascertain repeatability and reproducibility of the retention times. The obtained data is shown in Table 2. It is worth mentioning that used chromatographic equipment is without thermo conditioning.

The method linearity was tested in the range of 1–200 μM for AA and in the range of 0.1–20 μM for PA and CA using seven different concentrations with five replicates of each point. Calculations for the calibration graphs and regression equations were based on the peak-area ratios between the components and internal standard. The obtained r values were in range from 0.994 to 0.999 with exception of His and Met with $r = 0.974$ and $r = 0.988$, respectively (see Table 2).

The repeatability of the analytical procedure was measured using standard sample and human plasma sample. For each sample, five replicates of the analytical procedure were applied. The relative standard deviation (RSD%) was found to be in the range of 1.1–4.6% for standard and 0.4–7.6% for plasma sample as it is shown in Table 2.

The limits of detection (LOD) and quantification (LOQ) were calculated on the basis of calibration graphs using S/N = 3 and S/N = 10 ratio, respectively. As it could be seen in Table 2, LOQ amounts for CA and PA are below usually observed levels in plasma and urine samples.

The recovery study was carried out using real plasma sample by spiked techniques. PA and CA were examined because they are usually in low concentrations and are sensitive to acidic treatment. Three spiked levels were investigated (1 μmol, 2 μmol and 4 μmol). The obtained data (Table 3) show good recovery values for all PA and CA. The values for ME and NME are relatively low. This fact could be the result of susceptibility to acid treatment [26] or the influence of the sample matrices. Moreover, the investigated samples contained unknown com-

Table 3
Recovery data for polyamines, catecholamines and metanephrines

	Found concentration in sample[a] ($n = 5$)($\mu M L^{-1}$)	Added amount 1 μmol ($n = 5$)		Added amount 2 μmol ($n = 5$)		Added amount 4 μmol ($n = 5$)	
		Found ($\mu M L^{-1}$)	Recovery (%)	Found ($\mu M L^{-1}$)	Recovery (%)	Found ($\mu M L^{-1}$)	Recovery (%)
Put	4.03	5.06	101	6.12	104	8.01	99
Cad	1.24	1.97	88	3.02	89	4.68	86
Spd	0.25	1.19	95	2.07	91	3.68	92
Spm	0.36	1.33	98	2.23	94	4.21	96
NME	0.96	1.39	71	2.42	73	3.73	69
ME	0.51	1.10	73	2.07	78	3.31	70
NE	0.22	1.24	102	2.50	104	4.82	105
E	0.40	1.22	87	2.24	92	3.69	89
D	0.20	1.16	97	2.26	103	4.04	96

[a] The studied sample was from patient with diagnosis of carcinoma of bladder and was obtained before surgery intervention (stress conditions) so probably resulted in relatively high CA and MN levels.

pounds that partially overlapped the peaks of ME and NME (Fig. 2).

3.4. Method application

The applicability of the reported method for simultaneous quantification of AA, PA, CA and MN has been demonstrated for the analysis of human plasma and urine samples. The results of this survey were presented in separate form during poster session on 28th Congress of the Societe International d'Urologie [27].

4. Conclusion

A new chromatographic method for simultaneous determination of diagnostically important groups of compounds – amino acids, polyamines, catecholamines and metanephrines, in human body fluids is proposed.

The most important part of this study was the design of the mobile phase composition. Baseline resolution of almost all Fmoc derivatives was achieved by successful realization of simultaneous gradient of pH and organic solvent during the chromatographic analysis. This was feasible due to the combination of excellent properties of dibutylamine and perchloric acid as mobile phase modifier and ion-pairing agent.

According to our knowledge, such method that utilizes controlled formation of simultaneous gradient of organic solvent and pH during the chromatographic run has never been demonstrated in literature up to date. We believe that the proposed mobile phase design for formation of simultaneous gradient of pH and organic modifier should be practical for the analysis of complex samples containing different ionogenic compounds.

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