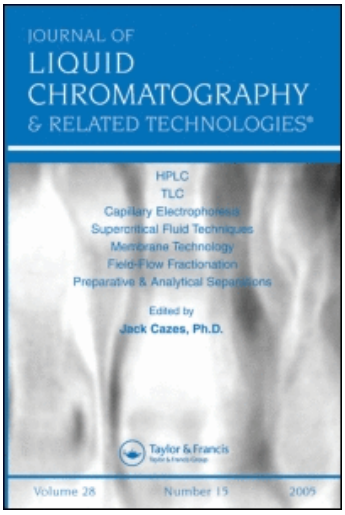

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REVERSED-PHASE ION-PAIRING LIQUID CHROMATOGRAPHIC SEPARATION AND FLUORIMETRIC DETECTION OF POLYAMINES

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ABSTRACT

A rapid and specific reversed-phase ion-pairing high performance liquid chromatographic procedure for putrescine, spermidine and spermine is reported. The ion-pairing reagent, heptanesulfonate, was employed and o-phthalaldehyde and 2-mercaptoethanol were used for on-line post-column derivatization and subsequent fluorescence detection. Experiments were carried out to determine the effects of several variables such as pH, concentration of the aqueous buffer, counter-ion concentration, and the percentage of organic modifier in the moving phase. The minimum detection limits for the polyamines ranged from 120 pmoles for spermine to 12 pmoles for putrescine. The method includes a gradient program which provides complete separation from amino acids and specificity for the three polyamines. The procedure was applied successfully to urine and serum samples.

INTRODUCTION

During recent years there has been a great deal of interest in the physiological and clinical significance of certain polyamines as clinical markers. Urinary and serum polyamines have been reported to be useful biochemical indicators of cancer (1-4). It has also been reported that polyamine metabolism may play an important role in the pathophysiology of psoriasis (5-7).

The biologically significant polyamines of interest in this study are putrescine (Pu), spermidine (Sd), and spermine (Sp).

TABLE 1

Polyamine Structures

Name (Abbrev.)	
Putrescine (Pu)	$H_3\overset{\oplus}{N}(CH_2)_4\overset{\oplus}{N}H_3$
Spermidine (Sd)	$H_3\overset{\oplus}{N}(CH_2)_3\overset{\oplus}{N}H_2(CH_2)_4\overset{\oplus}{N}H_3$
Spermine (Sp)	$H_3\overset{\oplus}{N}(CH_2)_3\overset{\oplus}{N}H_2(CH_2)_4\overset{\oplus}{N}H_2(CH_2)_3\overset{\oplus}{N}H_3$

Table 1 shows the structures of these molecules in their protonated forms.

Previous analytical methods for these and other biogenic amines have included gas chromatography (8-10), thin layer chromatography (11), ion-exchange chromatography (12-18), and more recently, reversed-phase high performance liquid chromatography (HPLC) utilizing pre-column derivatization (19-25).

Most of these methods, however, possess certain disadvantages. The gas chromatographic procedures tend to require tedious sample preparations and sometimes lack accuracy. Ion-exchange techniques are also characterized by tedious sample preparations in addition to lengthy analysis times ranging from 60 to 120 minutes.

Reversed-phase HPLC techniques utilizing pre-column derivatization of amines currently appear to be quite popular. Several recent papers have been published describing pre-column fluorimetric derivatization with fluorescamine and o-phthalaldehyde (19, 20), dabsyl chloride (25), and the popular reagent, dansyl chloride (21-24). These techniques also require extensive sample preparation and analysis times and often yield complex chromatograms. A comprehensive review of analytical methods for polyamines in physiological fluids has been published by Seiler (26).

An increasingly popular approach to the separation of ionic species is ion-pairing HPLC. Several excellent reviews of the theoretical aspects of this technique have been published (27-32).

