ELSEVIER

Journal of Chromatography B, 688 (1997) 179–186

JOURNAL OF CHROMATOGRAPHY B

Urinary polyamine evaluation for effective diagnosis of various cancers

Ja Won Suh[b], Seon Hwa Lee[a], Bong Chul Chung[a,*], Jongsei Park[a]

[a]*Doping Control Center, Korea Institute of Science and Technology, P.O. Box 131, Cheongryang, Seoul, South Korea*
[b]*Dept. of Special Chemistry, Seoul Medical Science Institute (SCL), Seoul 140-230, South Korea*

Received 27 February 1996; revised 22 May 1996; accepted 10 June 1996

Abstract

With a newly modified analytical method, the concentrations of free and acetylated urinary polyamines were simultaneously determined in a control group (32 cases) and patients with various types of cancers (104 cases, 20 males and 84 females) by gas chromatography–nitrogen–phosphorus detection. Significant concentration differences between normal subjects and various cancer patients were found. The various types of cancers (advanced gastric carcinoma, ovarian cancer, acute myelocyte leukemia, non-Hodgkin's lymphoma) gave unique patterns of urinary polyamine profile as well as significant differences of concentration. To indirectly evaluate the possible involvement of enzymes, precursor-to-product concentration ratios were compared between controls and patients with various types of cancers.

Keywords: Polyamines; Putrescine; Spermidine; Spermine; *N*-Acetylputrescine

1. Introduction

Polyamines have been the subject of numerous studies due to their involvement in the processes of cell proliferation, cell differentiation and malignant transformation since the reports of Russell in the 1970's [1]. It is well-known that their concentration in tissues and biological fluids is regulated by numerous enzymes in their metabolism, and is elevated in urine of patients with a variety of tumors. However, this rise of polyamines in tissues and biological fluids is not only specific for malignant states but is also found in proliferating tissues in diseases such as pernicious anemia, hemolytic anemia, polymyositis, pulmonary tuberculosis and psoriasis [2]. For this reason, the role of urinary polyamines as biochemical markers to screen cancers or to monitor therapy has been investigated but the results have been conflicting, probably because total polyamines were measured instead of free and acetylated polyamines. Therefore, this lack of specificity has discouraged the measurement of polyamines as a method of cancer detection. However, in numerous recent studies the importance of polyamines has been reconfirmed owing to anticancer agents such as α-difluoromethylornithine (DFMO) and methyl glyoxalbis(guanyl)hydrazone (MGBG) which inhibit the accumulation of polyamines by blocking the related enzyme activity during cell proliferation [3]. The most recent studies on polyamines have focused on identification of chemotherapy efficacy and extended to early prediction of relapse [4].

Due to their potential usefulness as biochemical

*Corresponding author.

PII S0378-4347(96)00266-6

markers, many analytical methods have been developed for the analysis of urinary fractionated (free and conjugated) polyamines and particularly Van den Berg et al. [5] have established normal values for free, *N*-acetylated polyamines and their metabolites using a capillary gas chromatography with nitrogen–phosphorus detection (GC–NPD). However, improvements are needed in criteria establishments for more accurate cancer diagnosis. Therefore, our first task was to establish urinary polyamine values for accurate diagnosis criteria and the second was to investigate whether polyamine levels were increased in various types of cancers, and if so, whether they had a potential usefulness as tumor markers in each cancer case to some extent.

In this paper, we describe a newly modified (simple and convenient) extraction method for analysis of urinary (free and *N*-acetylated) polyamines, based on the method of Van den Berg et al. [5]. Also a specific pattern recognition of urinary polyamines is introduced to classify the various types of cancers and the relative concentration ratios of precursors to products in biochemical pathway of polyamines are presented to evaluate possible involvement of enzyme activity.

