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Determination of polyamines in human urine by precolumn derivatization with benzoyl chloride and high-performance liquid chromatography coupled with Q-time-of-flight mass spectrometry

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ABSTRACT

A simple and sensitive HPLC/Q-TOF MS method for simultaneous determination of 1,3-diaminopropane, putrescine, cadaverine, spermidine, spermine and acetyl-spermine in human urine was developed in electrospray-ionization source by positive ion mode. The samples were firstly pretreated by 10% $HClO_4$ and then derivatized by benzoyl chloride with 1,6-diaminohexane as internal standard. The derived polyamines were separated on a C_{18} column by a gradient elution with methanol–water, and then sensitively detected with Q-TOF MS. The limits of detection for polyamines ranged from 0.02 to 1.0 ng ml^{-1} with excellent linearity within the range from 1 to 1000 ng ml^{-1} except acetyl-spermine from 5 to 1000 ng ml^{-1}. The intra- and inter-day R.S.D. for all polyamines were 2.0–14.7% and 3.9–12.9%, respectively. The method was applied to determine the polyamines in human urine from 10 cancer patients and 15 healthy volunteers. Results showed that the mean levels of polyamines in urine of patients were all higher than those in healthy volunteers. The cluster analysis was used to establish the distinction mode between cancer sufferers and healthy individuals.

1. Introduction

Polyamines are the generic aliphatic compounds possessing two or more primary and secondary amino groups in their long chain, mainly including 1,3-diaminopropane (DAP), putrescine (PUT), cadaverine (CAD), spermidine (SPD), spermine (SPM) and acetyl-spermine (AcSPM). Polyamines as cations at physiological pH values can interact electrostatically with anionic macromolecules like DNA, RNA, acid phospholipids and proteins. Therefore, polyamines play an important role in growth and proliferation of cell, synthesis of proteins and nucleic acids. In 1971, Russell et al. reported the importance of polyamines in cancer diagnosis for the first time [1]. After that, a great number of literatures demonstrated that the polyamines' levels in body tissue and fluid correlated closely with cancer [2–7]. So the simultaneous determination of polyamines has become an important task for cancer diagnosis and antitumor drug monitoring, particularly in the study of metabonomics related with polyamines and cancer [8,9]. Thus, a sensitive analytical method for quantitative analysis of each polyamine in biological samples is prerequisite for the further investigation of the application of polyamines *in vivo*.

For the pre-treatment of biological specimens, many methods had been applied to separate polyamines from the endogenous substances including solid phase extraction (SPE), single hollow fiber supported liquid membrane (SLM) extraction and ion exchange resin procedure [10,11]. Unfortunately, these pre-treatment procedures were technically demanding and time consuming, so we sought to develop a simple, efficient and sensitive method in this study. The derivative procedure was selected since polyamines do not contain a suitable chromophore or fluorophore group and posses the properties of low volatility and low molecular weight. Among the numerous derivative reagents, benzoyl chloride has been widely adopted for its easy obtaining and high activity in acylate reaction. For example, Asotra et al. derived polyamines with benzoyl chloride in vertebrate and invertebrate tissues (mouse and sea star) [12], while Özdestan and Uren [13] and Dadáková et al. [14] used the same derivatization reagent for the determination of organic amines in wine and food samples, respectively. In order to detect the trace concentration of polyamines in biological specimens, many chromatographic methods had been used, such as HPLC separation with fluorometric detection, GC and HPLC separation with MS detection [10,15–18]. Although these methods had been applied to determine polyamines in some biological specimens, the simultaneous determination of very low concentration polyamines in complex biological samples without other biogenic amines or endogenous interfering was still a problem.

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Q-TOF mass spectrometry had been employed in comprehensive analysis of complex samples for its high resolution and accurate detection [19–22]. The coupling of HPLC and Q-TOF MS could acquire abundant data of analytes and extract the target ions from the total ion chromatogram (TIC) even they were totally hidden. Thus, by easy pre-treatment the polyamines in biological specimens could be analyzed selectively and accurately, which greatly saved the analytical time and made it possible to determine the low concentrations polyamines in biological samples. To the best of our knowledge, the method described for the simultaneous determination of these six polyamines in human urine had not been reported.