Very recently, Seiler and Knödgen reported the separation of natural polyamines and their monoacetyl derivatives by ion-pairing HPLC and post-column derivatization (33). In this paper, we report a rapid, sensitive, and specific reversed-phase ion-pairing HPLC separation of putrescine, spermidine and spermine followed by on-line post-column derivatization and fluorimetric detection. We believe our method is complementary to that of Seiler and Knödgen (33); it also includes a detailed study of the retention behavior of the three polyamines and provides a more rapid alternate analysis for naturally occurring polyamines. In addition, our detection limits for the polyamines appear to be slightly lower than those reported by Seiler and Knödgen. The method of detection involves the highly fluorescent product formed from the reaction of o-phthalaldehyde (OPT) and primary amines (34-36). A recent review of reaction liquid chromatography has been published by Frei (37) which outlines the principles involved in post-column derivatization.

MATERIALS AND METHODS

Apparatus

The chromatographic system used in this separation is illustrated in Figure 1. Pumps A and B were Model 6000A units (Waters Associates, Milford, MA 01757). These were controlled by a Model 660 Solvent Programmer (Waters Associates) and delivered the mobile phase through a 30 x 0.39 cm (I.D.) μBondapak C_{18} analytical column (Waters Associates). Samples were introduced into the system by use of a Model U6K injector (Waters Associates) supplied with a 100 μL sample loop.

Pump C was a Milton-Roy Mini-Pump 106-31 (Waters Associates). This pump delivered the OPT derivatizing reagent to the column effluent; the two streams were mixed in an on-line post-column reaction coil to form the fluorescent derivatives of the polyamines. The reaction coil was constructed of 305 x 0.023 cm (I.D.) coiled stainless steel tubing. The resulting fluorophores were then detected in a Schoeffel Model FS 970 Spectrofluoromonitor (Kratos,

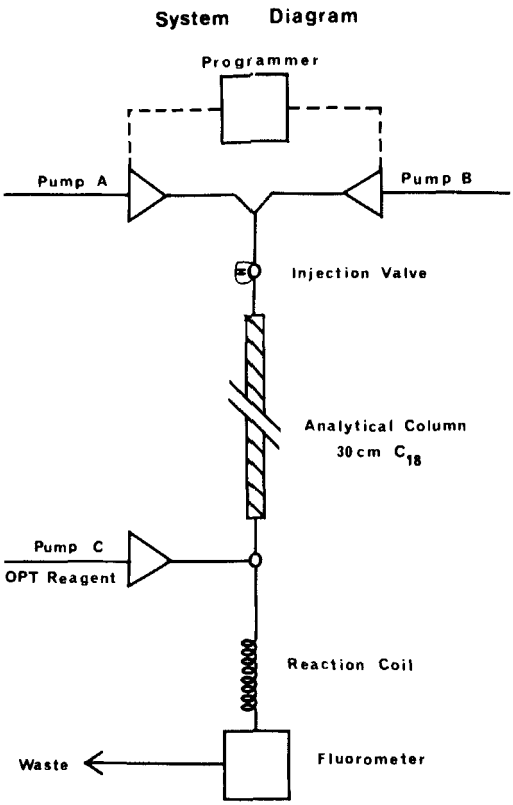

FIGURE 1. Schematic diagram of the chromatographic system.

Westwood, NJ 07675) with the excitation wavelength set at 340 nm and a Type 440 emission filter (Kratos). A Spectra-Physics, SP 4100 computing integrator (Spectra-Physics, Santa Clara, CA 95051) was used to record all chromatograms and perform data reduction. The post-column "T" connector was a Swagelok fitting (Allentown Valve & Fitting Co., Allentown, PA 18049).

Reagents and Chemicals

All polyamines were obtained in the hydrochloride salt forms. The putrescine salt was obtained from Eastman (Eastman Kodak Co.,

Rochester, NY 14650) and recrystallized from absolute ethanol. The spermidine and spermine hydrochlorides (Sigma Chemical Co., St. Louis, MO 63178) were used without further purification. OPT was also obtained from Sigma Chemical Co. and was used as received. Reagent grade 2-mercaptoethanol was purchased from Matheson, Coleman and Bell (Norwood, OH 45212). Sodium 1-heptanesulfonate was supplied by Eastman and used without further purification. Reagent grade tetrahydrofuran was obtained from Aldrich (Aldrich Chemical Co., Milwaukee, WI 53201). HPLC grade methanol was supplied by Burdick & Jackson Labs, Inc. (Muskegon, MI 49422). HPLC grade water was produced by a Milli-Q Reagent Grade Water System (Millipore Corporation, Bedford, MA 01730).

