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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[1]

Kyoko Hiramatsu,* Hiroko Miura,* Sachiko Kamei,† Kentaro Iwasaki,* and Masao Kawakita*,[2]

*Department of Physiological Chemistry, The Tokyo Metropolitan Institute of Medical Science (Rinshoken), 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8613; and †School of Allied Health Science, Faculty of Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519

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N^1,N^{12}-Diacetylspermine (DiAcSpm)-specific antibodies were raised in rabbits, using N-acetylspermine coupled to mercaptosuccinylated BSA *via* N-(4-maleimidobutyryloxy)-succinimide as an antigen. Highly DiAcSpm-specific antibodies were enriched from crude sera through a series of affinity-based fractionations. A competitive ELISA system, intended for measuring DiAcSpm in solution, was constructed using this antibody preparation, with N-acetylspermine coupled to a synthetic peptide *via* N-(8-maleimidocapryloxy)-succinimide as a solid phase antigen. The K_i value for DiAcSpm with this competitive ELISA system was 33 nM, and the cross-reactivity with DiAcSpm, AcSpm, DiAcSpd, N^1-AcSpd, and N^8-AcSpd was 100, 0.29, 0.20, 0.033, and 0.055%, respectively. This procedure can be applied to the determination of DiAcSpm in human urine samples, giving highly reproducible results. The coefficients of variation obtained were 6.7 and 4.2% for within-run and between-run precision, respectively. The correlation coefficient between DiAcSpm concentrations in urine estimated by ELISA and those by HPLC analysis was calculated to be 0.99, and the regression equation was expressed as $y=1.04x+0.026$ μM.

Key words: anti-polyamine antibody, diacetylspermine, ELISA, tumor marker, urine.

During the course of our comprehensive analysis of human urine for polyamines, using HPLC in combination with an enzymatic detection system involving polyamine-specific deacetylation and subsequent oxidation of individual polyamine components eluted from the HPLC column, we found that N^1,N^8-diacetylspermidine (DiAcSpd) and N^1,N^{12}-diacetylspermine (DiAcSpm) occur regularly in small quantities in urine from healthy persons (*1*). These diacetylated polyamine species had been largely ignored up to that time in the field of polyamine study (for reviews, see Refs. *2* and *3*) besides a few exceptions (*4, 5*). This is because their levels are very low in human urine and also because they can not be detected by commonly used post-column derivatization methods due to the absence of primary amino groups (*6, 7*).

Interestingly, on further analysis of these rediscovered polyamine components we found that diacetylpolyamines were markedly increased in patients with urogenital malignancies (*8*). We also demonstrated that the diacetyl polyamine levels in cancer patients rapidly decreased when they were subjected to effective treatments, and the levels after the treatments were very well correlated with their prognoses (*9*). Based on these observations we came to the conclusion that the urinary diacetylpolyamine level may serve as a tumor marker which is particularly useful in assessing the effectiveness of treatment given to a patient and in predicting the prognoses of patients in partial remission (*9*).

However, it seemed to us that HPLC analysis of polyamines is rather time-consuming and inconvenient, if these two diacetylpolyamines are to be used clinically as diagnostic and prognostic indicators of malignancy. This prompted us to develop an enzyme-linked immunosorbent assay (ELISA) for DiAcSpd and DiAcSpm as an alternative to HPLC analysis. As the outcome of this study, we previously reported the preparation of a highly DiAcSpd-specific antibody, and a procedure for sensitive and specific determination of DiAcSpd based on a competitive ELISA involving a specific antibody (*10*).