Here we described a new method of determining polyamines in human urine using benzoyl derivatization by HPLC/Q-TOF MS detection. It was applied to measure polyamines from 25 people including 10 cancer patients and 15 healthy volunteers. Based on contents of the polyamines, the difference between cancer sufferers and healthy individuals was investigated in this study by cluster analysis since the cluster analysis is more visible and distinct than other multivariate data classification [23–26].

2. Materials and methods

2.1. Chemicals, reagents and materials

DAP, PUT, SPM, 1,6-diaminohexane (used as an internal standard), CAD hydrochloride, SPD hydrochloride, AcSPM hydrochloride and benzoyl chloride were obtained from Sigma–Aldrich (St. Louis, MO, USA). Methanol and acetonitrile of HPLC grade were purchased from Fisher Chemicals (Fair Lawn, NJ, USA). All the other reagents were of analytic grade. Distilled and deionized water was used throughout the experiment.

The urine samples were collected from 15 healthy volunteers and 10 cancer patients. Fifteen healthy volunteers were from the authors' university and 10 patients were collected from a cancer hospital in China (from February 2010 to April 2010). Diagnoses of cancer were made on the basis of usual clinical and laboratory results and confirmed by tissue biopsy.

2.2. Preparation of standard and calibration solutions

Standards of polyamines were prepared for each analytical run. A stock solution was diluted in methanol to make a working mixture of 1 $\mu g\,ml^{-1}$. This working mixture was used to prepare calibration solutions with the ranging concentrations of 1, 5, 10, 50, 100, 500 and 1000 $ng\,ml^{-1}$ except that acetyl-spermine was from 5 to 1000 $ng\,ml^{-1}$. Then concentration of internal standard (IS) was 100 $ng\,ml^{-1}$. All solutions were stored at 4 °C prior to use.

2.3. Preparation of urine sample

0.5 ml IS solution (100 $ng\,ml^{-1}$) and 0.3 ml 10% perchloric acid were added to 0.5 ml urine, then mixed thoroughly for 3 min and centrifugated at 15,000 rpm for 5 min. The supernatant liquid was transferred into another clean tube and submitted to polyamines' derivatization procedure.

2.4. Derivatization procedure

The perchloric supernatants was alkalinized by adding 2 $mol\,ml^{-1}$ NaOH solution, followed by 250 μl 4% benzoyl chloride diluted with acetonitrile. After standing for 20 min under ultrasonication at 30 °C, excess benzoyl was removed by mixing with the equal volume of saturated sodium chloride solution. Polyamine derivatives were extracted into diethyl ether, followed by evaporating to dryness under nitrogen. The residue was reconstituted in 0.1 ml methanol, and the 20 μl supernatant was injected for HPLC/Q-TOF MS analysis.

2.5. Instrumentation and chromatographic conditions

HPLC/Q-TOF MS analytical procedures were performed on a Bruker Daltonics micrOTOF-Q mass spectrometer (Billerica, MA, USA) with an Agilent 1200 HPLC system. The software micrOTOF control was applied to system operation and date collection. Derivative polyamines were separated on a Kromasil ODS C_{18} column (5 μm, 250 mm × 4.6 mm id) held at 35 °C. The column was eluted with a gradient mixture of methanol (phase A) and water (phase B) at the flow rate of 1 $ml\,min^{-1}$. The gradient program was as follows: 55–74% A from 0 to 14 min and 74% A from 14 to 24 min. Q-TOF MS was operated in the positive ion mode with an electrospray-ionization source (ESI^+). The capillary voltage was set at 4500 V. Nitrogen was used as the desolvation and nebulizing gas. The desolvation gas temperature of 230 °C and the flow of 7.0 $l\,min^{-1}$ were employed. The nebulizer pressure was maintained at 1.3 bar. The analysis data was collected in full scan mode from m/z 50 to m/z 900.

