J. Biochem. 132, 783–788 (2002)

Two Enzyme-Linked Immunosorbent Assay (ELISA) Systems for N^1,N^8-Diacetylspermidine and N^1,N^{12}-Diacetylspermine Using Monoclonal Antibodies

Masaru Hamaoki,*,[1] Kyoko Hiramatsu,† Setsuo Suzuki,* Atsuo Nagata,* and Masao Kawakita‡

Immunology Laboratory, Diagnostics Department, Yamasa Corporation, 2-10-1 Araoi-cho, Choshi, Chiba 288-0056; †Department of Physiological Chemistry, The Tokyo Metropolitan Institute of Medical Science (Rinshoken), 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8613; and ‡Department of Applied Chemistry, Kogakuin University, 1-24-2 Nishi-shinjuku, Shinjuku-ku, Tokyo 163-8677

Received August 13, 2002; accepted September 3, 2002

We obtained monoclonal antibodies against N^1,N^{12}-diacetylspermine (DiAcSpm) and N^1,N^8-diacetylspermidine (DiAcSpd), and developed two systems of competitive ELISA that utilize the antibodies and a common enzyme-labeled antigen to measure these diacetylpolyamines. Cross-reactions with N^1-acetylspermidine in the assay of DiAcSpm and with N^8-acetylspermidine in the assay of DiAcSpd were as low as 0.26 and 0.6%, respectively, and were judged to be insignificant in clinical use for measuring urinary diacetylpolyamines. These assays were used to assess diurnal variations in diacetylpolyamine excretion in urine to show that the excretion of diacetylpolyamines after normalization for the concentration of creatinine is stable over a day with only minimal diurnal variation.

Key words: diacetylpolyamine, ELISA, monoclonal antibody, tumor marker, urine.

In the decades since Russell *et al.* (*1, 2*) reported that the amount of polyamines excreted in urine is higher in patients with cancer than in healthy persons, it has become recognized that the total amount as well as the amounts of individual free and monoacetylated polyamines in urine can not alone serve as a conclusive measure for the diagnosis of cancer because of fluctuations among individuals and the occurrence of many false negative as well as false positive cases (*3–6*).

Using high performance liquid chromatography (HPLC), we demonstrated that N^1,N^8-diacetylspermidine (DiAcSpd) and N^1,N^{12}-diacetylspermine (DiAcSpm) are present in healthy human urine with small fluctuations among individuals (*7*), and that these two diacetylpolyamines, rather than the total or other individual polyamine species, might be useful as novel tumor markers serving as diagnostic and prognostic indicators of cancers (*8, 9*). While analysis by HPLC is sensitive and versatile, it is time-consuming and inconvenient for handling large numbers of samples. We, therefore, developed an enzyme-linked immunosorbent assay (ELISA) procedure for the determination of DiAcSpd (*10*) and DiAcSpm (*11*) using affinity-purified polyclonal rabbit antibodies.

In this article, we describe the development of two systems of competitive ELISA using monoclonal antibodies, each intended for one of the two diacetylpolyamines. These systems both feature the use of a common horseradish peroxidase–labeled antigen. The antigen showed comparable affinities for the two antibodies which enabled us to construct ELISA systems with comparable sensitivities for DiAcSpm and DiAcSpd. The use of a common antigen significantly simplifies the overall design of the ELISA. The use of monoclonal antibodies assures a stable and unlimited supply of antibodies and serves to minimize variations among different lots of antibodies.

MATERIALS AND METHODS

Chemicals—The materials used in this work were as follows: free and acetylated polyamines from Sigma, MO, diacetylpolyamines from Chemistry Laboratory, Yamasa, disuccinimidyl suberate from Pierce, IL, bovine serum and bovine serum albumin (BSA) from GibcoBRL, NY, mouse serum albumin (MSA) from ICN, OH, and horseradish peroxidase (HRP) from TOYOBO, Osaka.