The previous work was further extended, and we have

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[2] To whom correspondence should be addressed. Phone: +81-3-3823-2101 (Ext. 5285), Fax: +81-3-3823-2965, E-mail: kawakita@rinshoken.or.jp

Abbreviations: Ac-Put, acetylputrescine; AcSpm, acetylspermine; BSA, bovine serum albumin; DiAcSpd, N^1,N^8-diacetylspermidine; DiAcSpm, N^1,N^{12}-diacetylspermine; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; ELISA, enzyme-linked immunosorbent assay; GMBS, N-(4-maleimidobutyryloxy)succinimide; HMCS, N-(8-maleimidocapryloxypropyl)succinimide; N^1-AcSpd, N^1-acetylspermidine; N^8-AcSpd, N^8-acetylspermidine.

now succeeded in obtaining an antibody preparation highly specific to DiAcSpm and have established a competitive ELISA system for measuring urinary DiAcSpm, which is described below. The affinity-purified anti-DiAcSpm antibody preparation obtained in the present study exhibited only 0.03% cross reactivity with N^1-AcSpd. Our previous measurements demonstrated that N^1-AcSpd is, on average, 30-times more abundant in human urine than DiAcSpm (*1*), and that it seriously interferes with the determination of DiAcSpm, causing indeterminate overestimation of the DiAcSpm content, if antibodies of insufficient specificity for DiAcSpm are utilized in the assay (*10*). Determination of the urinary DiAcSpm content using our highly purified anti-DiAcSpm antibody gave values agreeing very well with those obtained on HPLC analysis.

MATERIALS AND METHODS

Chemicals—Monoacetyl polyamines, *S*-acetylmercaptosuccinic anhydride, and bovine serum albumin (BSA) were purchased from Sigma, St. Louis, MO, USA. DiAcSpd and DiAcSpm were kind gifts from Dr. A. Shirahata of Josai University. *N*-(4-Maleimidobutyryloxy)succinimide (GMBS) and *N*-(8-maleimidocapryloxy)succinimide (HMCS) were from Dojindo Laboratories, Kumamoto, and AF-Carboxy-Toyopearl 650 from Tosoh, Tokyo, respectively.

Preparation of Monoacetylspermine-Carrier Conjugates—Acetylspermine (AcSpm) was conjugated to mercaptosuccinylated BSA *via* GMBS or to a synthetic peptide with the sequence, CEFLASVTKDTTSDSPAGIDN, *via* HMCS, as described previously (*10*). The resulting conjugates were designated as AcSpm-GMB-BSA and AcSpm-HMC-peptide, respectively (refer to Fig. 1 in Ref. *10* for the structures of these conjugates and the polyamine-conjugated resins described below).

Preparation of Polyamine Derivatives of AF-Carboxy-Toyopearl 650 with Acylamide Linkages—AF-Carboxy-Toyopearl 650 (2 g of swollen gel) was washed once with 0.5 M NaCl, mixed with 2 mM AcSpm or N^1-acetylspermidine (N^1-AcSpd) (7 ml), and then reacted with 40 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) at room temperature for 48 h at pH 5-6. The polyamine-conjugated resin was washed successively with 1 M NaCl and with 1 M NaCl in 10 mM HCl, and then finally stored in 10 mM HCl at 4°C.

Preparation of Antisera—Two New Zealand White rabbits were immunized according to the protocol described previously, using AcSpm-GMB-BSA conjugate as an antigen, and monitored as to the antibody titer (*10*). Each animal was finally bled from the heart, and the sera were separately stored at −80°C.