2.6. Validation of the method

To evaluate linearity, seven calibration solutions were prepared and assayed together with spiked amount of IS (100 $ng\,ml^{-1}$) in triplicate by derivative procedure as described above. The LOD was assessed by determining the concentration of polyamine at varying concentrations from 0.01 to 1 $ng\,ml^{-1}$. For intra- and inter-day assays, the samples were prepared individually in triplicate at low (50 $ng\,ml^{-1}$), medium (200 $ng\,ml^{-1}$) and high (1000 $ng\,ml^{-1}$) concentration in methanol. Likewise, the recovery was determined at three concentrations by comparing the recovered polyamine concentrations with the nominal concentrations. In order to evaluate the matrix effect on the ionization of analytes, i.e., the potential ion suppression or enhancement due to the matrix components, three concentration levels of polyamines were tested. The matrix effect was evaluated by comparing the peak area ratio of post-spiked derivative polyamines standards in the urinary derivatives and IS to that of the neat standard solutions. Stability was tested by analyzing the sample at 200 $ng\,ml^{-1}$. The samples were examined at 0, 2, 4, 8, 12 and 24 h after derivatization. During the analysis the samples were stored at 4 °C.

3. Results and discussion

3.1. Derivatization of polyamines

A standard solution of seven polyamines including IS (10 $mg\,ml^{-1}$ each, 10 μl) was used to investigate the optimum derivatization conditions. During the procedure, alkaline medium was necessary since hydrogen ion was continuously created during the acylate reaction of polyamines and benzoyl chloride. So the spiked volume of NaOH was of importance and examined first. In order to remove excess benzoyl chloride, saturated sodium chloride solution was then added. The peak areas of all polyamines reached maximum when the amount of derivative reagent was above 50 times of polyamines. The effect of the reaction time on benzoyl reaction was also tested at various temperatures from the room temperature to 50 °C. Various organic solvents, such as diethyl ether, acetidin, n-butyl alcohol and n-hexane were evaluated. Finally, diethyl ether was found to be optimal for yielding the highest recovery for polyamines.

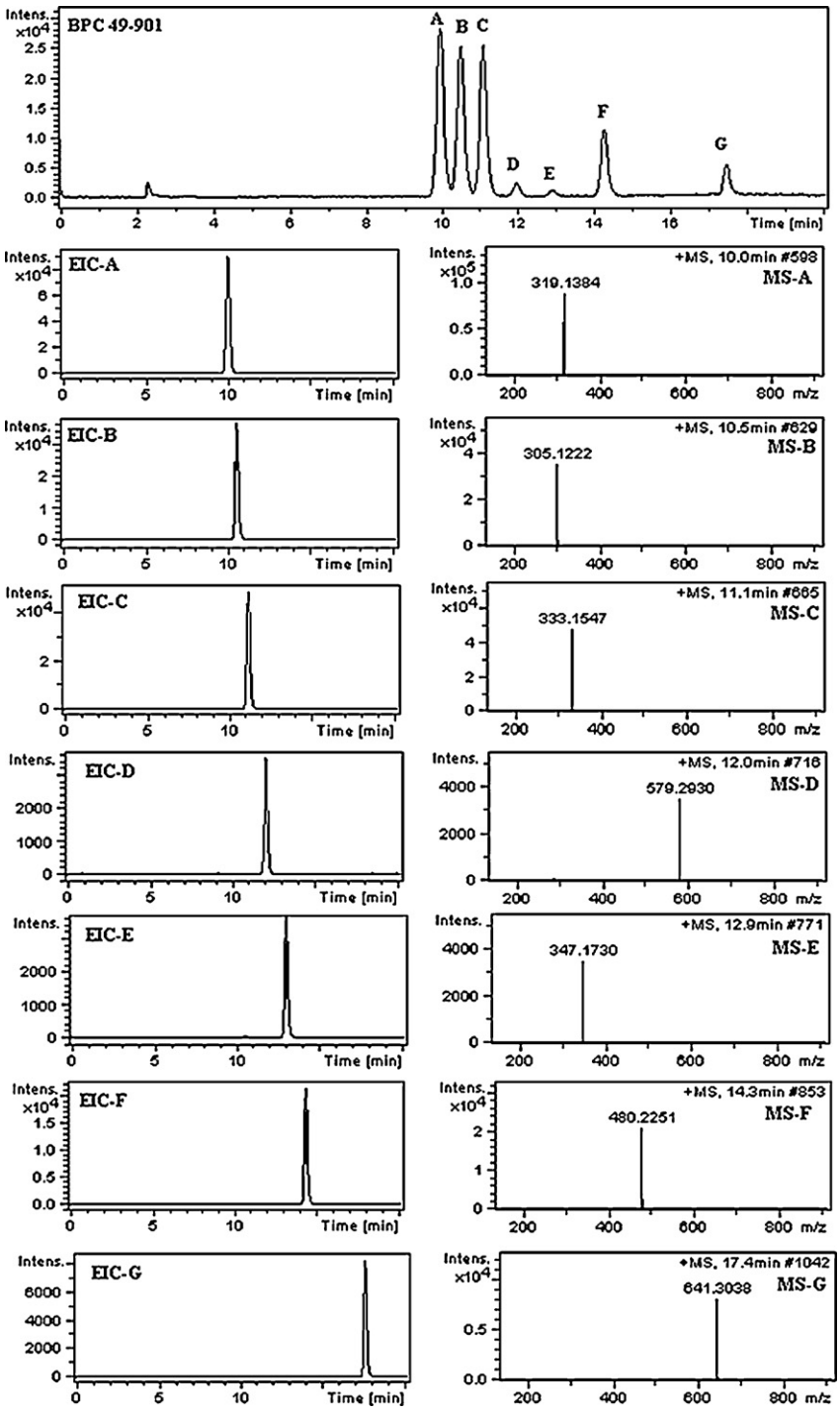

Fig. 1. Base peak chromatogram (BPC), extracted ion chromatogram (EIC) and MS spectra obtained from standard polyamines by HPLC/Q-TOF MS. (A) PUT; (B) DAP; (C)CAD; (D)AcSPM; (E) DAH (I.S.); (F)SPD; (G) SPM.

3.2. Mass spectrometry analysis and the optimal HPLC/Q-TOF MS conditions

Benzoyl chloride derivatization procedure significantly increased sensitivity as at least two benzoyl groups were added. Accordingly, the benzoyl polyamines were separated from the endogenous substance. Fig. 1 shows the base peak chromatogram (BPC), extracted ion chromatogram (EIC) and MS spectra obtained from the derivatives of standard polyamines. As shown in mass spectra, the sodium adduct ions $[M+Na]^+$ of all polyamines derivatives were identified. The $[M+Na]^+$ was most likely formed by combination of active nitrogen atom of benzoyl polyamines and sodium ion in reaction medium. The identified ions for quantify PUT, DAP, CAD, AcSPM, 1,6-diaminohexane, SPD and SPM were selected at *m*/*z* 319.1, *m*/*z* 303.1, *m*/*z* 333.2, *m*/*z* 579.3, *m*/*z* 347.2, *m*/*z* 480.2 and *m*/*z* 641.3, respectively.

To optimize the Q-TOF MS conditions, a single-factor test was performed. With the capillary voltage increased from 3700 V to 4500 V, the peak area increased gradually and had the maximum at 4500 V. Different dry gas flows were compared and the 7.0 l min^{-1} was found to be optimum. In the same manner, 230 °C was chosen for the optimum dry gas temperature. The nebulizer pressure was

Table 1
Validation data for the analysis of polyamines using the proposed method ($n = 3$).