Samples and Sample Preparation

Normal, pooled urine and serum samples were obtained from local hospitals and were kept frozen at -30°C until needed. In preparation for analysis, the physiological samples were thawed and hydrolyzed with an equal volume of concentrated hydrochloric acid at 100°C for a period of 10-12 hours. Following the hydrolysis, samples were cooled to room temperature. The pH of the resulting hydrolysate was adjusted to approximately 4.5 by addition of dilute base. The total volume of acid and base added to the original physiological fluid was recorded in order to calculate dilution factors.

Following adjustment of pH, the hydrolysate was filtered through a 0.20 μm Nalgene Filter (Sybron Corporation, Rochester, NY 14602) to remove particulate matter. These prepared samples were then stored at -30°C until assayed.

Standard stock solutions were prepared by dissolving weighed quantities of the polyamine hydrochloride salts in deionized, distilled water. Appropriate dilutions of these stock solutions were made to generate standard solutions at desired concentrations.

Mobile Phases and Derivatizing Reagents

All mobile phases used in this separation were prepared volumetrically. Solvent A consisted of 80% aqueous acetate buffer

(0.050 M acetic acid adjusted to pH 4.50 with sodium hydroxide) and 20% methanol. Solvent A was also 1.0×10^{-2} M in 1-heptanesulfonate. Solvent B consisted of 80% aqueous acetate buffer (0.10 M acetic acid adjusted to pH = 4.50 with sodium hydroxide), 18% methanol and 2% tetrahydrofuran.

The OPT derivatizing reagent was prepared by dissolving 800 mg of OPT in 10 mL of methanol. To this was added 600 µL of 2-mercaptoethanol. This mixture was diluted to a volume of 1.00 L with 0.50 M aqueous potassium borate buffer at pH 9.00. This reagent is stable at room temperature for a period of 24 hours.

Chromatographic Procedure

Pumps A and B, controlled by the solvent programmer, delivered the mobile phase through the analytical column at a rate of 2.0 mL/min. The mobile phase gradient consisted of an initial isocratic period at 0% B for 10 minutes followed by a linear gradient to 100% B over a 2 minute period. Pump C delivered the OPT derivatizing reagent to the analytical column effluent at a rate of 0.70 mL/min. After passing through the reaction coil where the derivatization process occurred, the mixture was monitored fluorimetrically (λ_{ex} = 340 nm; $\lambda_{em} \geq$ 440 nm).

RESULTS AND DISCUSSION

Retention Mechanism

The basis for this separation of the polyamines is ion-pairing chromatography. This technique allows charged species to be separated using reversed-phase HPLC. Equation 1 gives a very simplified representation of the process involved.

$$RNH_3^+{}_{(aq)} + HpS^-{}_{(aq)} \underset{}{\overset{K}{\rightleftharpoons}} (RNH_3^+HpS^-)_{(org)} \qquad (1)$$

In this example, $RNH_3^+{}_{(aq)}$ represents a protonated, positively charged amine in solution in the aqueous mobile phase. $HpS^-{}_{(aq)}$ represents the anionic counter-ion, 1-heptanesulfonate, also in

solution in the aqueous mobile phase. $(RNH_3^+HpS^-)_{(org)}$ represents the resulting neutral, hydrophobic ion-pair retained in the non-polar organic stationary phase. K is the overall equilibrium constant for the process.

If the equilibrium lies to the left, there is little or no affinity of RNH_3^+ for the non-polar stationary phase, thus little or no retention on the column. However, if the equilibrium is to the right, a neutral, hydrophobic ion-pair is formed which is retained in the non-polar organic stationary phase, thus permitting the separation of the protonated amines.

