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# RAPID CHROMATOGRAPHIC METHOD TO DETERMINE POLYAMINES IN URINE AND WHOLE BLOOD

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## SUMMARY

A procedure is described for the rapid determination of putrescine, spermine and spermidine in urine and whole blood. The samples are hydrolyzed with barium hydroxide and are neutralized with sulfuric acid. The polyamines are concentrated and separated from amino acids on a small bed of ion-exchange resin that then serves to load the samples on a two-channel, automated ion-exchange chromatography apparatus. As many as 100 samples can be analyzed in a 24-h period. The method has been shown to be applicable to the analysis of urine and whole blood samples, but further development is needed for application to serum samples.

## INTRODUCTION

Since the initial report by Russell and colleagues [1, 2] the evidence has become well known for elevated urinary excretion of polyamines (putrescine, spermine, and spermidine) by some cancer patients. Evidence is accumulating also that some cancer patients have larger than normal amounts of spermidine in their serum [3—6]. Much of what is known of the relation between polyamine levels and cancer was summarized at a 1973 symposium [7] and has been briefly reviewed more recently by Savory and Shipe [8].

A number of methods have been devised to determine the polyamines in biological samples. The analytical state-of-the-art is well summarized in the symposium volume [7] and the review by Savory and Shipe includes several references to analytical methods. Most of the recently described methods have been based on separation by gas chromatography or by ion-exchange liquid chromatography. The separation times by gas chromatography have been reasonably short (15—20 min), but the sample preparation steps have been tedious

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[9,10]. Many of the ion-exchange chromatography methods have also required tedious sample preparation, and column time per analysis has typically been from 90 to 120 min [3, 11, 12].

The limitations of the analytical methods have undoubtedly slowed the progress of clinical studies on the significance of polyamine levels in the diagnosis and therapy of cancer. Savory and Shipe [8] concluded their review by saying: "The relationship between polyamines and cancer appears to have been well established, and there are sufficient data to indicate that measurements of polyamines in biological materials could provide a valuable test in the diagnosis of cancer and for monitoring therapy. *At the present time, there is a need for the development of precise and accurate methods with ultimate emphasis being placed on simplicity in order that large scale studies can be carried out.* Normal values of polyamines in serum and urine need to be established and further extensive clinical correlations are required." (Italics ours.)

It was our purpose in the work reported here to develop a rapid method of analysis to facilitate the clinical studies. The method measures free or total (free plus conjugated) polyamines in urine or whole blood, and with some additional development it should be useful in the analysis of serum. It employs commercially available equipment entirely, except for one module assembled from commercial parts. The equipment makes it possible to analyze 100 or more samples per day.

## SUMMARY OF THE METHOD

The method has several features that distinguish it from earlier chromatographic methods for polyamine determination. These include novel procedures for sample hydrolysis and for separating and concentrating polyamines from the sample, in addition to a rapid ion-exchange column separation. The details of the procedure are given below, in the Experimental section. In this section, the general features of the method are described and discussed briefly. The sequence of steps in an analysis are: (1) sample hydrolysis; (2) pre-concentration and group separation; and (3) chromatography. The description here is in the inverse of that order, so as to make clearer the relations between the steps.

### *Rapid chromatography*

The primary objective of the method development program was to speed up the chromatographic analysis substantially from the state-of-the-art column time of 90—120 min per sample. (A newer method [13] requires only 35 min column time for the separation plus an unstated length of time to prepare the column for the next sample after each run.) In the method described here, the column time is 22 min including washing and regenerating the column for the next sample. The chromatographic separation is carried out on a dual-column analyzer [14] that is equipped for automated, unattended operation. It is possible to analyze six samples in little more than 1 h.

The column time was reduced by a combination of improvements over earlier methods:

(1) In the earlier methods [3, 11, 12] half or more of the column time was

needed to elute the amino acids in the sample. In the new method, the amino acids are separated from the sample before the chromatography.