Affinity Purification of Antibodies—The IgG fraction was prepared from crude antiserum using an immobilized Protein A column (Ampure PA; Amersham, Buckinghamshire, UK). One milliliter of the anti-DiAcSpm IgG thus obtained (0.5 ml crude serum equivalent) was diluted with 8 ml of 10 mM Tris-HCl buffer (pH 7.5), and then applied to a column (0.5 ml) of AcSpm-conjugated AF-Carboxy-Toyopearl 650. The column was washed successively with 5 ml each of 10 mM Tris-HCl buffer (pH 7.5) and 10 mM Tris-HCl buffer (pH 7.5) containing 0.5 M NaCl, and then the adsorbed proteins were eluted with 0.1 M glycine-HCl buffer (pH 2.5). The eluate was immediately neutralized by adding a 1/10 volume of 1 M Tris-HCl buffer (pH 8.0), and then the fractions containing partially purified anti-DiAcSpm antibodies were combined (Fr. 1) and saved. In the next step of purification, 2 ml of the Fr. 1 antibody (1 ml crude serum equivalent) was diluted to 10 ml with 10 mM Tris-HCl buffer (pH 7.5), and then applied to a column (0.5 ml) of N^1-AcSpd-conjugated AF-Carboxy-Toyopearl 650 equilibrated with 10 mM Tris-HCl buffer (pH 7.5). The flow-through fraction was collected (Fr. 2) and saved for further purification. The Fr. 2 antibody was then adsorbed again to a 0.5-ml column of AcSpm-conjugated AF-Carboxy-Toyopearl 650. The column was washed successively with 5 ml each of 10 mM Tris-HCl buffer (pH 7.5) and 10 mM Tris-HCl buffer (pH 7.5) containing 0.5 M NaCl, and then eluted with 10 ml of 2 mM N^1-AcSpd in 10 mM Tris-HCl buffer (pH 7.5). The column was washed successively with 10 ml each of 10 mM Tris-HCl buffer (pH 7.5) and 10 mM Tris-HCl buffer (pH 7.5) containing 0.5 M NaCl, and then the proteins remained adsorbed to the column were eluted with 0.1 M glycine-HCl buffer (pH 2.5). The eluate containing highly purified anti-DiAcSpm antibodies (Fr. 3) was immediately neutralized by adding a 1/10 volume of 1 M Tris-HCl buffer (pH 8.0), and then stored frozen in small aliquots at −80°C.

Determination of the Relative Specificity of Anti-DiAcSpm Antibodies for Polyamine Species—The wells of microtiter plates (Corning #25801) were coated with 0.05 ml of AcSpm-HMCS-peptide conjugate (1.0 μg/ml) in 0.1 M phosphate buffer (pH 7.4) containing 0.15 M NaCl for 1 h at room temperature, and then washed 3 times with 0.2 ml of a washing solution consisting of 0.14 M NaCl, 20 mM Tris-HCl (pH 7.6), and 0.02% Tween 20. The wells were then blocked with 0.2 ml of 5% skimmed milk in the washing solution overnight at 37°C, and washed 3 times with 0.2 ml of the washing solution. An appropriate concentration of competing polyamines (0.05 ml) was added to the wells, followed by anti-DiAcSpm antibodies, appropriately diluted in 0.1 M phosphate buffer (pH 7.4) containing 0.15 M NaCl (0.05 ml), and then the plate was incubated at room temperature for 1 h with constant shaking. The antibodies and competitors were removed, and the wells were washed 3 times with 0.2 ml of the washing solution. To each well was added horseradish peroxidase-labeled anti-rabbit IgG (Zymed, South San Francisco, CA, USA) at 1:5,000 dilution (0.1 ml), and then the plate was kept for 2 h at room temperature. The wells were washed 3 times with 0.2 ml of the washing solution, and then 0.1 ml of a H_2O_2-2,2′-azino-di[3-ethyl-benzothiazoline sulfonate (*6*)] (ABTS) solution (KPL, Gaithersburg, MD, USA) was added to each well. After keeping the plate for 30 min at room temperature, the absorbance at 405 nm was measured.

Determination of the Amount of DiAcSpm in Human Urine—The competitive ELISA, described above, was carried out using the Fr. 3 anti-DiAcSpm antibody at 1:3,000 dilution. Serially diluted urine samples (0.05 ml) were added to the microtiter wells in place of competing polyamine solutions. The dilution of a urine sample which gave 50% reduction of the binding of the antibody to the solid-phase antigen (D_{50}) was estimated by fitting the data points to the standard curve obtained with DiAcSpm as the competitor. Then, $K_{I,DIAcSpm}$ multiplied by D_{50} gives the

concentration of DiAcSpm in the urine sample. To examine the analytical recovery, appropriate amounts of authentic DiAcSpm were added to selected urine samples. The samples were then serially diluted and analyzed for DiAcSpm as above.