Polyamine-Protein Conjugates—*N*-Acetylspermine (AcSpm) and N^8-acetylspermidine (N^8-AcSpd) were conjugated separately to BSA using disuccinimidyl suberate as a cross-linker according to the manufacturer's instructions to serve as antigens for raising anti-DiAcSpm and anti-DiAcSpd antibodies, respectively. AcSpm and N^8-AcSpd were also conjugated to MSA in the same way to serve as antigens for screening hybridomas and used to coat the bottom of microtiter plate wells. AcSpm was conjugated to HRP (AcSpm-HRP) according to the procedure described by Nakane, in

[1]To whom correspondence should be addressed. Tel: +81-479-22-9840, Fax: +81-479-22-9845, E-mail: hamaoki@yamasa.com

Abbreviations: AcSpm, *N*-acetylspermine; AcSpm-HRP, HRP conjugated with AcSpm: DiAcSpd, N^1,N^8-diacetylspermidine; DiAcSpm, N^1,N^{12}-diacetylspermine; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; MAb, monoclonal antibody; MSA, mouse serum albumin; N^1-AcSpd, N^1-acetylspermidine; N^8-AcSpd, N^8-acetylspermidine; TMB, 3,3′,5,5′-tetramethylbenzidine; PBS, phosphate buffered saline.

which a Schiff base formed by the reaction between the primary amino group of AcSpm and the aldehyde group of oxidized HRP is reduced with sodium borohydride (*12*) and utilized in competitive ELISAs of DiAcSpm (M assay) and DiAcSpd (D assay) as described below.

Development of Hybridomas—BALB/c mice were immunized intraperitoneally with 50 μg of either of the conjugates emulsified with Freund's complete adjuvant at 2-week intervals. After six immunizations, the mice that gave the highest titer for each conjugate were treated intravenously with 5 μg of the respective antigen in saline 3 days before fusion. Splenocytes obtained from the immunized mice were fused with mouse myeloma cells (Sp2/0-Ag-14) (*13*) using 50% polyethylene glycol 1,000 as described by Kohler and Milstein (*14*). The culture supernatants of hybridomas were screened for anti-DiAcSpm antibodies in microtiter plate wells coated with AcSpm-MSA and for anti-DiAcSpd antibodies in wells coated with N^8-AcSpd-MSA.

Monoclonal Antibodies—Monoclonal antibodies (MAbs) were produced in ascitic fluid of BALB/c mice by priming with pristane, and purified by protein A affinity chromatography. Subclasses and types of the three MAbs used in this work were γ1κ as determined with a MONOAB-ID EIA kit (Zymed Laboratories, CA).

M and D assays—MAb 9G9 was used alone for the M assay. For the D assay, MAb 1D8 and a non-cognate MAb CAT1H12 were mixed at a ratio of 1:3. The wells of microtiter plates were coated with 100 μl of either of the immunoglobulins (10 μg/ml) in PBS overnight at 4°C, and washed 2 times with PBS. The wells were blocked with 150 μl of 0.5% skim milk, 5% sucrose, and 0.1% procline for 2 h at room temperature and then the solution was removed. The plates were vacuum-dried and stored at 4°C until use. Samples were diluted appropriately to 100 μl with dilution buffer (10 mM potassium phosphate buffer, pH 6, 0.15 M NaCl, and 0.1% procline). AcSpm-HRP was diluted at 50 ng/ml in 80% bovine serum, 0.1 M tricine-NaOH buffer, pH 8.6, 0.8 M NaCl, and 0.1% procline. A diluted sample (100 μl) was mixed with an equal volume of diluted AcSpm-HRP solution, and the mixture was applied to the wells of the antibody-coated microtiter plate for either the M or D assay. After incubation for 2 h at room temperature with shaking, the wells were washed 4 times with PBS containing 0.05% Tween-20, and then 100 μl of TMB color reagent (80 μg/ml 3,3′,5,5′-tetramethylbenzidine, 0.1 M sodium citrate buffer, pH 5.0, 0.005% H_2O_2) was added. After further incubation for 15 min, the reaction was stopped by the addition of 100 μl of 1 N H_2SO_4 and the color intensity was determined at 450 nm.