(2) A combination of factors were optimized to speed up the chromatography generally. These included the use of small, uniform resin particles [15], a short column, moderately fast pumping rates (possible without excessive pressure because of the short column and the properties of the resin [15]), increased column temperature, and high ionic strength buffers.

(3) The elution times of the polyamines were so spaced as to minimize "dead" time between peaks. This was done with buffer gradient elution, using an ionic strength gradient.

(4) By gas-segmenting the column effluent stream, band spreading is considerably limited as the stream passes through the hydraulic system where it is reacted with ninhydrin reagent. In unsegmented systems that allow some spreading of the peaks, the separation achieved in the column must be greater than what is needed at the colorimeter (cf. discussion in ref. 19).

The amino acid analyzer [14] incorporates features that permit reliable unattended operation, and it can be left running with up to 80 samples in position for automatic loading and analysis. It can chromatograph 100 samples, plus a standard after each tenth sample, in only 20 h, leaving up to 4 h a day for routine maintenance. (In practice, only a few minutes should be needed for daily maintenance.) The instrument will stop itself after the last sample or if certain malfunctions occur.

*Pre-concentration and group separation*

The functions of this part of the procedure are to save time in the chromatography by avoiding the introduction of amino acids to the ion-exchange column, to enhance sensitivity by concentrating the polyamines before the sample is loaded on the column, and to protect the column from degradation by extraneous compounds and particulates in the samples. In doing this, advantage is taken of the cartridge that is the means of automated sample loading in the amino acid analyzer [14].

The cartridge holds a small amount of an ion-exchange resin similar to that in the column. A 2-ml portion of the sample prepared for analysis is pumped through the resin in the cartridge under conditions of pH and ionic strength selected for quantitative retention of the polyamines on the resin. Some of the amino acids in the sample will also remain on the resin, but they are removed by rinsing the resin with a flow of buffer.

*Sample hydrolysis*

In most of the prior analytical methods, the samples were prepared for chromatographic analysis by hydrolysis at high temperature in 6 *N* hydrochloric acid followed by neutralization with sodium hydroxide, or by hydrolysis with potassium hydroxide and neutralization with hydrochloric acid. Samples prepared by either procedure would not be satisfactory for the new method, because the high ionic strength of the hydrolysate would adversely affect the retention of the polyamines on the resin in the pre-concentration and group separation step. Hydrolysis of samples containing protein also produces amino acid contents large enough to prevent quantitative concentration of the polyamines in the cartridge.

It is a common procedure to follow hydrolysis by extraction of the polyamines into *n*-butanol, followed by evaporation of the butanol and dissolution of the residue in aqueous buffer. This procedure is effective in controlling the ionic strength of the sample and in concentrating the polyamines. However, it is time consuming, and it does not remove the amino acids from the sample.

In the present work, the samples were first deproteinized with sulfosalicylic acid and then hydrolyzed in the presence of barium hydroxide. Neutralizing the hydrolysate with sulfuric acid formed insoluble barium sulfate, so that the final solution did not have a high ionic strength. Freshly precipitated barium sulfate is well known to co-precipitate other compounds, and we found that as much as half of the polyamines were being lost in this way. We circumvented this potential problem by the conventional expedient of allowing the precipitate to stand overnight before taking off the clear supernatant.

Basically the same sample preparation method has been used to determine total polyamines in urine, serum or plasma, and whole blood samples, except that the whole blood samples are hemolyzed before being deproteinized. Data given below suggest that the procedure must be modified for the analysis of serum samples. When the free polyamines are to be determined, the only modification in the method is the elimination of the hydrolysis and neutralization steps.

## EXPERIMENTAL

### *Equipment*

The chromatography was conducted on a Technicon TSM Amino Acid Analyzer [14, 15] under the conditions listed in Table I. A buffer gradient was established as shown diagrammatically in Fig. 1. The chromatograms were integrated with an Autolab System AA Amino Acid Data Analyzer with two Analyzer Modules.

The apparatus shown in Figs. 2 and 3 was employed in loading the cartridges in the pre-concentration and group separation step. It was built up from the turntable assembly, positive displacement pump and pressure gauge of the

TABLE I

CHROMATOGRAPHY CONDITIONS

Resin: Technicon C-2, 8% cross-linked, sulfonic acid type, 8—12 μm diameter (Technicon Part No. T15-0356-42).

Column: 45 x 4 mm I.D.

Temperature: 80°.

| Time (min) | Solution pumped | Flow (ml/min) | Volume in gradient flask (ml, start/end) |
|---|---|---|---|
| 0—2 | Buffer 1 to column | 0.9 | 5.6/5.6 |
| 2—13.5 | Buffer 2 to gradient | 0.8 | 5.6/4.4 |
| | gradient to column | 0.9 | |
| 13.5—14 | 1.5 *M* NaOH to column | 0.9 | 4.4/0 |
| | gradient to waste | 10 | |
| 14—22 | Buffer 1 to gradient | 1.6 | 0/5.6 |
| | gradient to column | 0.9 | |

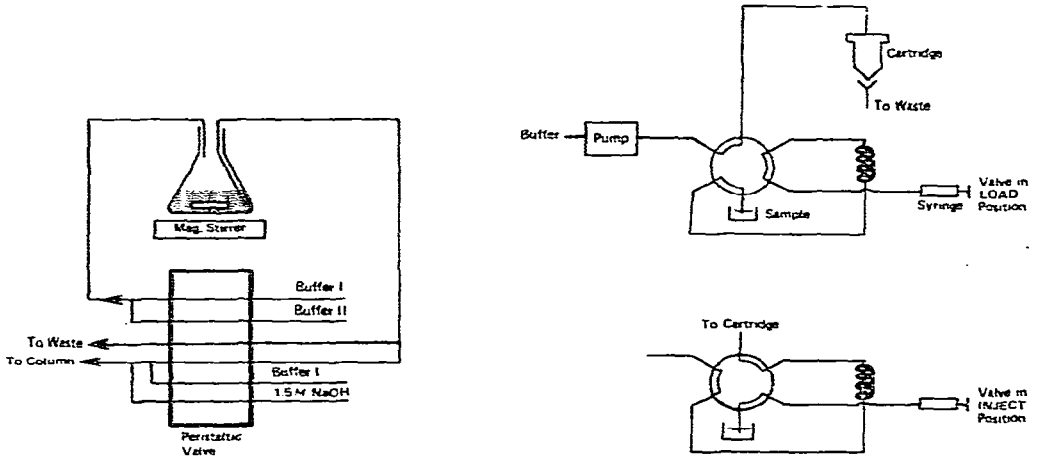


Fig. 1. Hydraulic diagram of the gradient buffer system. The peristaltic valve is a standard part of the amino acid analyzer.

Fig. 2. Hydraulic diagram of the cartridge loader, showing the two valve positions.

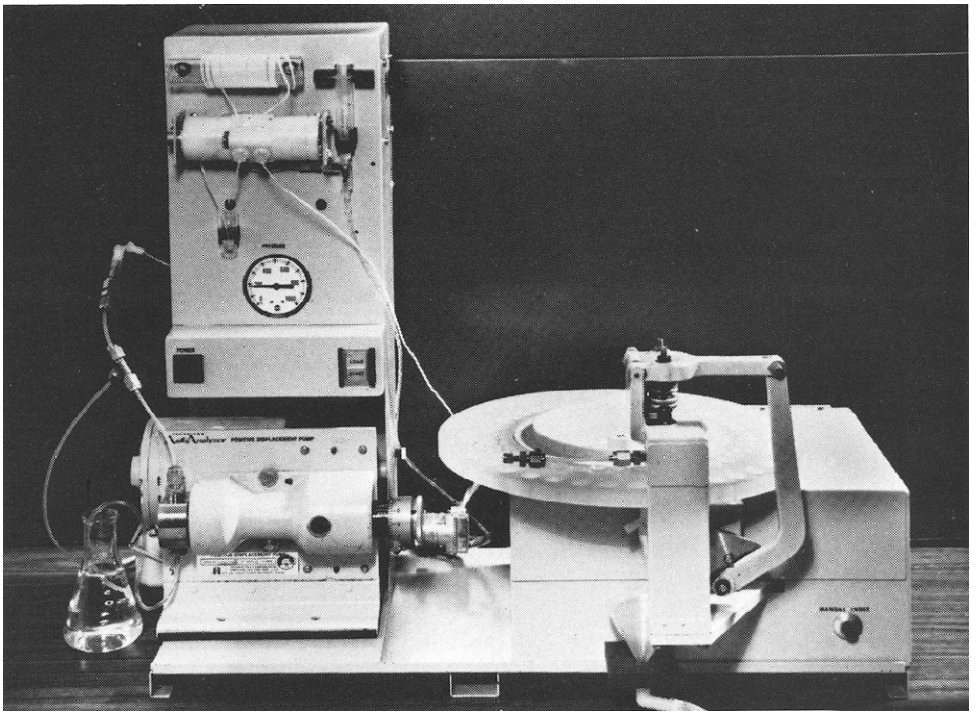

Fig. 3. The cartridge loader. The turntable for the cartridges is on the right, the pump at lower left, and the valve and sample loop at upper left.

Technicon Amino Acid Analyzer, a Chromatronix pneumatically actuated sampling valve, and a mechanical timer to control the sequence of operations. In using the cartridge loader, the operator sets the valve in the LOAD position and fills the sample loop by means of the syringe. The sample loop's length and diameter are chosen so that when the system is filled and the valve moved to the INJECT position it will contain 2.0 ml of sample. After the sample loop is filled, the operator presses the START button, with the valve still in the LOAD position. The pump starts, and buffer passes through the cartridge at 2 ml/min for 30 sec. The timer then causes the valve to switch to the INJECT position, and the sample is pumped through the cartridge in 1 min. The pump operates for 4 min more, passing buffer through the cartridge to remove the amino acids from the resin. The timer then causes the pump to turn off, the turntable assembly to move the next cartridge into position, and the system to stop in the STANDBY mode until the operator is ready to load the next sample.

*Reagents*

*Stock citrate.* Dissolve 420 g citric acid monohydrate and 165 g sodium hydroxide in approx. 1.5 l water, cool, and make up to 2.0 l with water.

*25% Phenol.* Mix 25 g phenol with sufficient water to give a total volume of 100 ml. This forms a two-phase liquid, that must be shaken well before use.

*Buffer 1 (pH 5.25, 2.0 M $Na^+$).* Dissolve 210.4 g sodium chloride in 600 ml water, add 200 ml stock citrate and 2 ml 25% phenol, and adjust to pH 5.25. Add 2 ml Brij-35 wetting agent (Technicon Part No. T21-0110-15) and make up to 2.0 l with water.

*Buffer 2 (pH 5.25, 3.5 M $Na^+$).* Dissolve 362.2 g sodium chloride in 600 ml water, add 200 ml stock citrate and 2 ml 25% phenol, and adjust to pH 5.25. Add 2 ml Brij-35 and make up to 2.0 l with water.

*Buffer for the cartridge loader (pH 5.25, 0.35 M $Na^+$).* Dissolve 17.5 g sodium chloride in 600 ml water, add 200 ml stock citrate and 2 ml 25% phenol and adjust to pH 5.25. Add 2 ml Brij-35 and make up to 2.0 l with water.

*Sulfosalicylic acid.* 3% (w/w) in water.

*Triton X-100.* Technicon Part No. T21-0188-06.