Analysis by HPLC was carried out exactly as described previously (*11*).

The DiAcSpm concentrations in the urine samples are presented throughout this study without normalization as to the creatinine content.

Urine Samples—Urine samples were collected from 16 healthy children, ranging from 2 to 10 years old, and 15 adults comprising 4 healthy volunteers, 6 patients with benign urogenital disorders, and 5 cancer patients in remission (1 with prostate cancer, 3 with testicular cancer, and 1 with bladder cancer). The samples were collected and stored as described previously (*9*).

RESULTS

Preparation of Anti-DiAcSpm Antisera and Purification of DiAcSpm-Specific Antibodies by Affinity-Chromatography—Antisera were raised in rabbits by immunization with AcSpm-GMB-BSA conjugate. The IgG fraction obtained from these antisera showed distinct specificity for DiAcSpm (Table I). The K_i value for DiAcSpm on competitive ELISA at this stage was 0.12 nM, and the cross-reactivity with DiAcSpm, AcSpm, N^1-AcSpd, DiAcSpd, and N^8-acetylspermidine (N^8-AcSpd) was 100, 23, 6.7, 1.1, and less than 0.24%, respectively (cross-reactivity with substance "s" is defined as $100 \times K_{i,\mathrm{DiAcSpm}}/K_{i,s}$). These values imply that polyamine components other than N^1-AcSpd would not significantly interfere with the determination of the urinary DiAcSpm content, if this preparation were used as such for the competitive ELISA. On the other hand, it was clear that cross-reactivity with N^1-AcSpd would cause considerable overestimation of the amount of DiAcSpm in urine, since human urine contains approximately 30-times more N^1-AcSpd than DiAcSpm, on average, as determined previously by HPLC (*1*). AcSpm would not cause much trouble, since it only occurs in a trace amount in human urine.

The anti-DiAcSpm IgG preparation was subjected to a series of affinity-based purification steps to improve the specificity of the antibody preparation for DiAcSpm relative to other polyamine species. Thus, in the first step of purification, antibodies were selected based on their ability to be adsorbed on an AcSpm-Carboxy-Toyopearl column, which has specific ligands mimicking DiAcSpm. While in the second step, those trapped by an N^1-AcSpd-Carboxy-Toyopearl column were discarded, the unadsorbed ones being saved. The K_i values for various polyamines in the standard competitive ELISA involving the Fr. 1 and Fr. 2 antibodies are listed in Table I. At first glance, these steps did not significantly improve the specificity of the antibodies to DiAcSpm. The K_i values in Table I show only modest changes through these steps. However, we noted an interesting change in the shape of the competition curve with N^1-AcSpd between the Fr. 1 and Fr. 2 antibodies, as shown in Fig. 1. With the Fr. 1 antibody, the binding of the antibody to the solid phase antigen, AcSpm-HMC-peptide, was completely abolished when the competing N^1-AcSpd was increased to 100 μM (Fig. 1A). In contrast, with the Fr. 2 antibody, N^1-AcSpd could not completely abolish the binding of the antibody even at concentrations above 100 μM, leaving about 10% of the bound antibody attached to the plate (Fig. 1B). Competition with DiAcSpm was similar