Retention Behavior of the Polyamine Ion-Pairs

In the process of determining optimum separation parameters, studies of the retention characteristics of the polyamine ion-pairs were performed. This was done by observing the capacity factors (k') of the ion pairs as a function of several variables. These variables included the percent of organic modifier in the mobile phase, the counter-ion concentration, the pH of the aqueous mobile phase buffer, and the acetate concentration of the mobile phase. The results of these studies are presented graphically in Figures 2-5 as plots of k' *vs* the various variables.

Figure 2 illustrates the change in capacity factors of the polyamine ion-pairs as a function of the percent organic modifier in the mobile phase. The modifier used in all of these studies was a 1:1 mixture of methanol and tetrahydrofuran. The acetate concentration was held at 0.050 *M*, the pH was 4.50, and the counter-ion concentration was fixed at 6.0×10^{-3} *M*. The retention behavior of the polyamine ion-pairs was as expected. As the percent organic modifier was increased, the capacity factor of each of the polyamine ion-pairs decreased. We believe that there are two reasons for the large difference in the plots for each of the ion-pairs. First, the spermine has four available ionic sites for ion-pairing, while spermidine has only three, and putrescine two. Secondly, spermine contains more methylene groups than spermidine

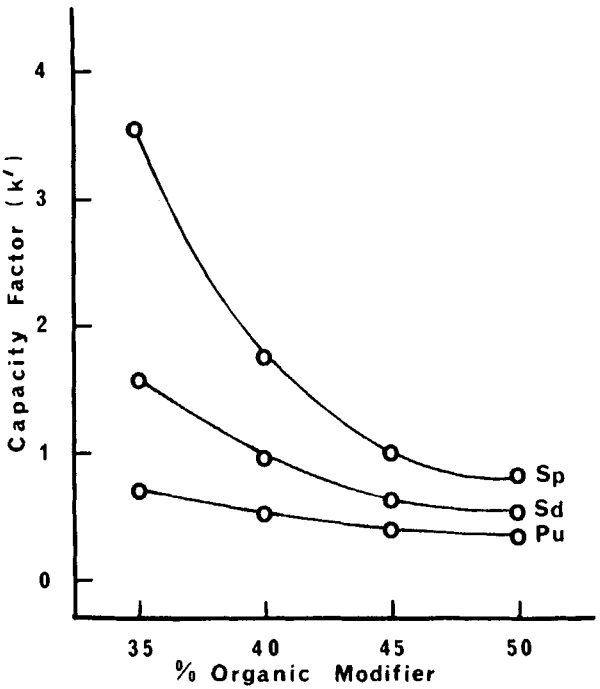

FIGURE 2. Effect of percent organic modifier (1:1 methanol and tetrahydrofuran) on the retention of the polyamine ion-pairs (0.050 M acetate; pH = 4.50; $[HpS^-]$ = 6.0×10^{-3} M).

or putrescine. These two factors combine to cause spermine to be considerably more hydrophobic with a larger retention time than either spermidine or putrescine.

Figure 3 illustrates the changes in capacity factors of the three polyamine ion-pairs as a function of the counter-ion concentration. The data were obtained using a mobile phase consisting of 60% aqueous acetate buffer (0.050 M acetic acid adjusted to pH = 4.50 with sodium hydroxide) and 40% organic modifier. As can be seen from the plots, an increase in counter-ion concentration results in increased retention. This observation is consistent with published results (31,32) and is reviewed here briefly.

