

Direct Determination of Polyamines in Human Serum by Radioimmunoassay¹

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SUMMARY

Antipolyamine antibodies were produced following immunization of New Zealand white rabbits with thyroglobulin spermine conjugate, using multiple-site injection and macrophage-harvesting techniques. The antispermine antibody in a radioimmunoassay system cross-reacted with spermidine (22%), putrescine (1%), diaminopropane (6%), and cadaverine (0.16%). No reaction with L-lysine, ornithine, or histamine was noted. Using this antibody, the radioimmunoassay for direct determination of serum polyamines was developed. A sensitivity of 200 pg was achieved on human serum samples. This radioimmunoassay would appear to be a useful tool for the detection and monitoring of a variety of pathophysiological states associated with abnormal polyamine metabolism, *i.e.*, cancer.

INTRODUCTION

The important role of polyamines in normal and abnormal biological processes has gained recognition in recent years (2, 13, 16, 19). The rapidly accumulating biomedical literature has stimulated several major reviews devoted to clarification of the biochemical and physiological behavior of those organic polycations (2, 13, 16). Published reports by Gehrke *et al.* (3), Marton *et al.* (7), and Russell *et al.* (14), indicating abnormalities in blood and urine polyamine levels in cancer, point to the possible clinical utility of the polyamines as markers for screening, diagnostic, and therapeutic monitoring programs.

Presently available methods for determination of polyamine levels in serum require acid hydrolysis of the sample, followed by extraction procedures in preparation for column separation (17). The nmole sensitivity may be achieved with a 3- to 5-ml serum sample. While autoamino analysis (7, 17) and gas-liquid chromatography techniques (3) enable discrete separation of the individual polyamines, the results of these procedures on serum and urine hydrolysates provide composite values for the individual polyamines which are, presumably, the sums of the protein-bound, free, and conjugated forms in the initial sample.

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The application of radioimmunoassay to polyamine biochemistry offers a new dimension to information gathering about polyamines in biological systems. Quash *et al.* (9-12), who first reported the production of antipolyamine antibodies, used them in a number of immunological and oncological experiments, with techniques other than radioimmunoassay. With the successful development of antipolyamine antibodies, a radioimmunoassay could then be designed that theoretically might be able to (a) be carried out directly on specimens of native fluid, (b) minimize sample collection volumes, (c) achieve pg sensitivity, and (d) provide information about free polyamines in cell-free systems.

In this paper we detail our results in developing a radioimmunoassay method for the direct determination of polyamines in serum samples, using rabbit antipolyamine antibodies.

MATERIALS AND METHODS

Biologicals

New Zealand white male rabbits, approximately 6 pounds in weight, were housed in separate cages in an air-conditioned, temperature-controlled room. Purina laboratory chow and water were provided *ad libitum*. Animal conditions were observed daily, and body weights were checked before injections.

Materials

The reagents used were as follows: [³H]spermine tetrahydrochloride (870 mCi/mmol), New England Nuclear, Boston, Mass.; putrescine, spermine, and spermidine hydrochlorides, L-lysine, histamine, ornithine, cadaverine, 1,3-diaminopropane, Calbiochem, San Diego, Calif.; bovine thyroglobulin, Sigma Chemical Co., St. Louis, Mo.; human γ -globulin (165 mg/ml), Cutter Laboratories, Inc., Berkeley, Calif.; Dextran 150, Pharmacia Laboratories, Inc., Piscataway, N. J.; charcoal SG-Extra, Norit Company, Jacksonville, Fla.; 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl, Ott Chemicals Co., Muskegon, Mich.; human serum albumin, crystalline, bovine thyroglobulin, Calbiochem.

Solutions

Borate buffer, 0.05 M, pH 8.0, containing 20 mg KCl,

20 mg CaCl_2 , and 21 mg $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ in 1000 ml buffer.

Working [^3H]spermine solution in borate buffer containing 2400 dpm/0.1 ml was prepared fresh daily from purified stock solution. The polyamines were purified every 3 weeks in silica gel column (5). Before use, polyamine hydrochlorides were recrystallized twice from ethanol. Spermine tetrahydrochloride in distilled water was used for the preparation of standard solutions in concentration range from 100 to 5000 pg/0.1 ml calculated as spermine base. Stored refrigerated in nalgene bottles, the stock solution was kept no longer than 1 month.