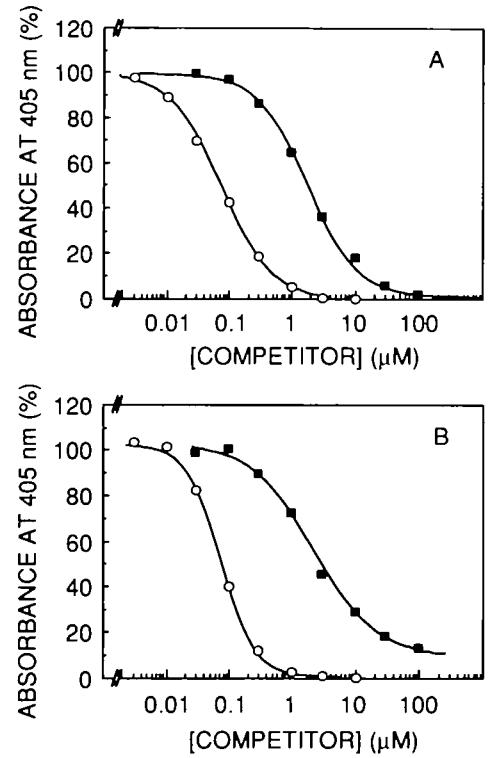

Fig. 1. **Cross-reactivity of N^1-AcSpd with the Fr. 1 and Fr. 2 antibodies, as assessed by competitive ELISA with AcSpm-HMC-peptide conjugate as the solid-phase antigen.** Competitive ELISA was carried out as described under "MATERIALS AND METHODS" with AcSpm-HMC-peptide conjugate as the solid phase antigen, using the Fr. 1 antigen at $1:9 \times 10^5$ dilution (A) or the Fr. 2 antigen at $1:1.3 \times 10^5$ dilution (B). DiAcSpm (○) and N^1-AcSpd (■), at the concentrations indicated on the abscissa, were used as competitors.

TABLE I. **Summary of the properties of the anti-DiAcSpm antibodies at different steps of purification.** The antibody titer is expressed in terms of the dilution ratio, at which the bound antibody gave an A_{405} value of 1.0 under the standard ELISA conditions, as normalized as to the volume of the crude antiserum. The concentration of each polyamine component (K_i), at which the A_{405} value decreased to 50% of that in the absence of the competitor, is presented.

	Titer	K_i(μM)					
		DiAcSpm	AcSpm	N^1-AcSpd	DiAcSpd	N^8-AcSpd	AcPut
IgG	1×10^6	0.12	0.53	1.78	11.2	>50	≫100
Fr. 1	9×10^5	0.071	0.33	1.58	7.9	>50	≫100
Fr. 2	2×10^5	0.089	0.42	2.51	10.0	>100	≫100
Fr. 3	6×10^3	0.033	11.5	100	16.2	60	≫100

and complete with either Fr. 1 or Fr. 2.

The shape of the competition curve with N^1-AcSpd in Fig. 1B suggested that the Fr. 2 antibody was heterogeneous with respect to the relative affinities to N^1-AcSpd and the solid-phase antigen, and that a part of this fraction showed only very weak cross-reactivity with N^1-AcSpd. To determine whether this is the case or not, we performed the competition experiment in the presence of 10 μM N^1-AcSpd. The results shown in Fig. 2 clearly indicate that the population of antibodies in Fr. 2 that could still bind to the solid-phase antigen in the presence of 10 μM N^1-AcSpd exhibits extraordinarily high specificity for DiAcSpm over N^1-AcSpd as well as other polyamine species. This particular population was extracted in the third step of purification. In this step, antibodies were first bound to an AcSpm-Carboxy-Toyopearl column, and then the unwanted majority of the bound antibodies was eluted with N^1-AcSpd. Finally, the antibodies remaining on the column were recovered to yield a highly specific anti-DiAcSpm antibody preparation (Fr. 3).

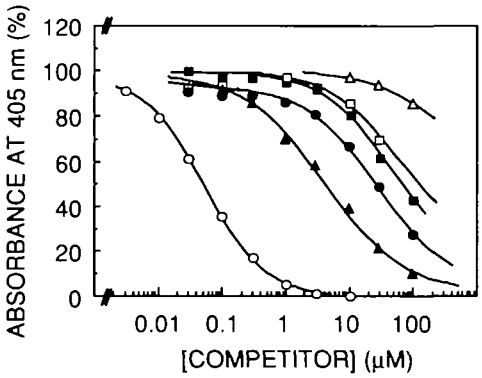

Fig. 2. **Specificity of the Fr. 2 antibody to DiAcSpm, as assessed by competitive ELISA in the presence of 10 μM N^1-AcSpd with AcSpm-HMC-peptide conjugate as the solid-phase antigen.** Competitive ELISA was carried out as in Fig. 1, using the Fr. 2 antibody at 1:15,000 dilution, except that 10 μM N^1-AcSpd was added to each well together with an appropriate competitor. DiAcSpm (○), AcSpm (▲), N^1-AcSpd (■), DiAcSpd (●), N^8-AcSpd (□), and AcPut (△), at the concentrations indicated on the abscissa, were used as competitors.