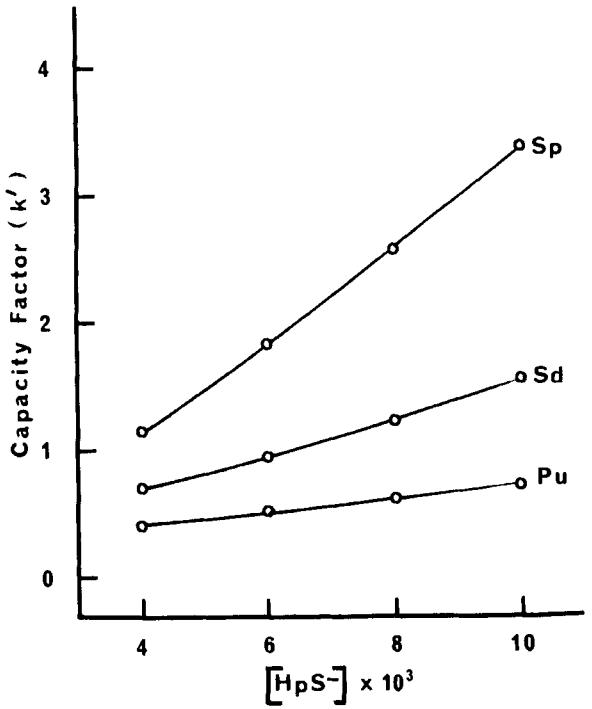

FIGURE 3. Effect of the counter-ion (HpS^-) concentration on the retention of the polyamine ion-pairs (0.050 M acetate; pH = 4.50; 40% organic modifier).

Equation 1 has already described the distribution of the ionic amine in the mobile phase and the extraction of the ion-pair into the organic stationary phase. The equilibrium constant for the extraction into the stationary phase is represented in Equation 1 as K. This extraction constant can be written as:

$$K = \frac{[RNH_3^+HpS^-]_{org}}{[RNH_3^+]_{aq}[HpS^-]_{aq}} \qquad (2)$$

In order to relate the extraction constant K to the capacity factor k', the following equations are required (31):

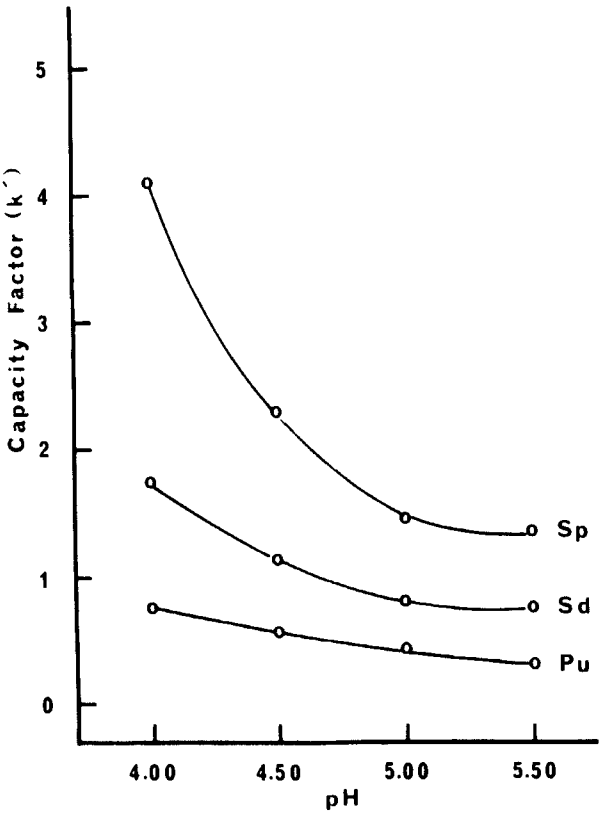

FIGURE 4. Effect of mobile phase pH on the retention of the polyamine ion-pairs (0.050 M acetate; $[HpS^-]$ = 6.0×10^{-3} M; 40% organic modifier).

$$k' = \frac{[RNH_3^+HpS^-]_{org}}{[RNH_3^+]_{aq}} \frac{V_S}{V_M} \quad (3)$$

$$k' = [HpS^-]_{aq} \; K \; \frac{V_S}{V_M} \quad (4)$$

In Equations 3 and 4, V_M represents the void volume of the chromatographic column and V_S represents the volume of the stationary