A protein-containing solution was prepared from known amounts of human serum albumin and human γ -globulin for development of standard calibration curves. The 1.1 g of albumin was dissolved in 18 ml borate buffer and mixed with 2 ml of human γ -globulin (for injection, 165 mg globulin per ml). Refrigerated overnight, the mixture was then centrifuged, divided into 1-ml aliquots, and stored frozen. A charcoal/dextran solution containing 5 g charcoal SG-Extra and 0.1 g Dextran-150 in 100 ml of borate buffer was prepared fresh daily for use.

Rabbit antiserum was diluted before assay with borate buffer to obtain the concentration at which 0.1 ml diluted antiserum bound 40% of [^3H]spermine. Scintillation fluid contained 8 g Omnifluor and 100 g naphthalene in 1000 ml of dioxane. Polyethylene and nalgene laboratory ware, including the counting vials, were used for all procedures because polyamines strongly adsorb to glass.

Preparation of Spermine-Thyroglobulin Conjugate

Linear conjugation was accomplished by coupling the terminal amino group of spermine molecules with the carboxyl groups of thyroglobulin, using the carbodiimide reaction as described by Goodfriend *et al.* (4) and Skowsky and Fisher (15). To determine the molar ratio of hapten/macromolecular carrier, a trace of ^3H -labeled spermine was added in the beginning to the reaction mixture. A molar ratio of 131/1 for the spermine/thyroglobulin conjugate was obtained.

Immunization

Antisperm antibodies were produced by immunizing the rabbits with 1 mg of conjugate dissolved in 1 ml of 0.9% NaCl solution and emulsified in an equal volume of complete Freund adjuvant. A multiple site-injection technique was used for injections at biweekly intervals. An additional group of rabbits was immunized by macrophage-harvesting technique (6) with 300 μg of conjugate per animal as the initial peritoneal immunization dose, followed by multiple-site booster injections of 1 mg conjugate in the same manner and on the same time schedule as was used with the 1st group.

Titration of Antibody

Antibody titers were measured by incubating 0.1 ml of serially diluted rabbit serum with 2400 dpm of [^3H]spermine in borate buffer (0.05 M, pH 8.0) at 4° in a final volume of 1

ml for 2 hr. Separation of free [^3H]spermine from antibody-bound [^3H]spermine was accomplished by the addition of 0.1 ml 5% charcoal/dextran solution in borate buffer and centrifuging for 20 min at $1000 \times g$. Eight-tenth-ml aliquots of the supernatants, after the addition of 10 ml scintillation fluid, were counted in the liquid scintillation counter. With each determination, additional tubes for total counts (no charcoal added) and nonspecific binding (no antiserum added) were used. All samples were assayed at 4° and in triplicate. The percentage of bound [^3H]spermine was calculated and plotted against the dilution of the antiserum. A working dilution of antiserum was chosen at which 0.1 ml of diluted antiserum gave 40 to 50% binding.

After 8 months of biweekly multiple-site injections, 1 rabbit developed a titer that gave 50% binding in final dilution of 1/900. The rabbits given injections by the macrophage-harvesting technique developed the same titer after 2 months of biweekly injections. The affinity constant (K_a) for the serum antibody with the highest titer was $K_a = 1.9 \times 10^9$ liters/mole.

Specificity

The specificity of antisperm antiserum was tested against putrescine, spermidine, and chemicals with a similar structure. The results were expressed as percentage cross-reactivity, according to the method of Abraham (1). The antisperm antiserum was incubated in the presence of increasing amounts of the test compound (from 100 pg to 1 μg) in borate buffer with constant amount of [^3H]spermine (2400 dpm). Calibration curves showing the relationship between concentrations of unlabeled compounds and the percentage of bound labeled spermine were plotted. The amount of each competitive compound (X) corresponding to 50% displacement was determined, and the percentage of cross-reactivity for the X was calculated [% cross-reactivity = (pg spermine at 50% bound)/(pg compound X at 50% bound) \times 100]. Results of these specificity tests of antisperm rabbit antiserum are shown in Chart 1 and Table 1.