Urine Samples—Urine samples used in experiments other than the correlation analysis were collected from healthy volunteers and stored below −35°C without NaN_3. Urine samples used in the correlation analysis contained 3 mM NaN_3. Before measuring the diacetylpolyamines in these samples by the proposed ELISA procedures, NaN_3 was removed by adding acetic acid to the samples at a concentration of 174 mM followed by vacuum-drying. The samples were then reconstituted with dilution buffer at appropriate concentrations. Analysis by HPLC was carried out by the procedure described previously after pretreatment of the urine samples by a procedure involving adsorption to and elution from cation exchange resin (Muromac CR-70) to remove substances interfering with the HPLC analysis of polyamines (*15*). Creatinine was determined enzymatically using the CRE(E) TEST (MIZUHO MEDY, Saga).

Statistical Analysis—Statistical calculations and evaluations for comparing the HPLC and ELISA procedures were performed with the aid of StatView software (SAS Institute, NC).

RESULTS

Generation of Monoclonal Antibodies—The conjugation of AcSpm and N^8-AcSpd to proteins using disuccinimidyl suberate results in the formation of an acylamide linkage at the linker–monoacetylpolyamine junction, giving a structure that closely resembled the corresponding portion of diacetylpolyamines (*10*). We, therefore, chose these conjugates as antigens for raising antibodies against diacetylpolyamines.

Human urine usually contains some 30- and 10-fold molar excess of N^1- and N^8-AcSpd over DiAcSpm and DiAcSpd, respectively (*7*). Cross-reactions with these monoacetylpolyamines should be minimal if an anti-diacetylpolyamine antibody is to be used in an ELISA to determine the quantity of diacetylpolyamines in human urine without interference.

The supernatants from the 48 wells that gave a positive reaction with AcSpm-MSA in the first screening of 1,410 wells were examined further for DiAcSpm-specificity by comparing the ability of DiAcSpm and N^1-AcSpd to compete with AcSpm-MSA to bind the antibody in the supernatant fluid. Finally, MAb 9G9, which showed the highest preference for DiAcSpm over N^1-AcSpd, was selected as an anti-DiAcSpm monoclonal antibody. Similarly, MAb 1D8, which showed the highest preference for DiAcSpd over N^8-AcSpd, was established as an anti-DiAcSpd monoclonal antibody. The latter antibody was chosen from 71 wells that

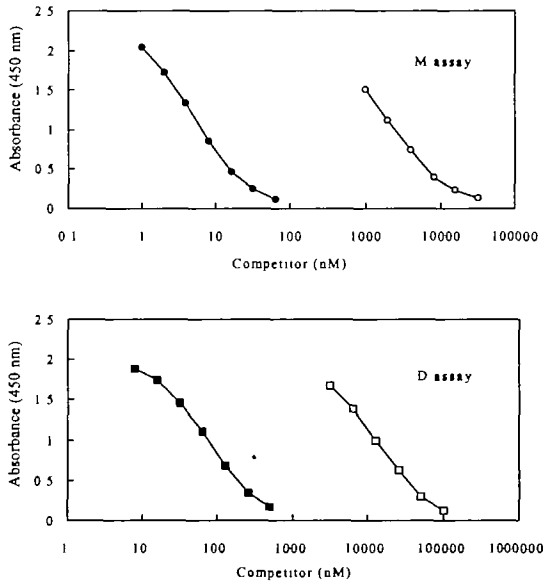

Fig. 1. **Cross-reactivities of N^1-AcSpd in the M assay (upper) and N^8-AcSpd in the D assay (lower).** (●), DiAcSpm; (○), N^1-AcSpd; (■), DiAcSpd; (□), N^8-AcSpd.

gave a positive response in the first screening of 940 wells coated with N^8-AcSpd-MSA.

Competitive ELISA for DiAcSpm and DiAcSpd—AcSpm-HRP reacted well with both the 9G9 and 1D8 antibodies adhered to the bottom of microtiter plate wells due to its limited cross-reactivity with diacetylpolyamine-specific antibodies. Since this binding of AcSpm-HRP to these antibodies was competed efficiently by DiAcSpm and DiAcSpd, respectively, this provided a convenient basis for a competitive ELISA procedure to measure these diacetylpolyamines. When the wells were coated with 1D8 alone, however, the amounts of AcSpm-HRP bound to the wells were greater than those appropriate for the measurement of color development after the peroxidase reaction under standard conditions. Coating the wells with a mixture of 1D8 and a non-cognate MAb CAT1H12 at a ratio of 1:3 made it possible to obtain comparable binding of AcSpm-HRP to either antibody, which was effectively competed by a cognate diacetylpolyamine. The competitive ELISA systems thus constructed were sensitive enough to determine DiAcSpm in normal urine samples using about 5 μl per well in the M assay, and to determine DiAcSpd using about 10 μl per well in the D assay.

Analytical Performance of the Assays—Figure 1 shows the competition by DiAcSpm and the cross-competition by N^1-AcSpd with HRP-AcSpm for binding to the anti-DiAcSpm antibody in the M assay and by DiAcSpd and N^8-AcSpd with HRP-AcSpm for binding to the anti-DiAcSpd antibody. The cross-reactivity of various polyamine species in the M and D assays, including these polyamines, is summarized in Table I with the reactivities of DiAcSpm and DiAcSpd in the M and D assays, respectively, taken as 100%.

The within-run and between-run variations of the assays were determined with values obtained in 8 measurements in duplicate of 4 samples, and the results are summarized in Table II. The precision and reproducibility of the assays are satisfactory except for a lower DiAcSpd concentration range in the D assay.

The recovery of diacetylpolyamines in these ELISAs was determined to examine whether human urine samples contain any substance that interferes with the determination. The recoveries ranged from 80–115% when authentic polyamines were added at various concentrations (4 and 8 nM DiAcSpm in the M assay and 32 and 64 nM DiAcSpd in the D assay) to urine samples as shown in Table III. The inclusion of 5 and 10 μl of either 4 M NaCl or 4 M urea in the M and D assays, respectively, did not interfere with the results of the diacetylpolyamine measurements (Table IV). The linearity of the assays was confirmed in the range of 3–50 nM for DiAcSpm and 30–500 nM for DiAcSpd, as shown in Fig. 2, by measuring the diacetylpolyamine concentra-

TABLE I. **Cross-reactivity of various polyamines in the M and D assays.** Cross-reactivity (%) for each compound was estimated by averaging the points showing competition comparable to 2–32 nM of DiAcSpm in the M assay and 16–256 nM of DiAcSpd in the D assay. Competition curves for each polyamine species paralleled that of DiAcSpm in the M assay and that of DiAcSpd in the D assay.

Compound	Cross-reactivity (%)	
	M assay	D assay
Putrescine	$<1.9 \times 10^{-5}$	5.6×10^{-4}
Acetylputrescine	2.4×10^{-5}	1.2×10^{-2}
Cadaverine	2.2×10^{-5}	1.2×10^{-3}
Acetylcadaverine	2.8×10^{-5}	8.5×10^{-3}
Spermidine	2.2×10^{-4}	4.4×10^{-3}
N^1-Acetylspermidine	2.6×10^{-1}	1.2×10^{-1}
N^8-Acetylspermidine	5.2×10^{-4}	6.0×10^{-1}
N^1,N^8-Diacetylspermidine	1.3	100
Spermine	2.8×10^{-3}	2.5×10^{-2}
N-Acetylspermine	5.0	6.8
N^1,N^{12}-Diacetylspermine	100	7.6

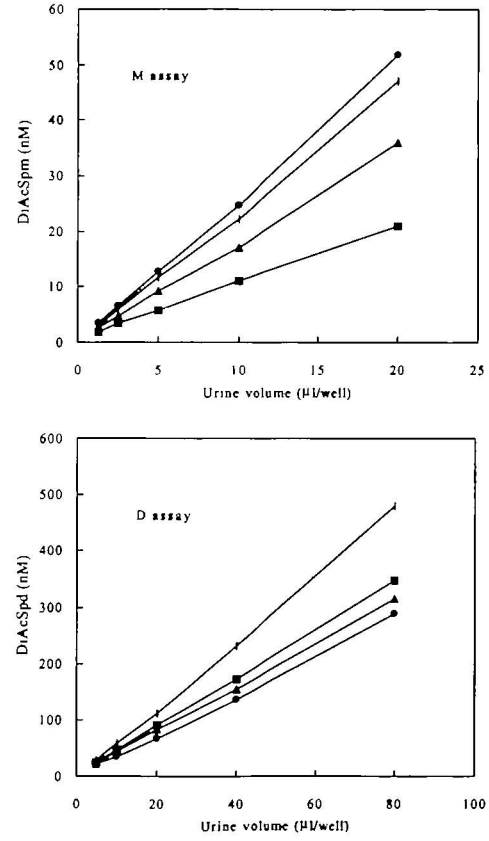

Fig. 2. **Dilution test.** Urine samples from four healthy volunteers were serially diluted with dilution buffer and the concentrations of DiAcSpm (upper) and DiAcSpd (lower) were determined by the M and D assays, respectively.

TABLE II. **Precision of the assays.** CVs were determined from 8 measurements in duplicate of 4 samples. Aliquots of 5 μl and 10 μl per well of urine sample were used in the M and D assay, respectively.

Sample	M assay			D assay		
	Mean (μM)	Within-run CV (%)	Between-run CV (%)	Mean (μM)	Within-run CV (%)	Between-run CV (%)
A	0.059	7.6	10.4	0.195	10.7	20.3
B	0.149	4.3	9.9	0.450	7.9	11.1
C	0.586	4.0	6.9	0.500	6.7	9.9
D	0.537	4.3	8.0	2.537	4.9	6.1

TABLE III. **Recovery of diacetylpolyamines in the assays.** Diacetylpolyamines were added to the wells to give the concentrations indicated. Aliquots of 5 and 10 μl per well of urine sample were used in the M and D assay, respectively.

Sample	M assay		D assay	
	Added (nM)	Recovery (%)	Added (nM)	Recovery (%)
A	4	80.6	32	81.8
	8	87.0	64	86.6
B	4	86.8	32	92.1
	8	87.8	64	100.8
C	4	109.0	32	95.4
	8	91.0	64	90.9
D	4	99.3	32	93.0
	8	115.3	64	96.7

TABLE IV. **Interference by NaCl and urea with the assays.** Aliquots of 5 and 10 μl per well of urine sample were used in the M and D assay, respectively. A corresponding volume of 4 M NaCl or urea was added to the wells. The data are expressed based on the concentration of each sample without interference as 100%.

Sample	Added	M assay (%)	D assay (%)
A	NaCl	94.0	98.8
	Urea	103.5	107.5
B	NaCl	100.6	93.1
	Urea	100.8	104.2
C	NaCl	105.1	104.3
	Urea	109.7	115.0
D	NaCl	98.5	86.1
	Urea	103.7	96.0

tions of 4 urine samples diluted serially.

The diacetylpolyamine content estimated by the proposed ELISA procedure was compared with that obtained by HPLC analysis, for which the reliability is well established. The results presented in Fig. 3 were characterized by correlation coefficients (r), slopes and y-intercepts with 95% confidence intervals as follows: r = 0.952–0.973–0.985, slope = 0.745–0.803–0.861, y-intercept = (–0.015)–0.060–0.134 for DiAcSpm and r = 0.693–0.820–0.897, slope = 0.676–0.861–1.046, y-intercept = (–0.052)–0.181–0.414 for DiAcSpd. The correlation between the values obtained by ELISA and HPLC was satisfactory especially as to DiAcSpm.