Polyamines	Calibration range (ng ml^{-1})	Linearity (r)	LOD (ng)	Urine recovery (%)			
				50 ng ml^{-1}	200 ng ml^{-1}	1000 ng ml^{-1}	Mean
PUT	1–1000	0.9995	0.02	91.0	89.0	90.5	90.1
DAP	1–1000	0.9991	0.3	85.0	83.9	80.1	83.0
CAD	1–1000	0.9992	0.1	84.9	87.5	84.5	85.7
AcSPM	5–1000	0.9992	1.0	73.31	71.84	70.13	71.76
SPD	1–1000	0.9992	0.2	84.3	88.5	82.5	85.1
SPM	1–1000	0.9993	0.4	74.80	75.29	78.10	76.06

Polyamines	Intra-day precision (R.S.D.%)			Inter-day precision (R.S.D.%)		
	50 ng ml^{-1}	200 ng ml^{-1}	1000 ng ml^{-1}	50 ng ml^{-1}	200 ng ml^{-1}	1000 ng ml^{-1}
PUT	13.2	11.7	6.9	12.9	10.1	11.3
DAP	10.3	2.1	2.0	11.1	8.5	4.2
CAD	14.7	4.1	4.4	6.9	9.3	7.4
AcSPM	11.0	4.6	7.6	11.0	5.4	7.6
SPD	7.2	3.3	8.8	4.7	7.4	5.3
SPM	10.8	7.3	7.9	7.7	4.6	3.9

tested from 1.2 to 1.4 bar for polyamines to obtain the highest ion intensity, and 1.3 bar was selected as the incoming pressure of the nebulizer gas.

3.3. Method validation

As shown in Table 1, the results of method validation were summarized. The calibration curves were obtained by plotting the peak area ratios of polyamines relative to IS from seven different (i.e., 1, 5, 10, 50, 100, 500, 1000 ng ml^{-1}) concentrations expect that acetylspermine from six different (i.e., 5, 10, 50, 100, 500, 1000 ng ml^{-1}) and the calibration regression coefficients were ranged from 0.9991 to 0.9995. The LOD was 0.02 ng ml^{-1} for PUT, 0.3 ng ml^{-1} for DAP, 0.1 ng ml^{-1} for CAD, 1.0 ng ml^{-1} for AcSPM, 0.2 ng ml^{-1} for SPD, 0.4 ng ml^{-1} for SPM at S/N = 3. The intra- and inter-day precisions were within 15% and 13%, respectively. Furthermore, the recoveries ranged from 70.13% to 91.0% for human urine. In terms of matrix effect, all the ratios defined in Section 2.6 were between 86.4% and 98.3%, which meant no significant matrix effect in this method. The stability of derivatization polyamines was found to be excellent with R.S.D. less than 1.5% within 24 h.

3.4. Determination of polyamines in human urine samples

The proposed method was applied to determine the concentrations of six polyamines in human urine samples obtained from 10 cancer patients and 15 healthy volunteers. 10% $HClO_4$ was chosen as extraction and deproteinization solvent for it could be combined with basic group of polyamines to separate from other endogenous substances of samples. The typical mass chromatograms and mass spectra of polyamines in human urine from cancer sufferers and healthy volunteers were shown in Fig. 2. The derivative polyamines were identified from the comparison of MS characteristic of standard polyamines. The amount of urinary polyamines was calculated with creatinine of each sample. As shown in Table 2, the mean levels of each polyamine (ng mg^{-1} of creatinine) and the total polyamines of patients were higher than those of healthy volunteers. The most abundant polyamine was putrescine followed by spermine in patients and the most abundant polyamine was spermine followed by putrescine in healthy volunteers. To classify the cancer sufferers and the healthy individuals, the SPSS (version 16.0) was applied. The between-groups linkage method was adopted and squared Euclidean distance was chosen to evaluate the similarity of samples. As displayed in Fig. 3, 25 samples were gathered into two groups, the samples of healthy volunteers were classified in Group I and cancer sufferers were classified in Group II. These results showed that the significant difference between cancer sufferers and healthy volunteers when using polyamines as indicators.

This significant difference of polyamines could be interpreted due to the appearance of tumor tissue. The most notable characteristic of tumor tissue was the rapid reproduction of

Table 2
Amounts of polyamines (ng mg^{-1} of creatinine) in urine form 10 cancer patients and 15 healthy volunteers.