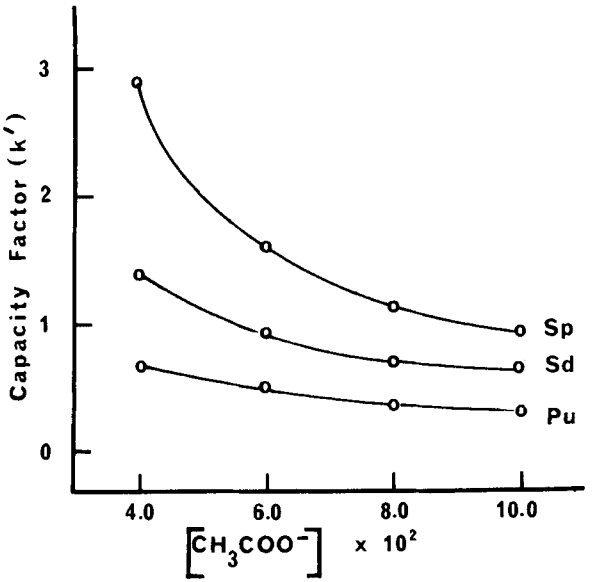

FIGURE 5. Effect of acetate concentration on the retention of the polyamine ion-pairs (pH = 4.50; $[HpS^-]$ = 6.0×10^{-3} M; 40% organic modifier).

phase contained in the chromatographic column. From Equation 4, it is seen that the capacity factor for the polyamine ion-pairs is proportional to the counter-ion concentration in the mobile phase. Figure 3 shows that this is indeed the case.

Figure 4 represents the data obtained in a study of the effect of the mobile phase buffer pH on the capacity factors of the polyamine ion-pairs. In this study the acetate concentration was fixed at 0.050 M, the percent organic modifier was held constant at 40%, and the counter-ion concentration was 6.0×10^{-3} M. It can be seen that a decrease in mobile phase pH resulted in an increase in k' values of the ion-pairs. The reason for this behavior may be due to the non-equivalent amine groups undergoing a greater degree of protonation with a resultant increase in ion-pair formation (and subsequent retention) as the mobile phase pH is decreased.

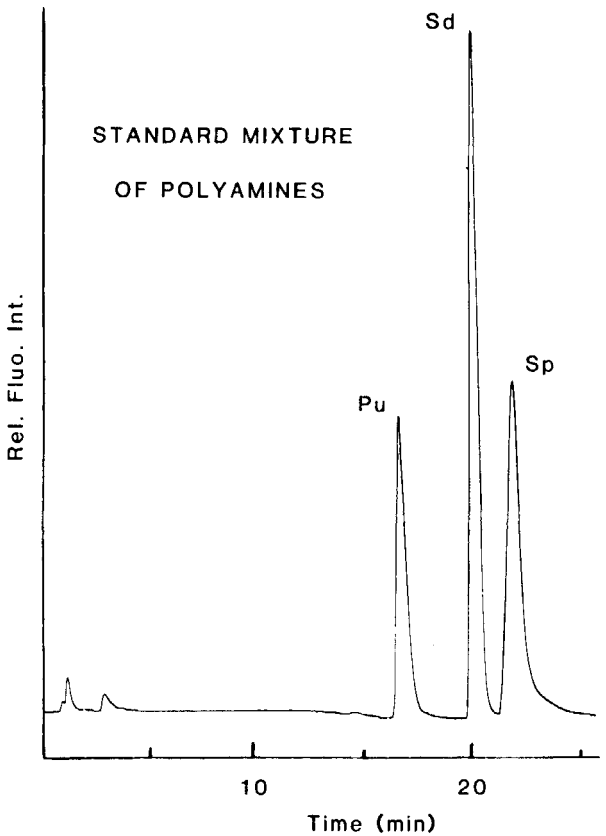

FIGURE 6. Chromatogram of a standard mixture of the three polyamines (1.6 nmol each).

The final study, shown in Figure 5, illustrates the dependence of k' of the ion-pairs as a function of the acetate concentration in the mobile phase. In this series of experiments, the percent organic modifier was 40%, the aqueous buffer pH was fixed at 4.50, and the counter-ion concentration was 6.0×10^{-3} M. It can be seen that as the acetate concentration increased, the k' values of the ion-pairs decreased. This behavior was expected, since previous studies (31,32) have shown that as the ionic strength of the mobile phase increases, the amount of ion-pair formation with the

