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SENSITIVE FLUORIMETRIC METHOD FOR THE DETERMINATION OF PUTRESCINE, SPERMIDINE AND SPERMINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND ITS APPLICATION TO HUMAN BLOOD

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SUMMARY

A fast and sensitive method for the determination of putrescine, spermidine and spermine by high-performance liquid chromatography is described. These compounds are converted to their fluorescent dansyl derivatives and are separated by a reversed-phase chromatographic system (Micropak CH-10) with water and acetonitrile as mobile phase. The sensitivity of the method is 30 pmoles.

The application of the method to the determination of polyamines in blood is described. It was found that most of the polyamines circulating in blood are localized in the erythrocytes, their content in normal human blood being spermidine 14.1 ± 3.1, and spermine 8.4 ± 2.8 nmoles/ml packed erythrocytes. Putrescine is not present in normal human erythrocytes. The polyamine level in serum is less than 0.1 nmole/ml.

The polyamine content of the erythrocytes from patients with malignant neoplasm was significantly elevated.

INTRODUCTION

Interest in the determination of the polyamines spermidine and spermine and their precursor putrescine in physiological material has been stimulated by the reports of Russell et al. [1,2], who described elevated levels of these compounds in the urine of patients with metastatic cancer. Following this finding, a number of papers for the estimation of polyamines in various biological samples such as urine [3—7], cerebrospinal fluid [8] and bone marrow cells [9] from cancer patients have appeared.

Since it was to be expected that polyamine levels in physiological fluids could be used as a clinical test in the diagnosis and follow-up of patients with

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cancer, it was desirable to develop a fast, simple and efficient method of analysing polyamines. A number of improved techniques have recently been developed, which are based on automated ion-exchange chromatography [10—14], gas chromatography [15—17], combined gas chromatography—mass spectrometry [18], thin-layer chromatography [19—21], or high-performance liquid chromatography (HPLC) [22—25] of polyamines and their derivatives.

In the course of our studies on haematopoietic malignancies we were interested in analysing polyamine levels in the blood of patients. Among the methods for polyamine analysis, the fluorescence method is the most sensitive one. However, hitherto reported methods are somewhat complicated and time-consuming. At the beginning of our studies we had to develop a less complicated, highly sensitive and reproducible method for polyamine analysis, especially suitable for analysing polyamines in blood.

This present work describes a quick and highly sensitive method for the determination of polyamines in blood as their dansyl derivatives by HPLC. The use of the method for the determination of polyamines in blood from normal subjects and patients with malignant neoplasm will be demonstrated.

EXPERIMENTAL

Materials

Dansyl chloride was obtained from Tokyo Kasei Kogyo (Tokyo, Japan) and recrystallized from *n*-hexane. Putrescine, spermidine and spermine as their hydrochlorides were purchased from Sigma (St. Louis, Mo., U.S.A.). