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ION-EXCHANGE CHROMATOGRAPHIC SEPARATION AND FLUOROMETRIC DETECTION OF URINARY POLYAMINES

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SUMMARY

An ion-exchange chromatographic procedure has been developed for the separation and fluorometric detection of the urinary polyamines 1,3-diaminopropane, putrescine, spermidine, cadaverine, and spermine. The polyamines were separated on a 15×0.45 cm cation-exchange column (70°) employing a combined pH–salt gradient. The separation conditions were designed to enable utilization of the sensitive reagent fluorescamine as a means of monitoring these amines in physiologic fluids. This non-fluorescent reagent forms a fluorophor with most primary amines. Experiments were carried out to determine the effects of several variables such as pH, reagent concentration, and flow-rate. The method was successfully applied to both normal and pathologic samples of human urine.

INTRODUCTION

Various methods have been proposed for the early diagnosis of malignancy in humans. Those which have received increased attention during the last few years are based on the determination of polyamines in physiologic fluids. Recent experiments suggest that patients with active cancer may have elevated levels of these compounds in their urine[1–7].

The polyamines generally include the following compounds: 1,3-diaminopropane, $H_2N(CH_2)_3NH_2$; putrescine, $H_2N(CH_2)_4NH_2$; cadaverine, $H_2N(CH_2)_5NH_2$; spermidine, $H_2N(CH_2)_3NH(CH_2)_4NH_2$; and spermine, $H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$. The separation and/or determination of polyamines in various biological samples has been accomplished by several techniques including fluorometric assay[8,9], enzymic assay[10], gas chromatography[11,12], ion-exchange chromatography[13–16], and thin-layer chromatography[17]. In the ion-exchange procedures, the ninhydrin detection system is generally used, although quantitative data for reactions of the polyamines with this reagent have not been reported.

The purposes of this investigation were to develop an ion-exchange technique for separating polyamines and a means of detecting these compounds fluorometrically

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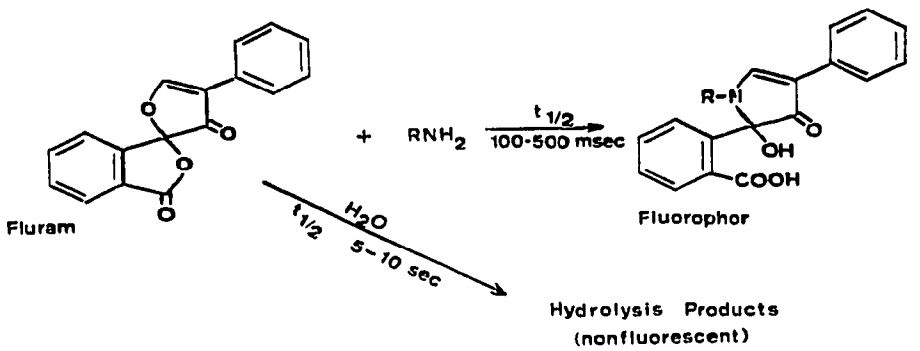
by utilizing the fluorophor that is formed when primary amines react with 4-phenyl-spiro [furan-2(3H),1'-phthalan]-3,3'-dione (fluorescamine or Fluram™). Fluorescamine (which itself is non-fluorescent) has been reported to form a highly fluorescent product with primary amines[18], and has been demonstrated to be two orders of magnitude more sensitive for the detection of picomole quantities of amino acids than the standard ninhydrin procedure[19]. A manual fluorometric assay for nanogram quantities of proteins utilizing fluorescamine has also been reported[20]. This reagent reacts directly with primary amines in aqueous medium (pH 9–10) at room temperature with a half-life of a fraction of a second. Excess reagent is destroyed by water, with a half-life of several seconds, to form non-fluorescent hydrolysis products[18]. The reactions are shown below.



MATERIAL AND METHODS

Chemicals

Putrescine dihydrochloride, cadaverine dihydrochloride, spermidine trihydrochloride, and spermine tetrahydrochloride were obtained from Sigma (St. Louis, Mo., U.S.A.). 1,3-Diaminopropane dihydrochloride was obtained from Aldrich (Milwaukee, Wisc., U.S.A.). Spectro-grade acetone was obtained from Eastman (Rochester, N.Y., U.S.A.). Fluorescamine (Hoffmann-La Roche) was purchased from Aminco (Silver Spring, Md., U.S.A.). Cross-linked sulfonated styrene and divinylbenzene copolymer Aminex A-5 cation-exchange resin were obtained from Bio-Rad Labs. (Richmond, Calif., U.S.A.).

Apparatus for manual procedures

Several experiments were carried out by manual procedures utilizing an Aminco-Bowman spectrophotofluorometer and a Sargent-Welch Model DR pH meter. A rotary evaporator was used to evaporate hydrolyzed urine samples to dryness.

Chromatographic system

Fig. 1 is a schematic flow diagram of the ion-exchange chromatograph which was constructed. Three minipumps (Milton-Roy) served to pump the column eluent, the buffering agent (0.1 *M* H_3BO_3) and the fluorescamine solution through the system. In the fluorescamine pump the rubber plunger seals were replaced by Rulon O-rings (Milton-Roy) in order to counteract the destructive effect of acetone. The

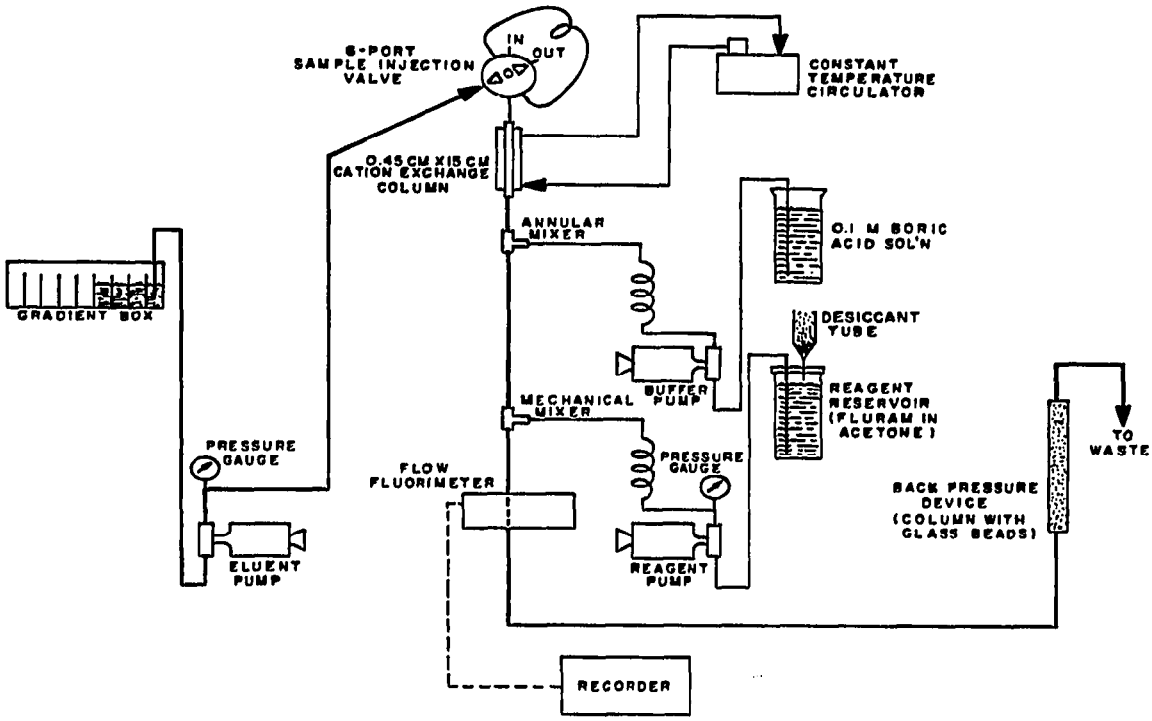


Fig. 1. Ion-exchange chromatograph for the separation and fluorescence detection of polyamines.

first four chambers of a nine-chamber gradient box supplied the eluent, which was pumped through a six-port sample injection valve supplied with a 0.450-ml sample loop. The design of this valve has been described previously[21]. A jacketed, nickel column, 15×0.45 cm I.D., was used in this investigation. Nickel was used to minimize the corrosive effects of sodium chloride solutions. The column was slurry-packed[22] with Aminex A-5 resin (13- to 20-μm particles), which was supported by a 1/4- to 1/16-in. reducer union containing a 0.5-μ stainless-steel frit. The column was operated at 70° utilizing a Haacke constant-temperature circulator.

The column effluent was first mixed with a stream of 0.1 *M* H_3BO_3 using an annular mixer. Details of the design and operation of this mixer have been reported previously[23]. The buffered stream was then fed to a T-mixer supplied with a small magnetic stirring bar, where the fluorescamine reagent stream was introduced.

A Laboratory Data Control fluoroMonitor was used to detect fluorescent components eluting from the column. The excitation lamp was a low-pressure, hot-cathode mercury lamp with a phosphor coating that emits near-UV energy peaking around 360 nm. Visible light was blocked by a Corning 760 primary filter; visible light emitted from the flow cell passed through a sharp cutoff UV blocking filter and impinged on the photosensitive elements of a dual photocell. The fluoroMonitor was equipped with a 2-mm-I.D. quartz flow cell with a volume of *ca.* 20 μl. The signal from the detector was fed to a 10-mV Leeds & Northrup Speedomax pen recorder which operated at a chart speed of 2 in./h.

The chromatographic system was provided with a back pressure device at

a point beyond the detector in order to prevent the formation of bubbles when acetone was mixed with the aqueous stream at the T-mixer. This device consisted of a 45×0.45 cm stainless-steel column which was packed with Type 500–5005 finely divided glass beads obtained from the Reflective Products Division of the 3M Co. (St. Paul, Minn., U.S.A.). A back pressure of 40–50 p.s.i. was sufficient to prevent bubble formation.

Operation of the chromatograph

The polyamines were separated on Aminex A-5 cation-exchange resin by using a combined pH–salt gradient. Prior to each run, the column was equilibrated with 0.03 *M* phosphate buffer (pH 9.2). The gradient was prepared by placing the following solutions in each of the first four chambers of the nine-chamber Phoenix Varigrad gradient box: Chamber 1, 25 g of 0.05 *M* NaCl, pH 11.20; Chamber 2, 25 g of 0.10 *M* NaCl, pH 11.80; Chambers 3 and 4, 25 g per chamber of 0.20 *M* NaCl, pH 12.00. The pH of each solution was adjusted by using a pH meter and adding a sufficient quantity of dilute sodium hydroxide to each salt solution. The column was operated at a flow-rate of 36 ml/h and a column inlet pressure of 150 p.s.i.

In order to form the fluorescamine derivatives of the polyamines at the optimum pH for fluorescence measurement, it was found necessary to readjust the pH of the column effluent by mixing it with 0.1 *M* boric acid solution. The boric acid was pumped into the annular mixer at a flow-rate of 5 ml/h.

Fluorescamine reagent solution was prepared in the reagent reservoir by dissolving 150 mg of the reagent in 1 liter of anhydrous spectro-grade acetone. The reagent was pumped into the mechanical T-mixer at a flow-rate of 16 ml/h.

Standard solutions of the polyamines were prepared by weighing appropriate quantities of the amine salts, dissolving the salts in triply distilled water and diluting to volume. Samples were introduced into the column by means of the sample injection valve. Before the sample was loaded, the 0.450-ml sample loop was flushed with distilled water and emptied; then about 1.5 ml of sample was passed through the loop to ensure that the sample was not diluted by residual water.

Urine samples

A number of "normal" and pathologic urine samples were analyzed for polyamines; these samples were stored at −60° prior to sample preparation. It was found necessary to hydrolyze each urine with HCl prior to analysis in order to release the polyamines from the conjugates which they form[2–4] with other components in urine. This was accomplished by refluxing 10 ml of urine for 10 h with sufficient HCl to make the final mixture 6 *M* in HCl. The hydrolyzed urine was then evaporated to dryness in a rotary evaporator at a temperature of 60° and a pressure of 1 mm in order to remove the excess HCl and thus prevent its adverse retention effects on the resin. Finally, the dried residue was diluted to a known volume with triply distilled water and filtered. The hydrolyzed urine samples were stored at −25° until they were ready for chromatographic analysis.

RESULTS AND DISCUSSION

Fluorescence properties of fluorescamine derivatives of the polyamines

A number of experiments was performed to determine the optimum conditions

for chromatographic operation. Activation and fluorescence spectra were recorded at a pH of 10.5 for each of the fluorescamine derivatives of the polyamines. In all cases, the excitation and emission maxima were 395 nm and 475 nm, respectively.

It has been reported[18–20] that the relative fluorescence intensity of the primary amine derivatives of fluorescamine is strongly dependent on pH. This parameter was investigated for the polyamines. A series of solutions was prepared in which the concentration of polyamine was kept constant at 1 μg/ml and the total amount of fluorescamine added (as an acetone solution) was such that the final concentration was 100 μg/ml. The pH of each solution was adjusted by use of phosphate buffers. A sufficient number of solutions was prepared to provide data for the pH range 8.0 to 12.0. The fluorescence intensity of each sample was measured with an Aminco-Bowman spectrophotofluorometer using an excitation wavelength of 395 nm and an emission wavelength of 475 nm. The data, illustrated in Fig. 2, show that the fluorescence intensities for spermine and spermidine are considerably less than for the other three compounds. The pH for obtaining maximum fluorescence from putrescine, cadaverine, and 1,3-diaminopropane derivatives ranges from 9 to 10, whereas the optimum pH for spermine and spermidine is 10.5 in each case. Using these data, it was determined that the column effluent should be buffered to a pH of about 9 to 10 prior to fluorometric detection.

The effect of excess reagent on the fluorescence intensity of the polyamine fluorophors was also studied. A series of solutions was prepared in which the poly-

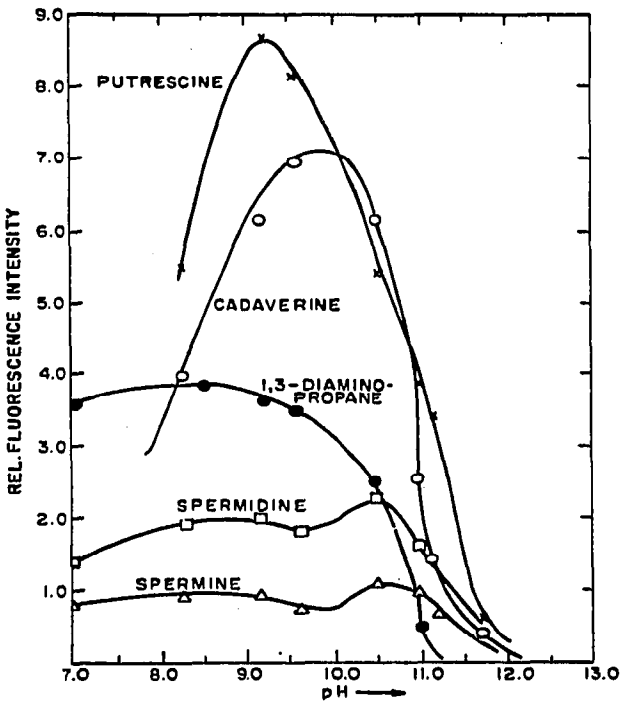


Fig. 2. Effect of pH on relative fluorescence intensity. Excitation wavelength, 395 nm; emission wavelength, 475 nm. Concentration of polyamines, 1 μg/ml (each); concentration of Fluram, 100 μg/ml.

amine concentration and the pH were kept constant at 1.0 μg/ml and 10.5, respectively, while the fluorescamine to polyamine weight ratio was varied. The results, shown in Fig. 3, showed that the curves of putrescine and cadaverine coincided. Maximum fluorescence intensities, in all cases, occurred at rather high fluorescamine to polyamine weight ratios (400 or 500: 1). Several chromatographic runs were carried out using the fluorescamine reagent at a concentration of 400 μg/ml; however, the gain in sensitivity was not sufficient to justify the high reagent cost. Accordingly, the fluorescamine reagent concentration utilized in the chromatographic system was 150 μg/ml.

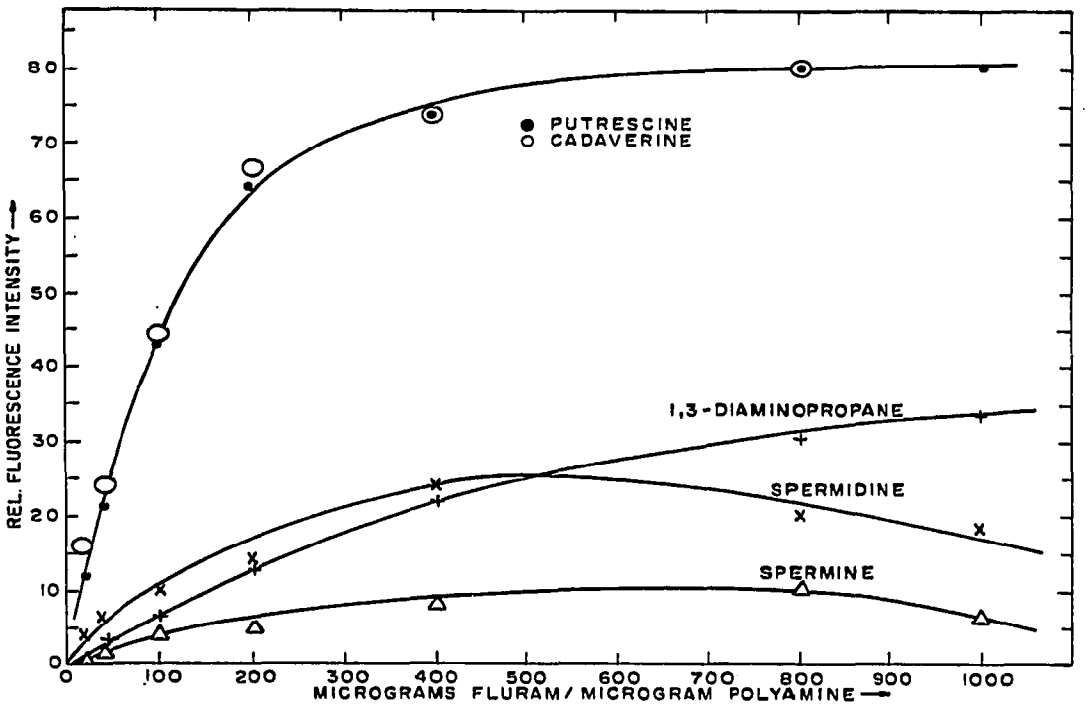


Fig. 3. Effect of fluorescamine concentration on the fluorescence intensity of several polyamines. Excitation wavelength, 395 nm; emission wavelength, 475 nm. pH = 10.5.

pH gradient and buffer stream

The pH gradient used to separate the polyamines was determined experimentally by monitoring aliquots of eluent at the column outlet. The resulting curve (curve 1) is shown in Fig. 4. Since the polyamines have retention volumes ranging from 30 to 57 ml, it is apparent from the data in Fig. 2 that the pH of the column effluent is above that necessary to achieve maximum fluorescence for the polyamine fluorophors. To lower the pH, and thus improve the sensitivity of the procedure, the column effluent was buffered with 0.1 *M* boric acid. At a boric acid flow-rate of 5.0 ml/h, the pH of the column effluent was lowered sufficiently that maximum fluorescence intensity for each component could be achieved. The pH of the buffered column effluent is shown in Fig. 4 (curve 2).

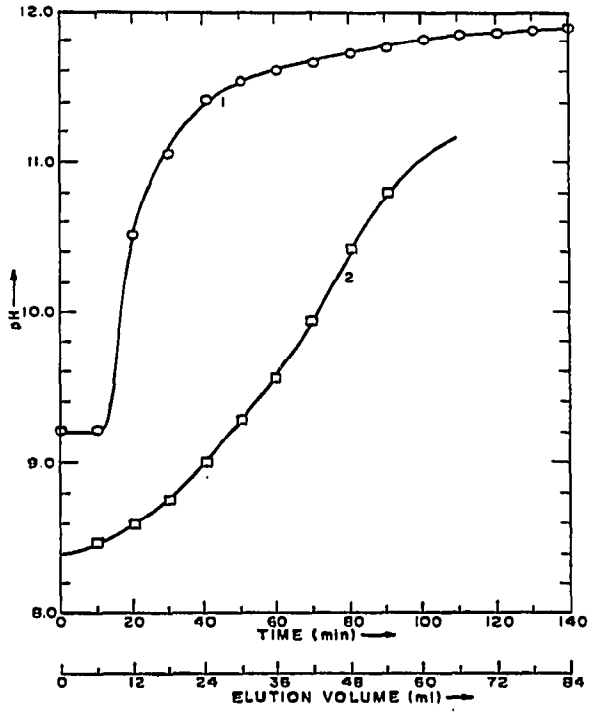


Fig. 4. pH gradient used for the separation of polyamines (curve 1) and pH of column effluent after buffering with 0.1 *M* boric acid (curve 2).

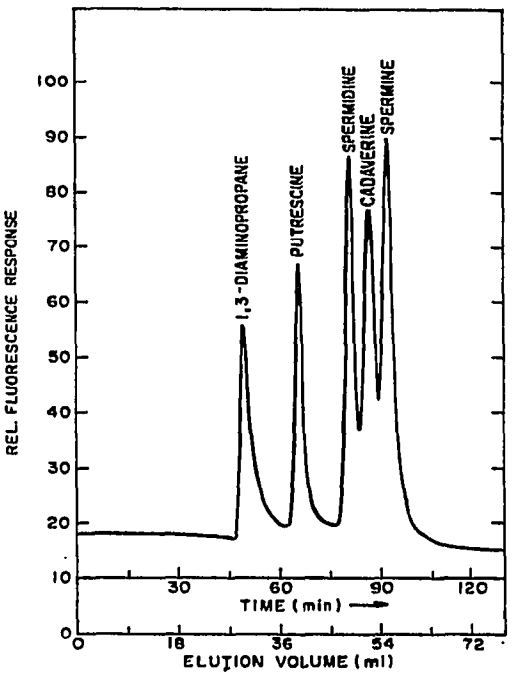


Fig. 5. Chromatogram of a standard mixture of 12.0 μg 1,3-diaminopropane, 3.6 μg putrescine, 15.0 μg spermidine, 7.0 μg cadaverine, and 35.0 μg spermine. (Fluorometer operated at lowest gain, × 64.)

Chromatographic separations and quantitative response

A typical separation of a standard mixture containing known amounts of the five polyamines is shown in Fig. 5. In this particular experiment, the boric acid stream was not employed. The resolution is satisfactory except for spermidine and cadaverine; however, quantitative measurements are possible for these two polyamines. The entire analysis can be completed in less than 2 h under the conditions described above. When the column was operated isocratically at a pH of 9.2 for 90 min after injection, and the gradient was started at this point, the elution of the polyamines was essentially unchanged, as shown for the four-component separation in Fig. 6. Such results indicate that the compounds are strongly retained on the resin at the lower pH until the gradient is begun. This procedure is useful because it automatically provides early elution of the less basic components in the front end of the chromatogram, prior to measuring the desired constituents.

The fluorescamine detection method is reasonably sensitive for 1,3-diaminopropane, cadaverine, and putrescine. The sensitivity for these compounds was greatly increased by the use of the boric acid stream to lower the pH of the column effluent, as shown in Fig. 7. The fluorescence response of each compound was measured with and without the boric acid flow. In all cases, peak heights were plotted.

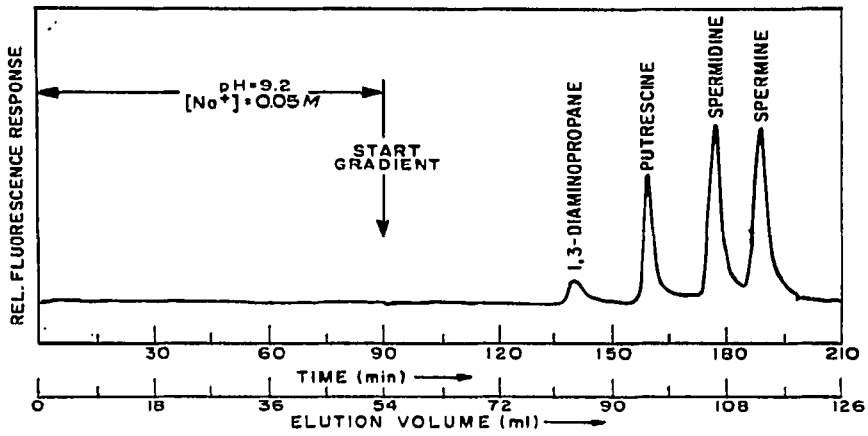


Fig. 6. Separation of a standard mixture of four polyamines using a delayed gradient.

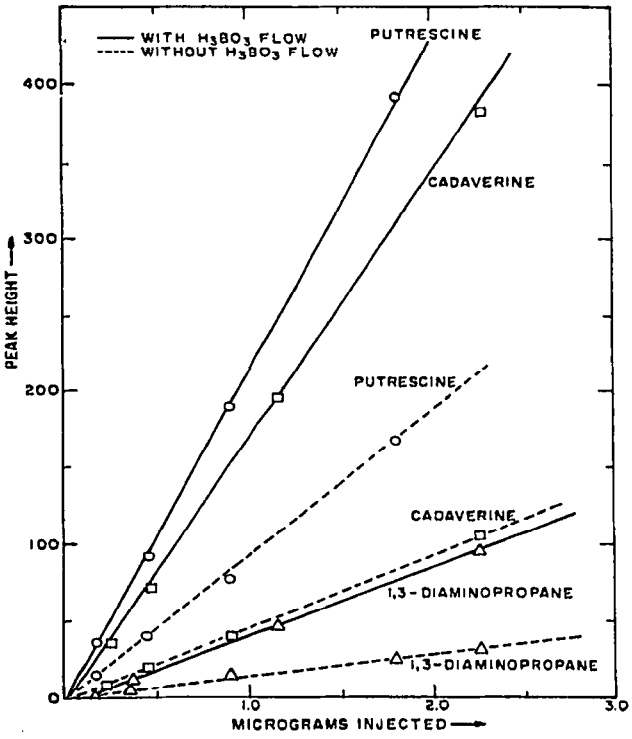


Fig. 7. Response plots for putrescine, cadaverine, and 1,3-diaminopropane with and without the effect of stream buffering with boric acid.

Decreasing the pH of the column effluent increases the response approximately twofold for each of the compounds. The minimum detectable quantity for each of these compounds was found to be 100 ng for 1,3-diaminopropane and cadaverine and 50 ng for putrescine. The sensitivity for spermidine and spermine is considerably less; the minimum detectable quantities were 1.0 and 3.0 μg, respectively. In the ninhydrin detection procedure, the limit of detection has been reported to be 0.5 μg for each polyamine[24].

Several urine samples were analyzed for polyamines. Typical examples of samples from a "normal" subject and from a cancer patient are shown in Fig. 8. Fig. 8a represents the chromatogram of a hydrolyzed, pooled urine sample obtained from eight normal males. Peaks 1, 3, 4, and 5 appear in the elution positions for 1,3-diaminopropane, putrescine, spermidine, and cadaverine, respectively. A smaller unknown peak (No. 2) also appears. The chromatogram in Fig. 8b represents a hydrolyzed urine sample from a cancer patient. Peaks 6 and 7 represent components located in the normal elution positions for putrescine and spermidine. Based on the previously established calibrations, the normal sample contains 0.7 μg/ml putrescine, 1.5 μg/ml spermidine, and a trace of cadaverine. The chromatogram in Fig. 8b shows the presence of more than 2.0 μg/ml putrescine (peak 6), and 4.0 μg/ml spermidine (peak 7). The major peak, eluting early in the chromatogram, contains the amino acids and other less basic components, leaving a reasonably clear region for the elution of the polyamines.

Slight changes in the elution position for standards were found after the

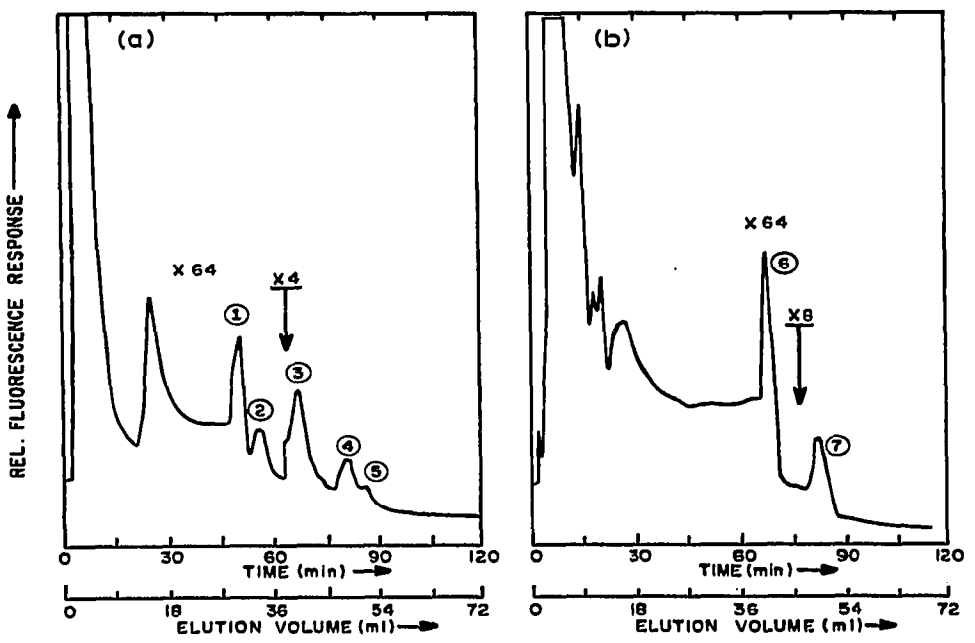


Fig. 8. (a) Chromatogram for 0.450 ml of a hydrolyzed, normal urine sample. The sample was diluted by a factor of 0.67. (b) Chromatogram for 0.450 ml of a hydrolyzed urine sample from a cancer patient. The sample was concentrated by a factor of 2.

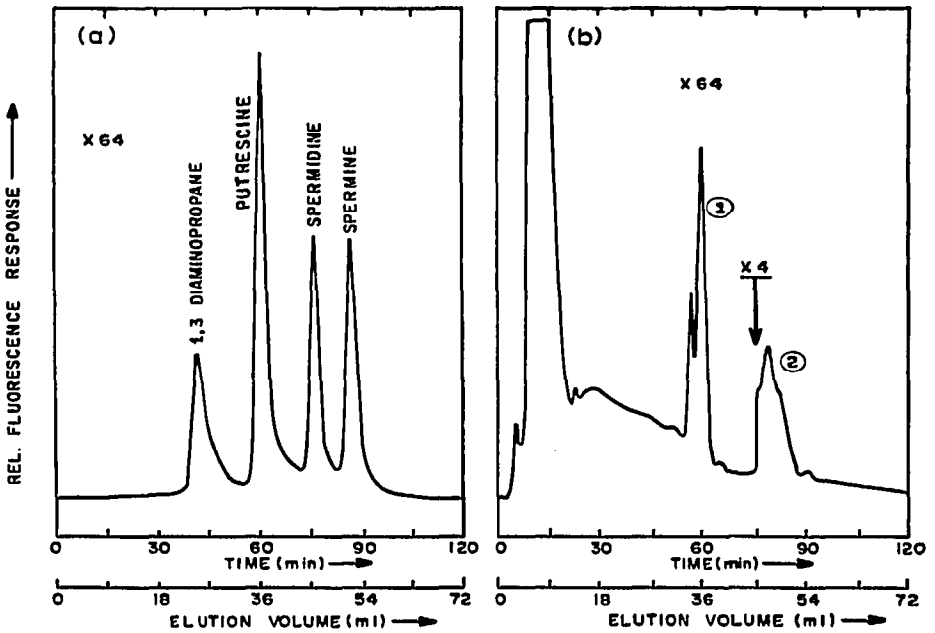


Fig. 9. (a) Chromatogram for a standard mixture of 1,3-diaminopropane, putrescine, spermidine, and spermine. (b) Chromatogram for 0.450 ml of a hydrolyzed urine sample from a cancer patient.

column had been repacked. This is illustrated in Figs. 5 and 9a. Fig. 9 compares the chromatogram for a standard mixture of 1,3-diaminopropane, putrescine, spermidine, and spermine (a) to that of a urine sample from a cancer patient (b). Putrescine (peak 1) and a smaller amount of cadaverine (peak 2) are present in somewhat elevated amounts.

CONCLUSION

The separation procedure described for the polyamines is rapid and gives satisfactory results. The sensitivity of fluorescamine toward 1,3-diaminopropane, putrescine, and cadaverine is acceptable for use in the analysis of physiologic fluids; however, the sensitivity for spermidine and spermine is not as good as had been anticipated from the earlier results obtained for amino acids[18,19]. It is interesting to note that the latter two compounds are the only ones with secondary amine groups (spermidine has one, spermine has two). Despite the presence of the two terminal primary amine groups in each of these molecules, the secondary amine groups apparently interfere with the fluorescamine reaction, or else they have a quenching effect on the activation of fluorescence in the fluorescamine derivatives of the polyamines.

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