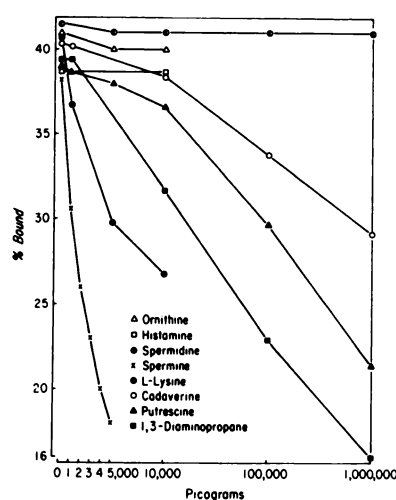


Chart 1. Inhibition curves of possible cross-reacting compounds with antisperm rabbit antiserum.

Table 1

Percentage cross-reactivity of polyamines and other similar compounds with antispermine rabbit and horse antiserum

Compound	% antispermine rabbit antiserum (dilution 1/900)	% antispermine horse antiserum (dilution 1/500)
Spermine	100	100
Spermidine	22.2	21.4
Putrescine	1.0	<0.006
Cadaverine	0.16	0.03
Diaminopropane	5.9	0.12
Histamine	0	0
Ornithine	0	0
L-Lysine	0	0

The antispermine rabbit antiserum showed 22.2% cross-reaction with spermidine, 5.9% with diaminopropane, and 1.0% with putrescine. Cadaverine cross-reacted only 0.6% in a very high concentration, while histamine, ornithine, and L-lysine did not cross-react.

Specificity of an antispermine horse antiserum, a kind gift of Dr. G. A. Quash, was also determined (Chart 2; Table 1). Antispermine horse antiserum cross-reactivity with spermidine was 21.4%, but lower cross-reactivity with diaminopropane, putrescine, and cadaverine was noted. Histamine, L-lysine, and ornithine did not cross-react with horse serum. Thus, the rabbit and horse antisera had similar cross-reaction properties. Antispermine antiserum received from our rabbits was also tested for cross-reactivity with human albumin, human γ -globulin, and bovine thyroglobulin. The results shown in Chart 3 indicate that the antispermine rabbit antiserum did not react with these proteins.

Radioimmunoassay

Antisera and standard solutions of [³H]spermine were diluted in borate buffer. The antiserum was used at a dilution that bound 40 to 50% of the added [³H]spermine in control tubes containing no unlabeled polyamine. The final dilution of [³H]spermine was 2400 dpm/sample tube.

The total assay volume was 1.0 ml; it contained 0.1 ml of [³H]spermine (2400 dpm), 0.1 ml of diluted antiserum, and 0.01 to 0.02 ml of serum samples. The calibration curve was prepared in the same borate buffer, with unlabeled spermine in an amount ranging from 100 to 5000 pg and 0.01 to 0.02 ml of protein solution.

After 2 hr incubation at 4°, the separation of labeled, bound, and free spermine was accomplished by the addition of 0.1 ml dextran-coated charcoal solution, followed by centrifugation in cold at 1000 × g for 20 min. After centrifugation, an 0.8-ml aliquot of supernatant and 10 ml of scintillation fluid were pipetted (automatic dilutor) in polyethylene counting vials (Packard Instrument Co., Downers Grove, Ill.) and counted. With the samples, the tubes for determination of nonspecific binding (no antibody added) were run in parallel. All samples have been done in triplicate. Subtraction of the nonspecific binding from

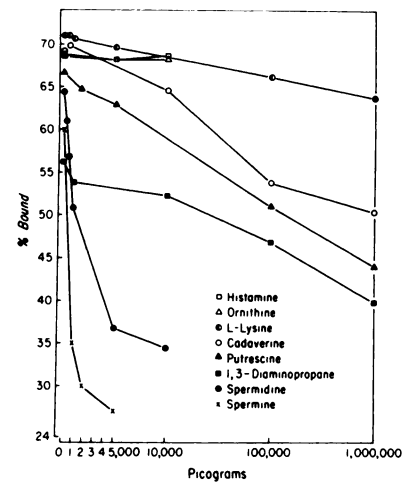


Chart 2. Inhibition curves of possible cross-reacting compounds with antispermine horse antiserum.