Diurnal Variation in Diacetylpolyamine Excretion— Urine samples were collected several times during a 24-h period from each of 5 individuals. Concentrations of DiAcSpm and DiAcSpd, normalized for creatinine concentration in each sample, are presented in Table V. The results indicate that the excretion of diacetylpolyamines is generally stable with only minimal diurnal variation, except for in the evening urine from a 5-year-old male (subject E).

DISCUSSION

We obtained monoclonal antibodies specifically recognizing DiAcSpm and DiAcSpd, and have used them to develop two ELISA systems to measure DiAcSpm and DiAcSpd. The assays are carried out in a 96-well microtiter plate coated with the appropriate antibody and are based on the competition between the diacetylpolyamine to be determined and a common enzyme-labeled antigen (AcSpm -HRP) for binding to the cognate monoclonal antibody on the plate. Under the conditions established in the present study, a comparable amount of enzyme-labeled antigen was bound to plates coated with either antibody, and the binding was competed effectively at similar concentrations of the appropriate diacetylpolyamine, giving a comparable sensitivity of measurement for both DiAcSpm and DiAcSpd. The use of a common enzyme-linked antigen makes it possible to measure the amount of two diacetylpolyamines in a very similar manner, which greatly simplifies the overall design of the procedure for the simultaneous determination of DiAcSpm and DiAcSpd.

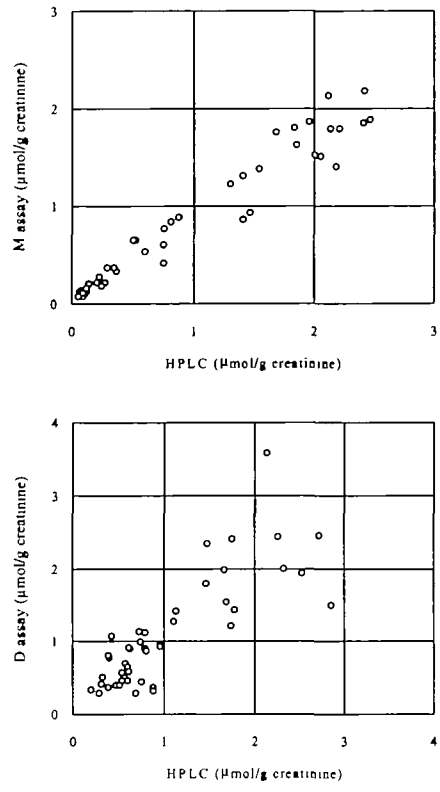

Fig. 3. **Comparisons between HPLC and the ELISAs.** The abscissas indicate the concentration of DiAcSpm (upper) and DiAcSpd (lower) in 45 urine samples as determined by HPLC, while the ordinates indicate the corresponding values as determined by the M assay (upper) and D assay (lower).

As human urine contains various amounts of free and acetylated polyamines, the possibility that cross-reactions might significantly distort the diacetylpolyamine measurements needs to be excluded. Overestimation of DiAcSpm due to cross-reaction with N^1-AcSpd and of DiAcSpd due to N^8-AcSpd should be assessed most carefully, since other polyamine species either show only limited cross-reactivity or are relatively less abundant in urine. Human urine contains, on average, 0.1, 2.7, 0.3, and 2.4 μmol/g creatinine of DiAcSpm, N^1-AcSpd, DiAcSpd and N^8-AcSpd, respectively (*7*). Based on the observed value for the cross-reactivity of N^1-AcSpd with anti-DiAcSpm antibody and that of N^8-AcSpd with anti-DiAcSpd antibody, overestimation due to N^1-AcSpd and N^8-AcSpd in healthy individuals would be approximately 7% in the M assay and 5% in the D assay. The former value is larger than the one expected in our previous ELISA for DiAcSpm that utilizes affinity-purified rabbit polyclonal antibodies (*11*), but is still within the range of precision of the present assay. The overestimation

TABLE V. **Diurnal variation in the excretion of diacetylpolyamines in human urine.** Aliquots of 5 and 10 μl per well of urine sample were used in the M and D assay, respectively. Data are calculated by taking the ratio of total polyamine excretion to total creatinine excretion per day for each person as 100%.