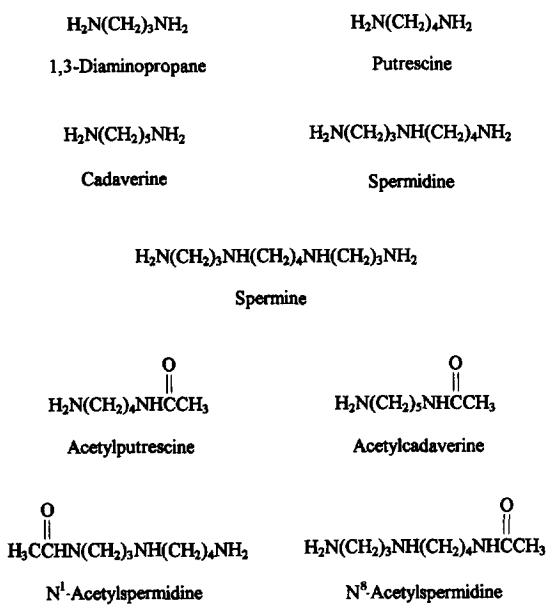

Fig. 1. Chemical structure of polyamines.

2. Experimental

2.1. Chemicals

All chemicals and solvents for gas chromatography with nitrogen–phosphorus detection were analytical grade. 1,3-Diaminopropane, putrescine, cadaverine, spermidine, spermine, *N*-acetylputrescine, *N*-acetylcadaverine, N^1-acetylspermidine, N^8-acetylspermidine, 1,6-diaminohexane and heptafluorobutyric anhydride were purchased from Sigma (St. Louis, MO, USA). Sep-Pak silica cartridges were from Waters (Milford, MA, USA). The chemical structures of polyamines tested in this study are shown in Fig. 1.

2.2. Patients and normal sample collection

Urine samples from 32 (16 male and 16 female) healthy volunteers ranging in age from 22 to 56 years old and from 104 (male and female) cancer patients from 26 to 72 years old were collected in polyethylene bottles. The collected urine samples were stored at −20°C until analysis. Creatinine was measured by the Jaffe method.

2.3. Gas chromatography–nitrogen–phosphorus detection

For gas chromatography with nitrogen–phosphorus detection, a Model 5890 gas chromatograph equipped with a Model 7673 automated sampler (both from Hewlett-Packard, Palo Alto, CA, USA) was used. The GC column was a 25 m×0.2 mm I.D. fused-silica capillary, coated with crosslinked 5% phenylmethylsilicone (film thickness, 0.33 μm). The carrier gas flow-rate (helium) was 0.8 ml/min, split ratio 1:15, detector temperature 300°C and injector temperature 300°C. The oven temperature program was a gradient system starting at 120°C, increased by 5°C/min to 270°C and held there for 5 min. The chromatographic peak areas were integrated by a Hewlett Packard 3392A networking integrator.

2.4. Gas chromatography–mass spectrometry

A Hewlett-Packard GC–MS system consisting of a Model 5890A gas chromatograph, a Model 5970B mass selective detector, an HP 5970C MS chemstation and a HP 7946 disc drive was used for this study. A fused-silica capillary column coated with HP-5 cross-linked 5% phenyl methyl silicone (Ultra-2, 34 m×0.2 mm I.D., 0.33 μm film thickness) was also used. The GC temperature was the same above. Transfer line temperature was 270°C and ion source temperature was 200°C. The mass spectrometer was operated at 70 eV in the electron impact (EI) mode using SCAN.

2.5. Sample extraction

For the extraction of urine samples, 0.5 μg of internal standard (1,6-diaminohexane) was added to a 1-ml volume of urine adjusted to pH 1. The mixture was extracted with 5 ml of ethylacetate and the organic phase was discarded. To the aqueous layer 2 ml of borate buffer (pH 9) was added, adjusted to pH 9 by adding about 80 μl of 4 *M* sodium hydroxide and vortexed. The supernatant was applied to a Sep-Pak silica cartridge preconditioned by washing with 6 ml of 0.1 *M* methanolic hydrochloric acid solution and distilled water. The Sep-Pak mini column was washed with 3 ml each of *n*-pentane and methanol, then the free and acetylated polyamines were eluted three times with 2 ml of a 0.1 *M* solution of hydrochloric acid in methanol. The eluate was evaporated to dryness in a rotary evaporator, then the polyamines were derivatized with a mixture of 100 μl each of ethylacetate and heptafluorobutyric anhydride by heating at 80°C for 1 h. After cooling, the solvent was evaporated at room temperature under a stream of air and the residue was dissolved in 50 μl of ethylacetate, and 2-μl volumes were injected onto the GC column, using an autosampler.

2.6. Quality control

For quality control, each series was analyzed using 1 ml of a pooled urine (blank samples) and of the same pooled urine fortified with 1, 3 and 5 μg of each analyte per ml. Quality control studies were performed three times for each polyamine.

3. Results and discussion

3.1. Extraction and analysis

Based on the extraction method of Van den Berg et al. [5], the effectively well-combined method of clean-up, including solvent partitioning and column chromatography, was established for the isolation of polyamines from biological fluids. In summary, the isolation of polyamines from endogenous interferences is based on specific elimination (below pH 1) and the unusual adsorption onto the silica gel mini column with borate buffer (pH 9). To enhance the specificity on the GC chromatograms, these were derivatized with heptafluorobutyric anhydride (HFBA) in ethylacetate. Under the above extraction method, the free putrescine (Put), cadaverine (Cad), spermidine (Spd), spermine (Spm) and conjugated [*N*-acetylputrescine (*N*-acPut), N^1-acetylspermidine (N^1-acSpd), N^8-acetylspermidine (N^8-acSpd), *N*-acetylcadaverine (*N*-acCad)] polyamines were simultaneously determined by GC–NPD and in a single run successfully separated within 30 min.

The intra-day and inter-day precision and accuracy of this method are presented in Table 1. The relative standard deviations (R.S.D.) were less than 10%, and the range of analytical recoveries was 48.6–101.2% as previously reported [6]. In acetylated polyamines (*N*-acPut, N^1- and N^8-acSpd), their recoveries were relatively lower than free polyamines (1,3-Dap, Put, Spd and Spm), but these phenomena may be explained as the lower polarity of acetylated forms compared to free forms. Also, the higher recoveries of diamines (Put: 101.2%, Cad: 84.5%) than tri- and tetra amines (Spd: 72.2%, Spm: 48.6%) may be due to affinity (polarity) with the silica gel column [7].

To identify the HFB derivatized polyamines, the structures of all HFB derivatives were verified by GC–MS. Fig. 2 shows mass spectra of some HFB derivatized polyamines. They contain the characteristic fragment ions such as $[M]^+$, $[CH_2NHCOC_3F_7]^+$, $[(CH_2)_3NHCOC_3F_7]^+$ and $[COC_3F_7]^+$: putrescine

Table 1
Precision, accuracy and recovery data for determination of polyamines

Concentration added (μg/ml)	Recovery (%)	Within-day		Day-to-day	
		Found (mean±S.D.) (μg/ml)	R.S.D. (%)	Found (mean±S.D.) (μg/ml)	R.S.D. (%)
1,3-Diaminopropane	85.1				
Blank		0.51±0.03	5.88	0.51±0.03	5.88
1.00		1.28±0.00	0.28	1.33±0.04	3.01
3.00		2.18±0.00	0.09	3.34±0.24	7.19
5.00		5.48±0.03	0.55	5.31±0.16	3.01
Putrescine	101.2				
Blank		0.97±0.01	1.03	0.22±0.01	4.55
1.00		1.65±0.04	2.42	1.26±0.10	7.94
3.00		3.37±0.04	1.19	3.20±0.15	4.69
5.00		5.85±0.01	0.17	5.34±0.43	8.05
N-Acetylputrescine	95.5				
Blank		1.76±0.07	3.98	1.94±0.14	7.22
1.00		4.44±0.34	7.66	3.74±0.29	7.75
3.00		7.91±0.18	2.28	4.73±0.34	7.19
5.00		10.05±0.80	7.96	5.80±0.49	8.45
Cadaverine	84.5				
Blank		0.07±0.00	5.71	0.10±0.01	10.00
1.00		1.06±0.00	0.28	1.02±0.05	4.90
3.00		2.96±0.01	0.34	2.94±0.03	1.02
5.00		5.27±0.02	0.38	5.07±0.19	3.75
N-Acetylcadaverine	95.8				
Blank		0.13±0.01	7.69	0.14±0.01	7.14
1.00		2.37±0.10	4.22	1.90±0.13	6.84
3.00		6.14±0.24	3.91	7.36±0.42	5.71
5.00		8.83±0.84	9.51	5.42±0.46	8.49
Spermidine	72.2				
Blank		0.17±0.01	5.88	0.23±0.00	0.60
1.00		1.08±0.02	1.85	1.25±0.12	9.60
3.00		3.32±0.00	0.03	3.32±0.00	0.05
5.00		6.03±0.06	1.00	6.08±0.51	8.39
N^8-Acetylspermidine	50.6				
Blank		0.75±0.03	4.00	0.82±0.08	9.76
1.00		2.10±0.11	5.24	1.86±0.18	9.68
3.00		4.35±0.39	8.97	4.71±0.46	9.77
5.00		7.15±0.62	8.67	5.94±0.05	0.84
N^1-Acetylspermidine	53.5				
Blank		0.63±0.02	3.17	0.80±0.03	3.75
1.00		1.83±0.07	3.83	1.76±0.09	5.11
3.00		3.84±0.29	7.55	4.13±0.40	9.69
5.00		6.10±0.59	9.67	5.31±0.20	3.77
Spermine	48.6				
Blank		0.55±0.05	9.09	0.65±0.04	6.15
1.00		1.40±0.09	6.43	1.43±0.11	7.69
3.00		3.21±0.15	4.67	3.94±0.28	7.11
5.00		4.01±0.37	9.23	6.15±0.06	0.98