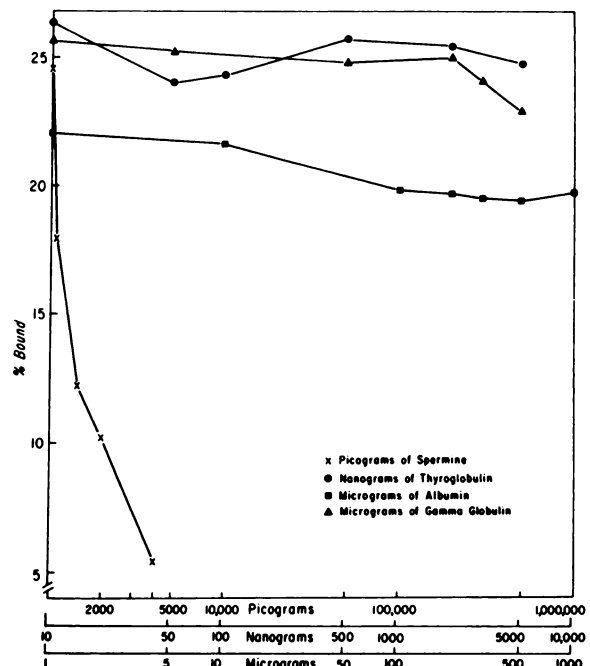


Chart 3. Inhibition curves of human serum albumin, γ -globulin and bovine thyroglobulin with antispermine rabbit antiserum.

standard curve and from individual samples improved the precision. The values for polyamines in unknown samples were read from the standard curve which was constructed from percentage bound [³H]spermine plotted semilog against the corresponding concentration of unlabeled spermine (Chart 4). The value for total free polyamines in the sample, mainly spermine and spermidine, was expressed as ng of spermine equivalent in 1 ml serum.

Precision. Intra- and interassay precisions were tested. Human serum samples were assayed several times in the same experiment and, on succeeding days, each experiment was repeated several times. The coefficient of variation for intraassay precision was 13.4% for the mean value of 125.7 ng/ml. Interassay precision was found to be 15.9% for the mean value 143.0 ng/ml human serum.

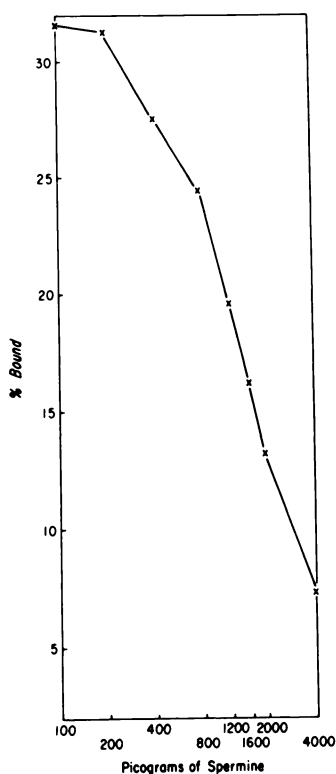


Chart 4. Standard curve for spermine.

Sensitivity. The percentage bound [³H]spermine corresponding to 200 pg was significantly different from the percentage bound corresponding to 0 pg in every standard curve. This allows measurements as low as 200 pg of spermine assayed per sample (*i.e.*, 0.01 ml serum).

Accuracy. To the serum sample containing known amount of polyamines, increasing amounts of unlabeled spermine standard were added. The amount of polyamines measured in the assay was found in good correlation (correlation coefficient, $r = 0.991$) with the amount of spermine added (Chart 5; Table 2).

DISCUSSION

The production of antisperm antibody has allowed us to develop a highly sensitive method for determining "free" polyamines in human serum. No extraction or fractionation of serum is necessary. The entire procedure requires approximately 5 hr for the completion of 20 clinical specimens, all done in triplicate. Using semiautomatic batch techniques, large numbers of samples could readily be processed.

On the basis of the cross-reactivity behavior of antisperm rabbit antibodies, it would appear that the polyamines determined by this radioimmunoassay in native human serum are primarily spermine and spermidine. This conclusion is based largely on the cross-reactivity data, *i.e.*, spermine, 100%; spermidine, 22.2%; putrescine, 1.0%; and cadaverine, 0.16%. Histamine, ornithine, and L-lysine were nonreactive.