*Ninhydrin reagent.* Dissolve 10 g ninhydrin (Technicon Part No. T11-0102-25) in 500 ml methyl cellosolve and 250 ml buffered sodium acetate (Technicon Part No. T21-0105-16, 4 *M*, pH 5.5), make up to 1000 ml with water.

*Hydrazine sulfate reagent.* Dissolve 0.2623 g hydrazine sulfate in 600 ml water, add 5 ml Brij-35, make up to 1.0 l with water, and add one drop conc. sulfuric acid.

*Standards*

Putrescine, cadaverine, spermidine and spermine were used as received from Aldrich, Milwaukee, Wisc., U.S.A. To 750 ml water were added 3.0 mg putrescine, 10.0 mg cadaverine, 1.5 mg spermidine and 2.0 mg spermine. The pH of the solution was adjusted to between 3 and 4 with hydrochloric acid, and the solution was made up to 1.0 l with water.

*Samples*

Whole blood, serum or plasma, and 24-h urine collections were obtained from Technicon employees. Patient samples were provided by Drs. M. K. Schwartz and Y. Hirshaut of the Memorial Hospital and Sloan-Kettering Institute, New York, N.Y., U.S.A.

*Procedure*

*Urine*. Test 1 ml of clear urine for protein with a few crystals of sulfosalicylic acid. If a precipitate forms, deproteinize 10 ml urine with 100 mg sulfosalicylic acid, mix, and centrifuge. For free polyamines, add 0.3 ml stock citrate to 3.0 ml urine or the deproteinized supernatant, and adjust to pH 3—4 with 0.1 *M* sodium hydroxide or hydrochloric acid solution. For total polyamines, hydrolyze the sample as follows: To 5.0 ml of clear urine or deproteinized supernatant in a 15 ml glass centrifuges tube add 1.0 g barium hydroxide, mix, and heat 4 h at 100° in an oil bath. Cool, add 2 or 3 drops of phenolphthalein solution, and neutralize with concentrated sulfuric acid (caution: add acid dropwise with cooling) to disappearance of pink color. Leave at room temperature at least overnight (24 h is better) and centrifuge. To 3.0 ml of the clear supernatant add 0.3 ml stock citrate and adjust to pH 3—4.

*Whole blood*. Add 1 drop Triton X-100 to 1.0 ml whole blood and agitate ultrasonically to effect hemolysis. Add 9.0 ml sulfosalicylic acid solution, mix, and centrifuge. Treat the clear supernatant according to the procedures described above for urine samples.

*Serum or plasma*. The procedure for serum or plasma is like that for whole blood, except that the hemolysis with Triton X-100 is omitted. If 1 ml of serum is avaiable, deproteinize and follow the rest of the steps exactly as for whole blood. If 2 ml of serum is available, deproteinize with 8 ml sulfosalicylic acid solution and proceed as for whole blood.

*Cartridge loading*. The cartridges contain a sulfonic acid-type ion exchange resin (Technicon Type C-4, Part No. T15-0361-42) similar to the one used in the chromatography column, but with a larger particle size. A description of the cartridge loader and its method of use are given above in the Equipment section. The cartridges may be re-used, but growth of algae in the resin bed may limit their useful life. We routinely add a small amount of phenol to the buffers to inhibit growth of algae, and store the cartridges in a phenol solution.

*Chromatography*. The conditions for chromatography are listed in Table I. The chromatography conditions are the same for all types of samples, and blood and urine samples can be intermixed. Because of the smaller polyamine concentrations in serum and plasma, the colorimeter controls are adjusted for a higher sensitivity, and serum or plasma samples cannot be intermixed with blood or urine samples.

## RESULTS

Fig. 4 is a chromatogram of a synthetic solution approximating the polyamine composition of hydrolyzed normal urine. The time scale on the abscissa is for the time of emergence of the eluate from the column, and the times above the peaks indicate when each arrives at the colorimeter; the time differ-

ence, about 10 min, is the residence time of the column effluent in the flow stream where it is mixed with reagents and incubated for color development. This residence time is nearly half the total analysis cycle, and it is not possible to change sample cartridges until the last peak has passed through the colorimeter. However, the interval is used to wash and regenerate the column. There would be little practical benefit if the chromatography conditions were changed to reduce the elution times. Cutting the elution time in half would only reduce the complete cycle from 22 to 16 min.