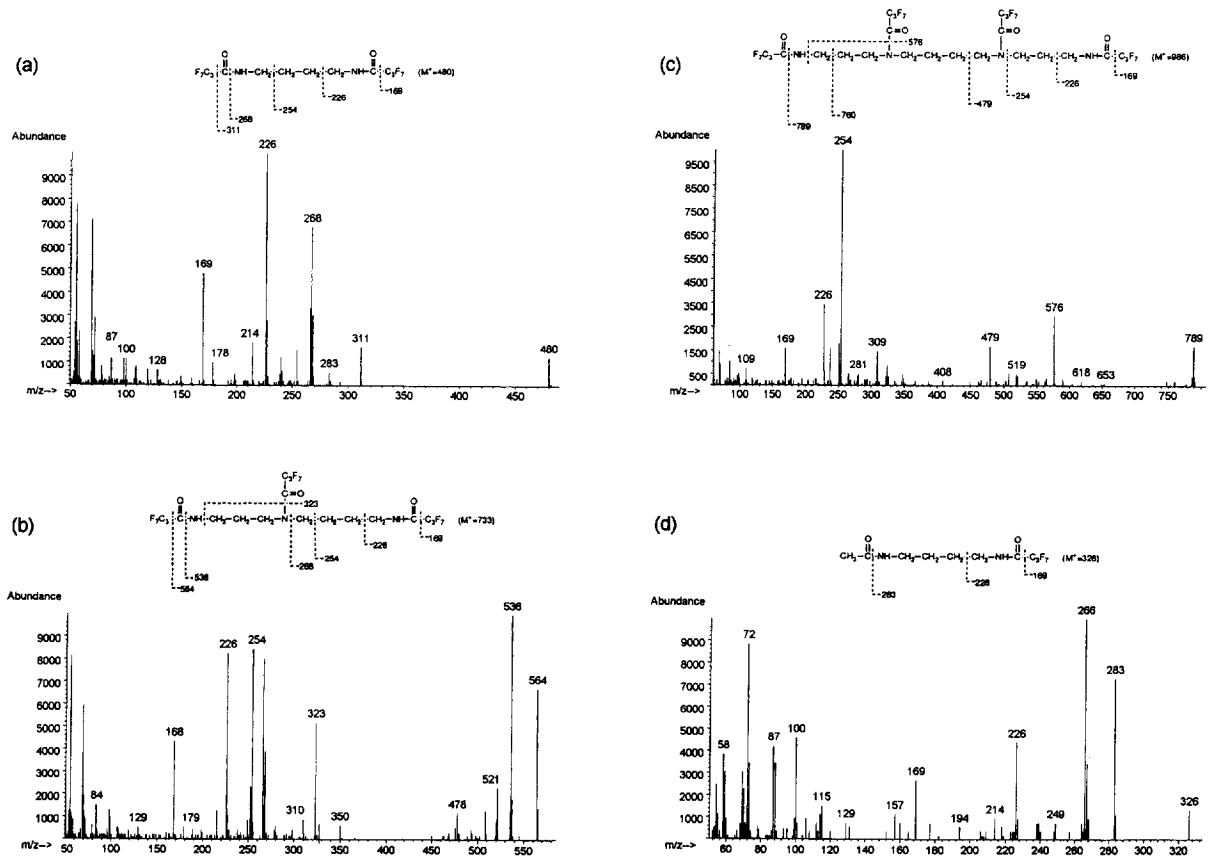

Fig. 2. Mass spectra of the heptafluorobutyryl derivatives of representative standard polyamines: (a) putrescine, (b) spermidine, (c) spermine and (d) *N*-acetylputrescine.

at *m/z* 480, 226, 254 and 268 ; spermidine at *m/z* 226, 254, and 536; spermine at *m/z* 226, 254, and 789; *N*-acetylputrescine at *m/z* 326, 226, 266 and 283. From these results, we concluded that all primary and secondary amino ($-NH_2$, $-NHR$) groups were substituted by HFB groups, but the amide NH groups were not.

3.2. Discussion on various types of cancers

Fig. 3 shows the typical gas chromatograms of normal and various types of cancers such as AGC (advanced gastric carcinoma), ovarian cancer, AML (acute myelocyte leukemia) and NHL (non-Hodgkin's lymphoma). As shown in Fig. 3 what is particularly noticed here is the significant differences in the patterns of urinary polyamines between normal and various types of cancer. First of all, in the case of AGC (advanced gastric carcinoma, $n=13$) as a representative of solid tumors (AGC, ovarian cancer), Fig. 3b shows significant elevations of all polyamines except for cadaverine compared to normal subjects, especially spermine. In the same way, in the case of ovarian cancer ($n=84$), Fig. 3c shows a similar pattern of urinary polyamine as AGC