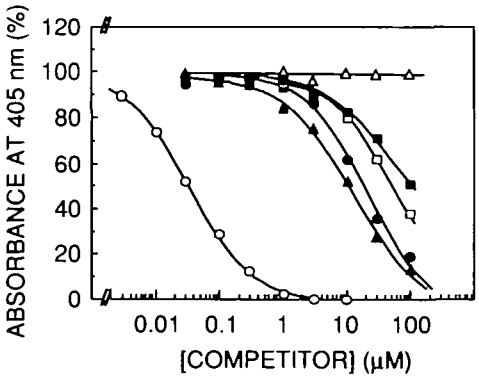

Fig. 3. **Specificity of the Fr. 3 antibody to DiAcSpm, as assessed by competitive ELISA with AcSpm-HMC-peptide conjugate as the solid-phase antigen.** Competitive ELISA was carried out as in Fig. 1, using the Fr. 3 antibody at 1:6,000 dilution. DiAcSpm (□), AcSpm (▲), N^1-AcSpd (▲), DiAcSpd (●), N^8-AcSpd (□), and AcPut (△), at the concentrations indicated on the abscissa, were used as competitors.

Competition curves with various polyamine species with the solid-phase antigen for the Fr. 3 antibody are presented in Fig. 3, and the corresponding K_i values are listed in Table I. Cross-reactivity with N^1-AcSpd was as low as 0.033%, which implies that N^1-AcSpd, occurring in a 30-fold in excess over DiAcSpm in human urine, would interfere with the determination of the latter by competitive ELISA by only less than 1%. Overestimation due to cross-reactions would not exceed 2.5%, when interference with N^8-AcSpd [24-fold in excess over DiAcSpm (*1*)] was also taken into account. Free polyamines, *i.e.* putrescine, cadaverine, spermidine, and spermine, did not cross-react at all with the Fr. 3 antibody at 100 μM (data not shown). It should also be noted that the K_i value for DiAcSpm, which limits the sensitivity of DiAcSpm-detection, decreased to 33 nM in this step, as shown in Table I. This K_i value is sufficiently low as compared to the average concentration of DiAcSpm in healthy human urine, which is approximately 100 nM (*1*).

Determination of DiAcSpm in Urine Samples—Based on these results, we considered that the competitive ELISA system involving the purified anti-DiAcSpm antibody (the Fr. 3 antibody) must be sensitive and specific enough to allow the determination of the amount of urinary DiAcSpm in the presence of interfering polyamine species including N^1-AcSpd. In fact, the validity of this procedure was demonstrated through a series of experiments, as described below.

We first examined whether or not human urine samples contain any substance that nonspecifically interferes with the determination of DiAcSpm. Appropriate amounts of authentic DiAcSpm were added to human urine samples from a healthy adult (No. 1) and a patient who had once suffered from testicular cancer but was currently in remission (No. 2), and the samples were analyzed for DiAcSpm. The recovery of DiAcSpm was practically quantitative, ranging between 99-106% (Table II).