	Urine of patients ($n = 10$)							Urine of healthy volunteers ($n = 15$)						
	PUT[a]	DAP[a]	CAD[a]	AcSPM[a]	SPD[a]	SPM[a]	Total polyamines	PUT[a]	DAP[a]	CAD[a]	AcSPM[a]	SPD[a]	SPM[a]	Total polyamines
	460.0	39.76	64.21	43.91	41.08	133.6	782.6	89.7	9.38	4.49	1.82	0.69	0.50	106.5
	444.5	37.83	29.89	43.82	38.27	93.3	687.6	3.51	6.70	3.42	3.77	4.52	36.62	58.54
	192.1	37.62	20.51	28.46	21.34	100.9	401.0	3.39	0.70	2.18	2.83	2.69	20.49	32.27
	179.3	27.62	41.84	22.05	40.14	87.1	398.0	6.95	1.35	9.54	4.65	3.25	38.21	63.96
	248.7	24.81	32.49	35.06	32.32	75.53	448.9	2.25	2.18	1.41	1.73	0.86	38.44	46.87
	332.7	37.76	35.74	15.00	26.63	40.12	487.9	7.96	7.39	6.49	3.19	2.33	16.39	43.73
	292.1	31.25	25.90	12.40	23.71	43.34	428.7	3.45	0.30	1.59	5.13	5.50	31.14	47.11
	229.5	30.99	26.44	22.01	28.18	52.89	390.0	4.67	1.65	9.06	3.53	1.76	18.33	38.98
	272.1	25.18	30.09	21.09	25.12	36.62	410.2	22.10	5.00	6.05	3.29	10.31	4.43	51.17
	191.6	22.01	20.48	17.65	26.08	43.93	321.7	1.07	2.40	2.30	6.10	1.21	29.79	42.88
								19.17	2.04	1.61	3.84	4.69	3.27	34.62
								48.10	5.55	1.46	3.61	9.87	9.38	77.98
								3.96	1.53	1.82	1.91	3.51	37.83	50.56
								5.44	0.62	2.62	2.43	4.60	37.89	53.59
								6.32	2.20	1.16	2.19	10.26	8.58	30.71
Mean	284.3	31.48	32.76	26.14	30.29	70.74	475.7	15.20	3.27	3.68	3.34	4.40	22.08	51.97

[a] Polyamines.