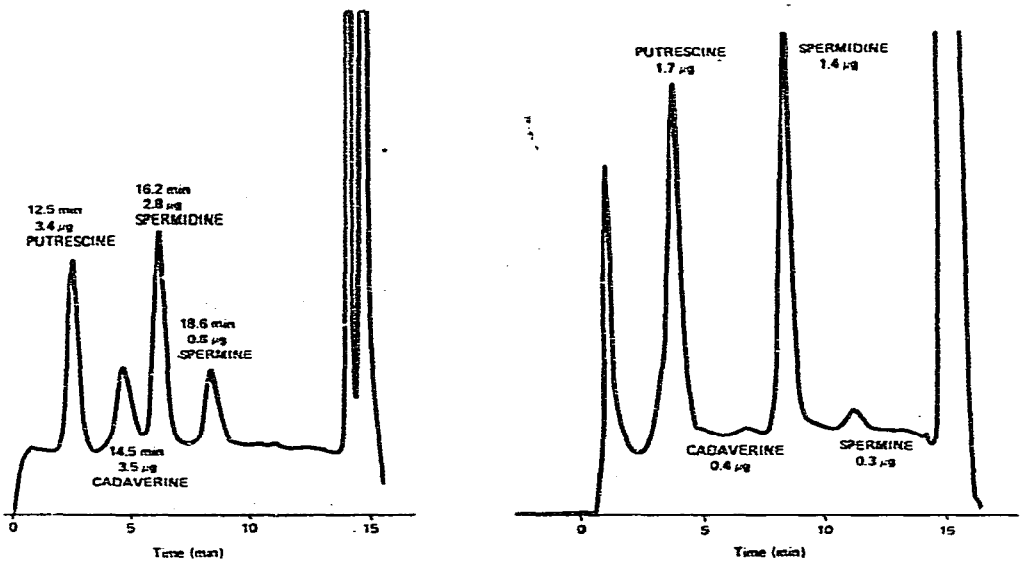


Fig. 4. Chromatogram of a standard solution.

Fig. 5. Chromatogram of a hydrolyzed urine sample.

Figs. 5 and 6 show examples of chromatograms of samples of urine and whole blood after hydrolysis. The only significant difference from the chromatogram in Fig. 4 is that residual amino acids that remain on the cartridge appear at the beginning of the trace. These represent only trace amounts of the amino acids; it is advisable to handle the cartridges carefully after loading or the amino acid peak may increase due to contamination by these compounds from the operator's fingers.

The chromatogram of Fig. 7 illustrates the performance of the instrument under high-sensitivity conditions. Normal serum levels of spermidine are reported [4, 5] to be near 1/3 nmole/ml, or about 50 ng/ml. The figure shows that this amount of spermidine would be detectable in the chromatogram. However, we have not found spermidine in normal plasma samples or samples from cancer patients. Some possible reasons for this are discussed later in this publication.

Occasional chromatograms have shown peaks other than those shown in Figs. 5–7. A peak is sometimes observed just before the peak for putrescine. Veening et al. [12] found 1,3-diaminopropane in some samples. When we ad-