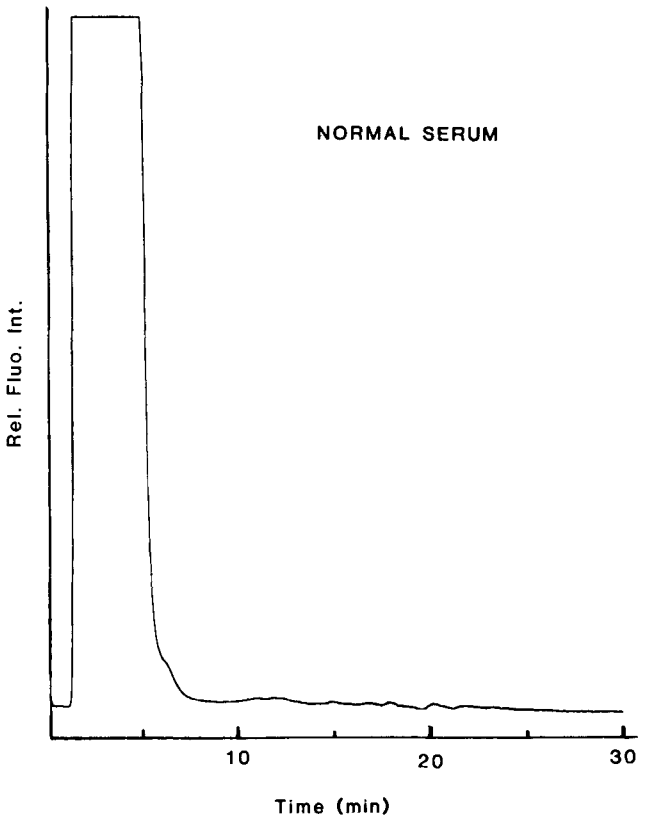

FIGURE 7. Chromatogram of pooled, normal serum.

counter-ion decreases. This results in a decrease in k' values of the ion-pairs. The reason for the decrease in the amount of ion-pair formation is due to the competition of secondary ions in the ion-pair formation process. In this case, it is likely that the anionic acetate ion is competing with the HpS^- counter-ion to form ion-pairs with the cationic polyamines.

Figure 6 shows the separation of a standard mixture of the three polyamines utilizing the previously listed mobile phase and chromatographic conditions. The chromatogram shows satisfactory resolution and symmetrical peak shapes. The total analysis time

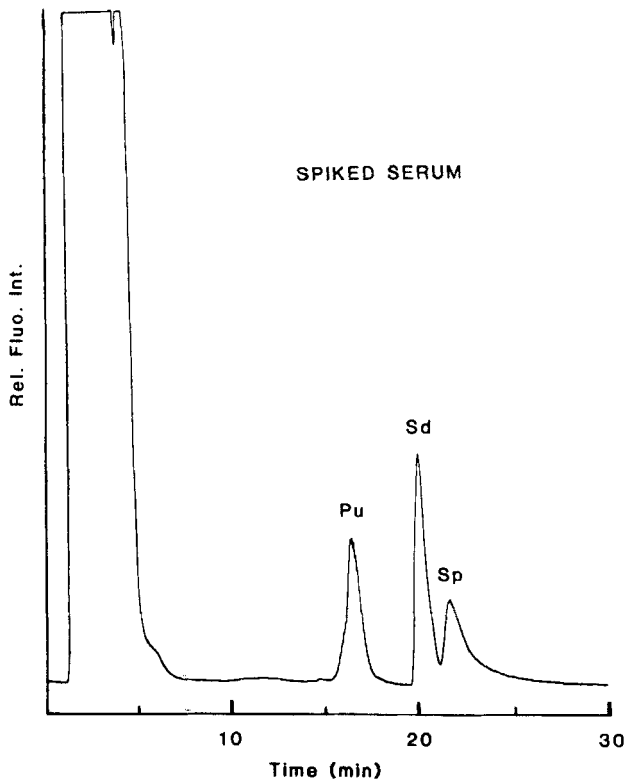

FIGURE 8. Chromatogram of pooled, normal serum spiked with ca. 1 nmol of each polyamine.

was ca. 26 minutes; this is significantly faster than most reported methods, including the most recently published procedure (33). The gradient which was used to produce the chromatogram actually changes three parameters during its operation; the acetate concentration is increased, the counter-ion concentration is decreased, and the strength of the mobile phase organic modifier is increased.