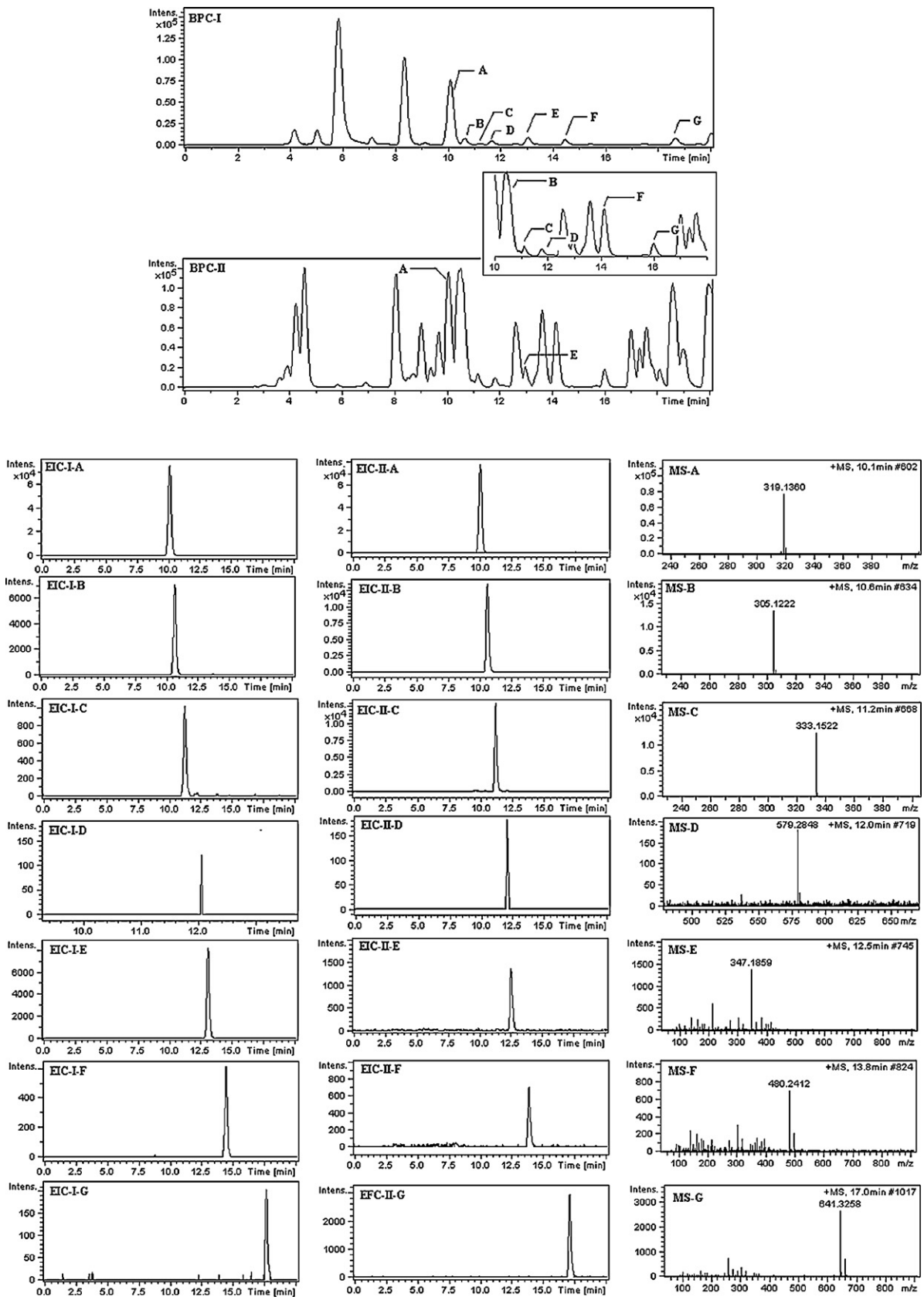

Fig. 2. Base peak chromatogram (BPC), extracted ion chromatogram (EIC) and MS spectra obtained from the human urine by HPLC/Q-TOF MS. The separation of derivatization polyamines in plasma within 10–18 min. (I) for healthy volunteers and (II) for cancer sufferers, (A) PUT; (B) DAP; (C) CAD; (D) AcSPM; (E) DAH (I.S.); (F) SPD; (G) SPM.

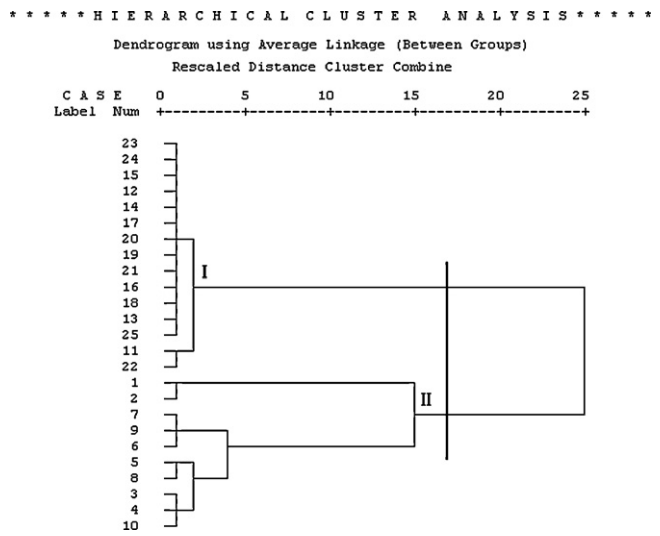

Fig. 3. Result of the cluster analysis, the between-groups linkage method was adopted and squared Euclidean distance was chosen as measurement. Group I for healthy volunteers and group II for cancer sufferers.

RNA. Polyamines could enhance RNA polymerase activity and involve in the regulation RNA metabolism. Therefore, putrescine as the precursor of spermidine and spermine, coupled with other metabolites like 1,3-diaminopropane, cadaverine and acetylspermine obviously increased rapidly in tumor tissue than in normal tissue. Our findings were compatible with the conclusion in application of other analysis methods in previous papers [13,27,28].