except for the elevation of spermine. From these findings, the spermine determination may be useful as a potential marker for the detection of AGC like CEA (carcinoembryonic antigen), the well-known marker of AGC [8].

However, in leukemia (acute or chronic) as a representative of hematological tumors (leukemia, NHL) there was a marked difference of two stereoisomers N^1-, N^8-acetylspermidine with the elevations

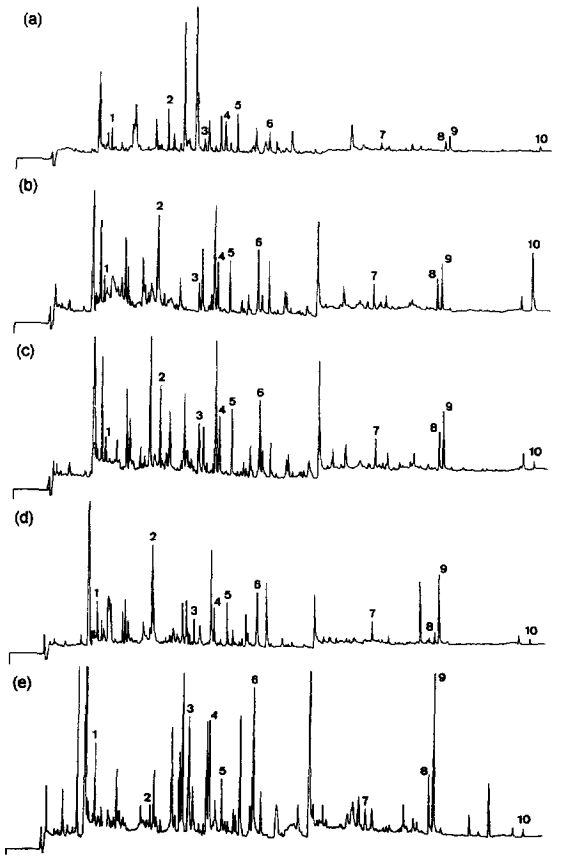

Fig. 3. GC–NPD chromatograms of urinary polyamines from (a) normal subjects and patients with (b) advanced gastric carcinoma (AGC), (c) ovarian cancer, (d) acute myelocytic leukemia (AML) and (e) non-Hodgkin's lymphoma (NHL). Peaks: 1=1,3-diaminopropane; 2=putrescine; 3=cadaverine; 4=*N*-acetylputrescine; 5=1,6-diaminohexane (ISTD); 6=*N*-Acetylputrescine; 7=spermidine; 8=N^8-acetylspermidine; 9=N^1-acetylspermidine; 10=spermine.

of all polyamines except for spermine like solid tumors (Fig. 3d). This difference of N^1-acSpd, a product of N^1-acetyltransferase in the reversible pathway of polyamine metabolism, seems to imply that N^1-acSpd may be a more specific marker of cancer than free or total polyamines. In the same way, in Fig. 3e NHL shows the same pattern as that of leukemia. This unique elevation of N^1-acSpd has been previously reported [5,9,10].