Person	Sampling time	DiAcSpm/Creatinine (%)	DiAcSpd/Creatinine (%)
A	7:30	88.0	94.2
(male, 42-year-old)	13:50	107.9	92.1
	19:40	103.3	105.0
	1:00	103.4	112.7
	Mean ± SD	100.6 ± 8.7	101.0 ± 9.6
B	7:40	102.8	92.6
(female, 39-year-old)	10:40	110.4	103.5
	14:00	102.6	102.4
	16:00	93.9	109.3
	21:00	91.0	102.4
	22:20	95.1	114.8
	Mean ± SD	99.3 ± 7.2	104.2 ± 7.5
C	7:40	101.0	105.7
(male, 11-year-old)	10:50	90.4	93.0
	14:20	104.6	98.0
	18:50	102.2	90.1
	19:30	97.9	107.6
	Mean ± SD	99.2 ± 5.5	98.9 ± 7.7
D	7:30	106.9	105.6
(male, 9-year-old)	12:30	93.9	96.2
	19:40	93.5	93.9
	Mean ± SD	98.1 ± 7.6	98.6 ± 6.2
E	7:30	123.2	119.6
(male, 5-year-old)	9:50	113.9	115.3
	12:50	113.8	107.4
	15:50	80.7	83.7
	18:50	62.9	64.5
	20:40	66.1	86.7
	Mean ± SD	93.4 ± 26.7	96.2 ± 21.4

of DiAcSpd in the D assay is also within the range of precision of the assay. It should also be noted that the interference due to monoacetylpolyamines would, in fact, be lower than the above estimate, since DiAcSpm and DiAcSpd levels tend to increase more markedly than monoacetylpolyamines in tumor patients (*8*). Based on these considerations we conclude that the proposed ELISA systems are specific enough for DiAcSpm and DiAcSpd to be used in clinical determinations of these diacetylpolyamines.

The assay systems show good linearity over a wide range of DiAcSpm and DiAcSpd concentrations, and tolerate a considerable amount of NaCl and Urea. The recovery of exogenously added diacetylpolyamines was thus satisfactory. Finally, the correlation between the values obtained by ELISA and HPLC was satisfactory, especially as to DiAcSpm. Altogether, the ELISA systems developed during the course of this study are suitable for measuring urinary diacetylpolyamines in that they are highly specific, reproducible, quantitative, and practically free from interference by other polyamine species as well as NaCl and Urea, which are abundant in urine.

The ELISA systems were used to assess diurnal variations in diacetylpolyamine excretion in urine. It was shown that the excretion of diacetylpolyamines after normalization as to the concentration of creatinine is stable over the period a day with only minimal diurnal variation. This validates the use of spot urine samples instead of pooled urine samples in estimating the daily excretion of diacetylpolyamines, which would help to simplify the protocol for clinical examination. Samples from a 5-year-old male showed a decrease in the amount of creatinine-normalized diacetylpolyamine excretion toward evening, but it should be noted that even in this case, the secretion of diacetylpolyamines in urine samples collected in the morning did not deviate extensively from the daily average.

The daily excretions of DiAcSpm from subjects A to E were 0.099, 0.144, 0.246, 0.254, and 0.283 μmol, and those of DiAcSpd were 0.352, 0.277, 0.287, 0.252, and 0.290 μmol, respectively. It may be that children tend to excrete specifically more DiAcSpm than adults, with the excretion of DiAcSpd almost independent of age, but this should be examined more carefully in future studies.

We thank K. Yamada, K. Kitano, and F. Kano (Chemistry Laboratory, Yamasa Corporation) for providing DiAcSpm and DiAcSpd.