The within-run precision of this procedure was then determined. A normal urine sample from a healthy adult was analyzed on a 96-well microtiter plate, and the mean value, standard deviation, and coefficient of variation were calculated, which are shown in Table III. To estimate the

TABLE II. **Analytical recovery of DiAcSpm externally added to urine samples.**

Urine sample	DiAcSpm added (μM)	DiAcSpm detected (μM)	Recovery (%)
1	0	0.090	—
	0.1	0.195	105
	0.5	0.620	106
2	0	0.298	—
	0.2	0.500	101
	0.5	0.794	99

TABLE III. **Precision of the competitive ELISA.**

	Mean (μM)	SD (μM)	C.V. (%)	*n*
Within-run precision	0.18	0.012	6.7	12
Between-run precision	0.18	0.0075	4.2	11

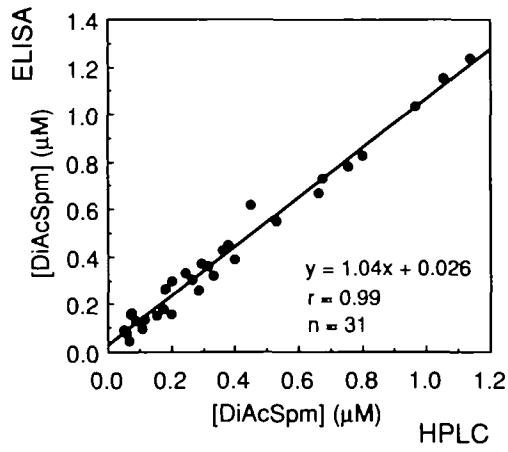

Fig. 4. **Comparison between the concentrations of DiAcSpm in human urine determined by the ELISA and HPLC procedures.** The abscissa indicates the concentration of DiAcSpm in urine determined by the standard HPLC procedure (*11*), while the ordinate indicates the corresponding values determined by competitive ELISA, as described under "MATERIALS AND METHODS."

between-run precision, the same urine sample was analyzed on a number of occasions on separate plates, and the relevant analytical variables are also shown in Table III. The coefficients of variation were 6.7 and 4.2% for within-run and between-run precision, respectively, indicating that the analytical values obtained are highly reproducible.

Comparison between the Analytical Values Obtained with ELISA and HPLC—Finally, we compared the DiAcSpm content estimated by competitive ELISA with that obtained by HPLC analysis, whose reliability is well-established (*1, 11*). Urine samples obtained from 31 subjects, whose DiAcSpm concentrations were distributed in the range of 0.03 to 1.15 μM, were analyzed by both methods, and the results are presented in Fig. 4. These samples included ones from children under 15 years old, whose urinary DiAcSpm content is usually much higher than that of adults (Hiramatsu, K., unpublished observation). From Fig. 4, the correlation coefficient, r, between the two values was calculated to be 0.99, and the regression equation was expressed as $y=1.04x+0.026$ μM. Based on these results, we can eliminate the possibility of overestimation of DiAcSpm due to any other polyamine components in the urine, and are convinced that the competitive ELISA involving the highly purified anti-DiAcSpm antibody may replace HPLC analysis for the reliable assessment of the DiAcSpm level in human urine.

DISCUSSION

An antibody preparation highly specific to DiAcSpm was obtained, and a sensitive and accurate competitive ELISA system for determining DiAcSpm in urine samples was developed. The principle of the molecular design of the hapten-carrier conjugate used in the present study is the same as that described and discussed in detail in our previous report on DiAcSpd-specific antibodies (*10*). AcSpm conjugated to GMBS utilized here closely mimics DiAcSpm, and AcSpm-GMB-BSA conjugate served as a potent antigen for raising DiAcSpm-selective antibodies. However, the specificity of the crude antibody preparation for DiAcSpm over N^1-AcSpd, in particular (15-20 times higher affinity for the former), was insufficient for our purpose. It should be recalled that the antibody is above all intended for practical application to the determination of DiAcSpm in human urine samples, to provide diagnostic and prognostic information that facilitates the treatment of cancer patients.