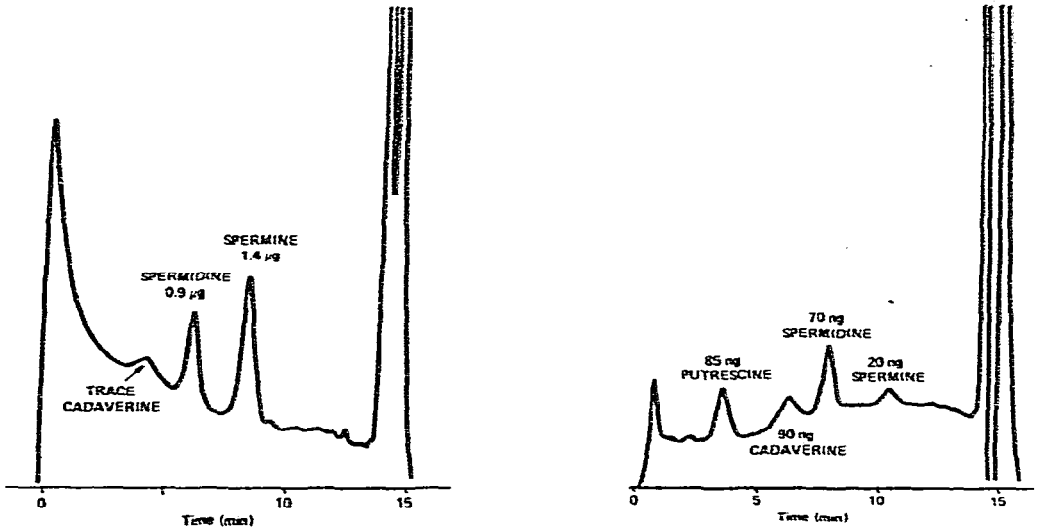


Fig. 6. Chromatogram of a hydrolyzed whole blood sample.

Fig. 7. Chromatogram of a standard with high-sensitivity settings.

ded this compound to standards, it gave a peak just before putrescine. In a few samples, the peak due to 1,3-diaminopropane has been larger than the putrescine peak and has interfered with its quantitation. Modifying the method by reducing the ionic strength of Buffer 1 would resolve these two peaks without greatly increasing total analysis time. We have also, quite rarely, observed an extra peak near the peak for cadaverine. This peak has not interfered with the determination of putrescine, spermine, or spermidine, and we have made no effort to identify the compound responsible.

*Hydrolysis conditions*

Hydrolysis with barium hydroxide was adopted as a means to avoid build-up of ionic strength of the hydrolysate, which would have interfered with the absorption of the polyamines in the ion-exchange resin in the sample cartridge. During the development of the method, it was found that recoveries of the polyamines were low when standard solutions were hydrolyzed with barium hydroxide, the base was neutralized with sulfuric acid, the clear supernatant was removed before the precipitate had aged. It was thought that the loss could be caused by either coprecipitation or by degradation of the polyamines during heating of the solution in the presence of the base. However, losses were found even when the heating step was eliminated; that is, when barium hydroxide was added to the polyamine solution and immediately neutralized with sulfuric acid.

Coprecipitation can be reversed by aging the barium sulfate precipitate. When the sample–precipitate mixture was held overnight at 100°, the polyamines were recovered quantitatively. Aging at room temperature is more convenient, and we found that overnight aging this way resulted in only small

losses (5—10%) that are rather constant from sample to sample and can thus be adequately compensated for in the calibration.

Other hydrolysis variables studied were the amount of barium hydroxide and the length of heating. Preliminary trials showed that hydrolysis is slow with only 0.5 g of base per 5 ml of sample. With 1 g of base per 5 ml of sample, heating for 4 h gave the best results. Hydrolysis was incomplete with 2 h heating, and with 6 h heating there was evidence of degradation of the polyamines; recoveries were found to be lower for putrescine and spermidine, and the cadaverine peak developed a shoulder.

*Polyamine levels found*

Only limited summary data are included in this report on analysis of samples from cancer patients and controls. The analysis results have been given to Dr. Hirshaut at Memorial Hospital for correlation with clinical information and eventual publication.