REFERENCES

1. Russell, D.H., Levy, C.C., Schimpff, S.C., and Hawk, I.A. (1971) Urinary polyamines in cancer patients. *Cancer Res.* **31**, 1555–1558
2. Russell, D.H. (1971) Increased polyamine concentrations in the urine of human cancer patients. *Nat. New Biol.* **233**, 144–145
3. Campbell, R.A., Hunt-Retzlaff, Z., and Russi, J.B. (1989) Polyamines in health and disease in *The Physiology of Polyamines* (Bachrach, U. and Heimer, Y.M., eds.) Vol. II, pp. 163–182, CRC Press, Florida
4. Bachrach, U. (1989) Polyamines as indicators of disease activity and response to therapy in *The Physiology of Polyamines* (Bachrach, U. and Heimer, Y.M., eds.) Vol. II, pp. 235–249, CRC Press, Florida
5. Löser, C., Fölsch, U.R., Paprotny, C., and Creutzfeldt, W. (1990) Polyamines in colorectal cancer, evaluation of polyamine concentrations in colon tissue, serum and urine of 50 patients with colorectal cancer. *Cancer* **65**, 958–966
6. Bachrach, U. (1992) Polyamines as markers of malignancy. *Prog. Drug Res.* **39**, 9-33
7. Hiramatsu, K., Sugimoto, M., Kamei, S., Hoshino, M., Kinosh-

ita, K., Iwasaki, K., and Kawakita, M. (1995) Determination of amounts of polyamines excreted in urine: demonstration of N^1,N^8-diacetylspermidine and N^1,N^{12}-diacetylspermine as components commonly occurring in normal human urine. *J. Biochem.* **117**, 107–112
8. Sugimoto, M., Hiramatsu, K., Kamei, S., Kinoshita, K., Hoshino, M., Iwasaki, K., and Kawakita, M. (1995) Significance of urinary N^1,N^8-diacetylspermidine and N^1,N^{12}-diacetylspermine as indicators of neoplastic diseases. *J. Cancer Res. Clin. Oncol.* **121**, 317–319
9. Hiramatsu, K., Sugimoto, M., Kamei, S., Hoshino, M., Kinoshita, K., Iwasaki, K., and Kawakita, M. (1997) Diagnostic and prognostic usefulness of N^1,N^8-diacetylspermidine and N^1,N^{12}-diacetylspermine in urine as novel markers of malignancy. *J. Cancer Res. Clin. Oncol.* **123**, 539–545
10. Hiramatsu, K., Miura, H., Sugimoto, K., Kamei, S., Iwasaki, K., and Kawakita, M. (1997) Preparation of antibodies highly specific to N^1,N^8-diacetylspermidine and development of an enzyme-linked immunosorbent assay (ELISA) system for its sensitive and specific detection. *J. Biochem.* **121**, 1134–1138
11. Hiramatsu, K., Miura, H., Kamei, S., Iwasaki, K., and Kawakita, M. (1998) Development of a sensitive and accurate enzyme-linked immunosorbent assay (ELISA) system that can replace HPLC analysis for the determination of N^1,N^{12}-diacetylspermine in human urine. *J. Biochem.* **124**, 231–236
12. Nakane, P.K. (1979) Preparation and standardization of enzyme-labeled conjugates in *Immunoassays in the Clinical Laboratory* (Nakamura, R.M., Dito, W.R., and Tucker, E.S., eds.), 3rd edition, pp. 81–87, Alan R. Liss Inc., New York
13. Shulman, M., Wilde, C.D., and Köhler, G. (1978) A better cell line for making hybridomas secreting specific antibodies. *Nature* **276**, 269–270
14. Köhler, G. and Milstein, C. (1975) Continuous culture of fused cells secreting antibody of predefined specificity. *Nature* **256**, 495–497
15. Hiramatsu, K., Kamei, S., Sugimoto, M., Kinoshita K., Iwasaki, K., and Kawakita, M. (1994) An improved method of determining free and acetylated polyamines by HPLC involving an enzyme reactor and an electrochemical detector. *J. Biochem.* **115**, 584–589