Summarising the above results, Fig. 4 shows the distribution of free and acetylated polyamine values of the control group and various cancer type patients. As shown in Fig. 4, most polyamine values are significantly greater in cancer patients than in normal subjects. In Fig. 4 (I, II, III, and IV), acetylated polyamines are shown to be more effective for discrimination of both groups than free polyamines. This agrees well with the facts that the majority of polyamines were excreted as acetylated forms [5].

To discuss possible involvement of related enzymes in the biochemical pathway of polyamines, Table 2 summarizes the precursor-to-product concentration ratios between the control group and various cancer patients. As shown in Table 2 both free putrescine to free spermidine (Put/Spd) and total (free plus acetylated) putrescine to total spermidine (t-Put/t-Spd) are significantly greater in all cancer patients than in normal subjects. This significant difference of ratio values supports the well-known fact that extracellular putrescine concentrations may reflect either rapid tumor growth or tumor-cell loss [4]. No difference is observed in (Put/*N*-acPut) and (Spd/*N*-acSpd) between the control group and cancer patients. This may be caused by

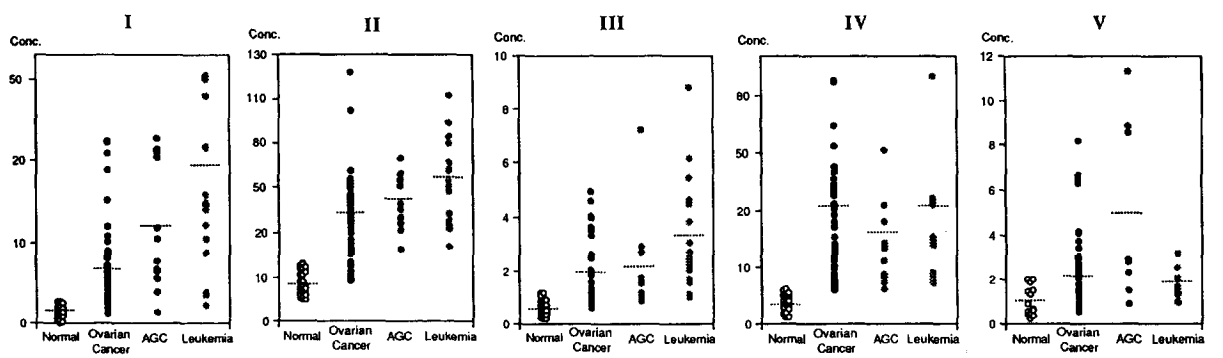

Fig. 4. Concentration distributions of urinary free and acetylated polyamines in normal subjects and patients with ovarian cancer, AGC and leukemia. I, Putrescine; II, *N*-Acetylputrescine; III, Spermidine; IV, *N*-Acetylspermidine; V, Spermine.

Table 2
The relative ratios of polyamines in normal subjects and various cancer patients

Relative ratios	Normal		Ovarian cancer		AGC		Leukemia	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
t-Put/t-Spd	1.82	0.99–3.94	2.44	0.68–5.25	5.89	3.13–15.47	4.29	0.53–7.39
Put/Spd	2.11	0.30–6.00	11.16	4.96–22.63	7.53	1.13–24.41	8.08	2.16–26.60
Put/*N*-acPut	0.15	0.01–0.43	0.14	0.08–0.28	0.27	0.06–1.07	0.43	0.07–0.98
Spd/*N*-acSpd	0.15	0.05–0.32	0.09	0.01–0.60	0.19	0.06–0.64	0.20	0.05–0.68
Spd/Spm	0.84	0.23–1.45	0.74	0.12–1.23	0.82	0.18–3.14	0.70	0.73–1.61
N^1-acSpd/ N^8-acSpd	0.78	0.63–1.21	0.98	0.82–1.84	1.09	0.53–2.03	2.25	0.96–6.41