The juxtaposition of the exposed primary and secondary amines of carrier-coupled spermine is probably responsible

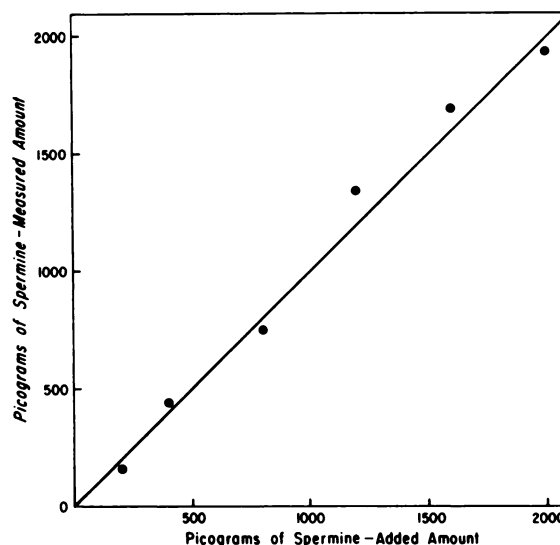


Chart 5. Correlation of spermine measured and spermine added to the serum sample.

Table 2
Recovery of known additions of spermine from a serum sample

Added amount of spermine (pg)	Calculated amount (pg)	Measured amount (pg)
		610
200	810	770
400	1010	1050
800	1410	1360
1200	1810	1950
1600	2210	2300
2000	2610	2550

for the degree of specificity. The kinds of cross-reactions with other polyamine congeners in human biological fluids require consideration. Monoacetylated polyamines, monoacetyl spermidine in particular, have been characterized in human urine (8, 18). While acetylation of polyamines may occur in biological systems as a mechanism to enhance disposal, methodological restrictions in the choice of chemical determination of polyamine derivatives have prevented their detection in serum to date. If acetylated polyamines are present in the serum, cross-reactivity data would suggest that our polyamine antibodies would very probably bind in significant amounts only monoacetylated spermine that has 1 propyl moiety free. That the bulk of human serum polyamines might not be either bound or conjugated is suggested by comparing our preliminary normal human serum values with values obtained by others who used acid hydrolysis, extraction, and column separation. The normal serum values for free polyamines determined by radioimmunoassay from 8 normal subjects ranged from 10 to 60 ng/ml of serum with a mean of 28 ng/ml and, for 8 cancer patients, it ranged from 76 to 390 ng/ml of serum with a mean of 159 ng/ml (Table 3). While absolute comparisons cannot be made because of differences in methodology, it is of interest that both hydrolyzed and intact serum sample values are within the same order of magnitude.

While the polyamines, as measured in normal human serum by radioimmunoassay with antisperm rabbit anti-

Table 3

Serum polyamine values in cancer patients and a group of normal male controls (expressed as mg of spermine)

Cancer patients			Normal controls	
Subject	Type of cancer	ng/ml	Subject	ng/ml
202	Metastatic sarcoma	264	1	30
13	Melanoma	100	2	10
15	Hepatoma	96	3	22
33	Undifferentiated carcinoma + basal cell carcinoma	114	4	15
54	Lymphoepithelioma	76	5	13
61	Gastric carcinoma	82	6	32
183	Wilm's tumor	390	7	42
203	Metastatic sarcoma	150	8	60
Mean		159.0		28.0
S.D.		39.9		6.0
S.E.		4.0		4.7

sera, are presumed to be free, it is theoretically possible that some are firmly bound to glycoproteins or other macromolecular compounds by ionic and/or hydrophobic interactions. In this case, it would be expected that the antibody would have a greater binding affinity for the polyamines and would therefore displace them from the other macromolecular carriers, especially at the higher pH range of the buffer. Another species, polyamines covalently linked to reactive groups on polypeptides and proteins, might be present without appropriately exposed primary and secondary amines. If present in large amounts, one would expect large differences between the values obtained after hydrolysis versus direct radioimmunoassay. This does not appear to be the case in normal serum. If polyamines covalently bound to the surface of proteins and polypeptides make a major contribution to the quantities of polyamine measured in normal human serum by radioimmunoassay, it is likely they would provide antigenic sites for rabbit antipolyamine antisera.

A number of these questions about the assay of biological fluids cannot be fully answered until various blood fractions are comparatively analyzed, using various methodologies, and until acetylated and aldehydic congeners of the polyamines become readily available to the biological investigators.

Meanwhile, the application of polyamine radioimmunoassay to the establishment of a library of normal serum values for children and adults and comparing these values with those obtained from more cancer patients and those with other diseases is appropriate. The simplicity and rapidity of the direct polyamine radioimmunoassay may make this method particularly suitable for screening and monitoring cancer patients.

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