*Urine.* Analyses for free and total polyamines were done on 24-h urine collections from 21 ambulatory controls. The results (mg excreted per 24 h) are given here as the range of total amounts found and the mean. Putrescine: 0.02—2.55, 0.89; cadaverine: trace—2.98, 0.55; spermidine: 0.02—1.32, 0.53; spermine; not detected (2 samples)—0.38, 0.14. Statistical treatment of the analysis results showed that the data are not normally distributed, and perhaps have a log-normal distribution. Individuals who excreted larger amounts of one polyamine did not necessarily excrete large amounts of others. The total amount of the four polyamines excreted per day ranged from 0.28 to 6.5 mg, with a mean of 2.0 mg. The fraction in conjugated form was also highly variable from one person to another, and no relation was apparent between the fractions of different polyamines present as conjugates nor between the total amount excreted and the fractions conjugated. Many of the cancer patients, but not all by any means, excreted considerably larger amounts of the polyamines than were found in the urine of the ambulatory controls.

The amounts of individual polyamines found are similar to results reported for the analysis of normal urines by gas chromatography [9], and somewhat lower than but overlapping the results by liquid chromatography [3]. Savory and Shipe [8] remarked: "Many more normal sera and urines need to be analyzed and a rigorous statistical analysis of the data made before a true normal range can be established."

*Whole blood.* We have analyzed whole blood from only a few ambulatory controls, besides the more extensive analyses on samples from hospital patients. Very little has been reported on polyamine levels in whole blood. Raina [16] reported the following means and standard deviations from the analysis of blood samples from 30 normal individuals: spermine, 1.34 ± 0.3, spermidine, 0.96 ± 0.16 μg/ml. (Note: Raina's publication lists the standard error of the mean, from which we have computed the standard deviation.) Iliev et al. [17] reported results nearly identical with those of Raina. Data from the present study are consistent with the earlier results. Spermine and spermidine were found in all blood samples. The concentrations for the few ambulatory controls were at the levels previously found, with considerable elevations in bloods from some of the cancer patients. The whole blood concentrations are so much

larger than what has been reported for serum [3—6] that it is apparent that polyamines in whole blood occur primarily in the cells. The data in the literature and our data have not been correlated with cell counts.

*Plasma and serum.* The results of analysis of plasma by this method are not in agreement with previously published results [3—6]. Analysis of synthetic solutions (Fig. 7) demonstrated that the method is adequately sensitive to detect the polyamines at the concentrations that have been reported in serum. Apparently, polyamines are lost during the sample preparation. We interpret the available evidence to suggest that polyamines in serum are bound to a specific protein — possibly an antibody or a carrier protein — firmly enough to be carried down with the protein when the sample is treated with sulfosalicylic acid.

We investigated non-specific coprecipitation of polyamines with protein during the development of the analytical method. Serum albumin was added to a standard containing all of the polyamines at normal urine levels, and the protein was then precipitated. The polyamines were recovered quantitatively in the supernatant. It is significant also that extraction with trichloroacetic acid is a common procedure to solubilize polyamines from tissue samples. Marton et al. [13] extracted polyamines from tissue samples with a solution of sulfosalicylic acid. We cannot account for the losses of the polyamines by assuming that volatilization occurs during hydrolysis, as this would have affected standards carried through the procedure.

It is well known that many small molecules in plasma are held by specific binding proteins, and this binding has been shown to cause serious errors in some radioimmunoassay procedures. Procedures are available to release small molecules from the binding proteins. For example, Fang and Refetoff [18] tested the release of triiodothyronine ($T_3$) from binding proteins by heat inactivation of the protein, by 8-anilino-1-naphthalenesulfonic acid, and by ethanol extraction. They also mentioned some other simple procedures that have been applied to release $T_3$ from binding proteins and that are presumably not specific to $T_3$. We have not been able to test the effectiveness of any of these procedures in the analysis of serum or plasma for polyamines.

## CONCLUSIONS

We believe that the procedures described here meet the need for a more rapid analysis method than has previously been available for the determination of the polyamines, so that normal and pathological levels of polyamines in urine and blood can be studied in detail. Some further work is needed to adapt the method to the analysis of serum or plasma samples.

## ACKNOWLEDGEMENTS

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