Figure 7 represents a chromatogram for a pooled, normal serum sample. The large peak which elutes early in the chromatogram is a result of the large quantities of amino acids produced in the

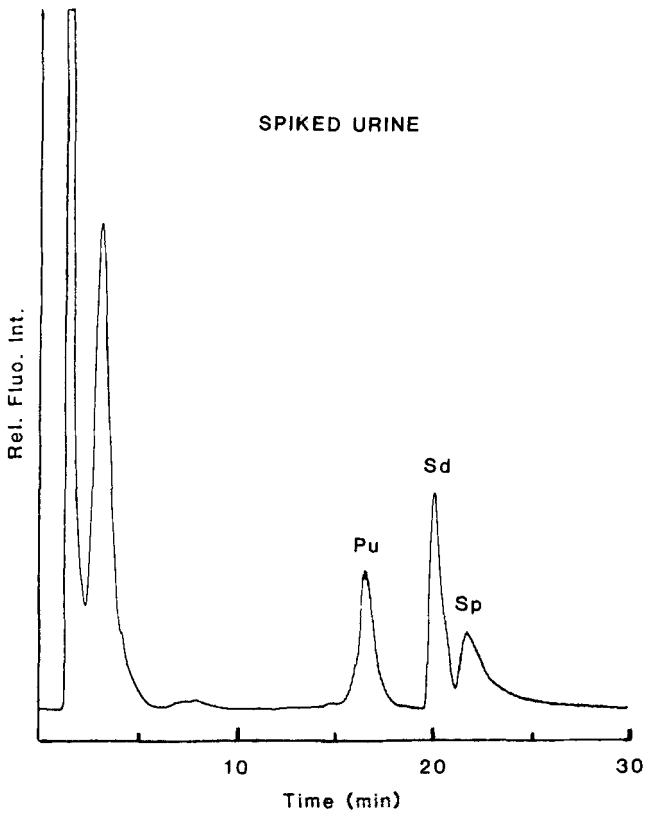

FIGURE 9. Chromatogram of normal urine spiked with ca. 1 nmol of each polyamine.

hydrolysis procedure. Since OPT reacts with primary amines, this response cannot be eliminated. However, under the chromatographic conditions used, the polyamines are strongly retained on the column during the initial isocratic period. The gradient portion of the program then elutes the polyamine ion-pairs after the interfering amino acids have passed through the column. Chromatograms for normal serum and normal urine samples spiked with the three polyamines are shown in Figures 8 and 9. These separations illustrate the non-interference of amino acids when analyzing physiological fluids for polyamines.

TABLE 2

Linear Ranges and Minimum Detection Limits

Compound	Ret. Time (min)	Min. Detection Limit	Linear Response Range	R^2
Pu	17.0±0.5	1.0 ng (12 pmole)	1.0 to 350 ng	0.999
Sd	20.0±0.5	6.0 ng (40 pmole)	6.0 to 290 ng	0.997
Sp	22.0±0.5	24 ng (120 pmole)	24 to 800 ng	0.998

There is a relatively long time period between the tailing edge of the amino acid front and the beginning of the first polyamine peak. The experimental parameters reported in this paper were tailored to provide optimum separations for our specific application. This separation method, however, is extremely flexible. If samples appear to have a consistently low amino acid content, then the initial isocratic period of the gradient may be shortened to decrease analysis time with no loss of resolution.

The linear ranges and minimum detection limits for each of the polyamines are presented in Table 2. The minimum detection limits listed are for the polyamines in the free form, $R(NH_2)_x$, and not in the protonated or ion-paired state. The linear correlation coefficients (R^2) for each of the polyamines indicate good linearity of detector response based on measurement of peak areas.

CONCLUSION

In summary, we have described a new, rapid, sensitive, and specific separation technique for putrescine, spermidine, and spermine utilizing reversed-phase ion-pairing HPLC coupled with on-line post-column derivatization and fluorimetric detection of

the polyamines. Analysis times are significantly shorter than most methods currently available. We are presently using this method to determine polyamines in physiological fluids obtained from psoriatic arthritis patients.

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