Human urine contains, on average, 0.1 and 2.7 μmol/g creatinine of DiAcSpm and N^1-AcSpm, respectively (*1*). This represents a serious problem in the immunochemical determination of DiAcSpm. DiAcSpm shows considerable structural overlapping with AcSpm and N^1-AcSpd. Consequently, we should expect that any antibody which recognizes DiAcSpm would also be cross-reactive with AcSpm and N^1-AcSpd, at least to some extent. Immunochemical determination of urinary DiAcSpm is thus highly prone to overestimation due to the cross-reaction with N^1-AcSpd, although AcSpm would not cause significant trouble, since its urinary content is almost negligible (*1*). A simple calculation, based on the average contents of DiAcSpm and N^1-AcSpd in human urine, revealed one should keep the cross-reactivity with the latter at less than 0.15% to keep this indeterminate error due to N^1-AcSpd below 5%. The specificity requirement should be more strict, perhaps less than 0.1% cross-reactivity, as it was noted that the N^1-AcSpd/DiAcSpm ratio fluctuates considerably among individuals.

With the above consideration in mind, we tried to isolate such a population of antibodies that meet the most strict requirement discussed above. Elution with N^1-AcSpd from the DiAcSpm-mimicking ligand turned out to be very effective, an antibody that fulfills the required quality specification being finally obtained. This is quite reasonable because antibodies must have been fractionated according to the relative affinity to N^1-AcSpd and DiAcSpm under the conditions used. As a result, the most DiAcSpm-preferring population remained on the column, and was recovered later as the Fr. 3 antibody. Other steps in the present purification protocol might not seem very effective, but were indispensable. When either of these steps was omitted, the antibody thus obtained invariably showed 0.5-0.6% cross-reactivity with N^1-AcSpd (Hiramatsu, K., unpublished observation).

The purified anti-DiAcSpm antibody, Fr. 3, showed only 0.29, 0.20, 0.033, and 0.055% cross-reactivity with AcSpm, DiAcSpd, N^1-AcSpd, and N^8-AcSpd, respectively. This is well above the quality specification required. It is also important that the K_i value for DiAcSpm in the competitive ELISA (33 nM) was low enough to allow the determination of DiAcSpm at 10-20 nM, which is well below its average urinary concentration. The antibody titer was substantially decreased during the affinity-purification, but we think the titer of the purified anti-DiAcSpm antibody (Fr. 3) was high enough to be practical, since we can use it at a dilution of 1:6,000, as normalized as to the volume of the crude antiserum, in the standard competitive ELISA.

The competitive ELISA involving the Fr. 3 antibody, with AcSpm-HMC-peptide conjugate as the solid-phase antigen, proved to be applicable to the determination of the amount of DiAcSpm in human urine. The recovery of authentic DiAcSpm, which was added to urine samples prior to the assay, was quantitative, and the reproducibility

of the analytical values was excellent. Moreover, the DiAcSpm concentration determined by ELISA agreed very well with that determined by HPLC. This definitely indicates that the values obtained by ELISA are free from possible error due to other polyamine species including N^1-AcSpd, and that the competitive ELISA system described here can replace HPLC analysis for the determination of the urinary DiAcSpm level.

Our analysis of DiAcSpm in human urine, so far as carried out by HPLC, has strongly suggested that DiAcSpm behaves as an informative tumor marker, which is particularly useful as an indicator of the effectiveness of clinical treatments and of the prognoses of patients in remission, in cases of malignant urogenital disorders. We pointed out previously that DiAcSpm may serve as a tumor marker generally applicable to a wide variety of neoplastic diseases, not only urogenital malignancies (*8, 9*). However, this possibility remains to be examined by determining the amounts of DiAcSpm in urine samples collected from a large number of patients with a wide variety of diseases. HPLC analysis, with which it takes 2 h to analyze one sample, is obviously not adequate for this purpose. It is important that the analytical procedure is much simpler in ELISA than in HPLC. In addition, it should be noted that the pre-treatment of urine samples involving a cation-exchange column, which was indispensable in the HPLC analysis (*1, 11*), is not necessary for the analysis by ELISA. Urine samples can be used directly for the competitive ELISA methods. This leads to another significant simplification of the overall procedure of the ELISA, as compared to HPLC analysis. The development of this simple ELISA procedure for the accurate determination of DiAcSpm in human urine will greatly help us establish the usefulness of DiAcSpm as an indicator of malignancy in cases of urogenital as well as various other diseases, and will facilitate its clinical application.

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