N-acetyltransferase activity, an enzyme of polyamine products (*N*-acPut, *N*-acSpd) in the polyamine biochemical pathway. This enzyme is probably little affected during malignant transformation. Also, no difference is observed in the concentration ratios of spermidine to spermine (Spd/Spm). This seems to be due to the normal activity of spermine synthetase, but has not yet been elucidated. Then, the relative ratios of two stereoisomers (N^1-acSpd, N^8-acSpd) for normal urine, AGC and ovarian cancer were almost unity, as Yamamoto et al. previously reported [10], while leukemia had ratios of above unity in comparison to normal and solid tumors (ratio>1). N^1-acSpd seems to be more sensitive for the detection of hematological tumors while N^8-acSpd is somewhat less sensitive.

From these results, we suggest the possibility of a different polyamine metabolic pathway for solid and hematological tumors. This seems to imply that the concentration ratio difference of two stereoisomers (N^1-acSpd/N^8-acSpd) may be one means to classify solid tumors (AGC, ovarian cancer) and hematological tumors (leukemia and NHL). Thus, for the elucidation of significant ratio differences between two stereoisomers, investigations on another possible biochemical pathway will be done at a later date.

4. Conclusion

Using the newly modified method described in this paper, free and acetylated polyamines were simultaneously determined in the urine of normal subjects and various cancer patients by GC–NPD. The concentration range for free and *N*-acetylated polyamines was established to be a useful criterion for more accurate cancer diagnosis. From the above results, significant differences were found in absolute urinary concentrations between cancer patients (104 cases) and normal subjects (32 cases), and it was shown that the various types of cancers had specific urinary polyamine patterns. This seems to support the theory that urinary N^1-acSpd, unlike other acetylated polyamines and free polyamines, may be a specific and sensitive marker for the confirmation of hematological tumors such as leukemia and NHL. Furthermore, we suggest that the concentration ratio differences of two stereoisomers N^1-, N^8-acetylspermidine may effectively be used to classify solid tumors and hematological tumors.

For more accurate diagnosis, this study will be extended further to recognize the specific urinary polyamine patterns in normal subjects and patients with various types of cancers, to accurately classify both groups with sufficient subjects and to establish new biochemical markers derived from the above investigations.

Acknowledgments

This study was supported in part by Grant 2N13691 from the Korea Ministry of Science and Technology.

References

[1] D.H. Russell, Nature, 233 (1971) 144.
[2] A. Garnica, T. Benton, P. Slanina, T. Miale and A.L. Stenke, Ann. Clin. Lab. Cancer, 11 (1981) 109.
[3] H.W. Herr, E.L. Kleinert, N.M. Relyea and W.F. Whitmore Jr., Cancer, 53 (1984) 1294.
[4] S. Kubota, Z. Yamaski, M. Yoshimoto, N. Murata, T. Wada, N. Ohsawa and F. Takaku, Cancer, 56 (1985) 1630.

[5] G.A. Van den Berg, F.A.J. Muskiet, A.W. Kingma, W. Van der Silk and M.R. Halie, Clin. Chem., 31 (1986) 1930.
[6] J.W. Suh, S.H. Lee, Y.H. Park, B.C. Chung and J. Park, J. Korean Soc. Anal. Sci., 8 (1995) 895.
[7] D.P. Grettie, D. Bartos, F. Bartos, G. Smith and R.A. Cambell, in R.A. Cambell, D.R. Morris, D. Bartos, G.D. Daves, Jr. and F. Bartos (Editors), Advances in Polyamine Research, Vol. 2, Raven Press, New York, 1978, p. 13.
[8] M.M. Abdel-Monem, J.L. Merdick and A. Thoologides, Cancer Res., 42 (1982) 2097.
[9] D.H. Russell, Clin. Chem., 23 (1977) 22.
[10] S. Yamamoto, M. Yokogawa, K. Wakamatsu, H. Kataoka and M. Makita, J. Chromatogr., 233 (1982) 29.