4. Conclusion

This paper described the simultaneous determination of six polyamines in human urine after derivatization with benzoyl chloride by HPLC/Q-TOF MS. In comparison with conventional methods of polyamines analysis, the proposed method was superior to use for its simple pre-treatment produce and perfect separation of polyamines and acetylated polyamines in biological samples. Meanwhile, derivative polyamines were effectively identified by highly selective and sensitive Q-TOF MS. Cluster analysis could divide samples into the cancer patient group and healthy people group. Data obtained in this study indicated that the polyamine levels in patients were higher than those in healthy volunteers. So this method may be a useful tool in early diagnosis of cancer in patients.

References

[1] D.H. Russell, C.C. Levy, S.C. Schimpff, Cancer Res. 31 (1971) 1555.
[2] D.H. Russell, Clin. Chem. 23 (1977) 22.
[3] G. Sulochana, R. Kalavathy, L. Padmanabhan, et al., Med. Chem. Res. 77 (1983) 758.
[4] Y. Horn, Beal S.L., N. Walach, et al., Cancer Res. 42 (1982) 3248.
[5] D.H. Russell, P.M. Gullino, L.J. Marton, et al., Cancer Res. 34 (1974) 2378.
[6] D.H. Russell, B.G. Durie, S.E. Salmon, Lancet 25 (1975) 797.
[7] C. Moinard, L. Cynober, J.P. Bandt, J. Clin. Nutr. 24 (2005) 184.
[8] R.M. Reguera, B.L. Tekwani, R. Balaña-Fouce, Comp. Biochem. Physiol. Part C: Toxicol. Pharmacol. 140 (2005) 151.
[9] N. Uehara, K. Kita, S. Shirakawa, et al., J. Cancer 71 (1980) 393.
[10] L. Li, K.J. Hara, J.T. Liu, et al., J. Chromatogr. B 876 (2008) 257.
[11] K. Dziarkowska, K. Dziarkowska, Anal. Chim. Acta 606 (2008) 184.
[12] S. Asotra, P.V. Mladenov, R.D. Burke, J. Chromatogr. A 408 (1987) 227.
[13] Ö. Özdestan, A. Uren, Talanta 78 (2009) 1321.
[14] E. Dadáková, M. Křížek, T. Pelikánová, Food Chem. 116 (2009) 365.
[15] T. Ekegren, C. Gomes-Trolin, Anal. Biochem. 338 (2005) 179.
[16] F. Gaboriau, R. Havouis, J.P. Moulinoux, Anal. Biochem. 318 (2003) 212.
[17] M.H. Choi, K.R. Kim, B.C. Chung, J. Chromatogr. A 897 (2000) 295.
[18] L. Hawel III, C.V. Byus, Anal. Biochem. 311 (2002) 127.
[19] M. Guihaus, V. Mlynski, D. Selby, Rapid Commun. Mass Spectrom. 11 (1997) 951.
[20] K. Sugiura, J.Z. Min, T. Toyo'oka, et al., J. Chromatogr. A 1205 (2008) 94.
[21] M.S. Dopico-Garcíab, J.M. López-Vilariño, G. Fernández-Martínez, Anal. Chim. Acta 667 (2010) 123.
[22] A.P. Stevens, K. Dettmer, G. Kirovski, J. Chromatogr. A 1217 (2010) 3282.
[23] R.M. Balabina, R.Z. Safievab, E.I. Lomakina, Anal. Chim. Acta 671 (2010) 27.
[24] R.M. Balabin, R.Z. Safieva, Fuel 87 (2008) 2745.
[25] Q.-K. Zhang, L.-K. Dai, Control Instrum. Chem. Ind. 32 (2005) 53.
[26] R.M. Balabin, R.Z. Safieva, Fuel 87 (2008) 1096.
[27] M.-J. Paik, S. Lee, K.-H. Cho, K.-R. Kim, Anal. Chim. Acta 576 (2006) 55.
[28] S.H. Lee, J.W. Suh, B.C. Chung, S.O. Kim, Cancer Lett. 122 (1998) 1.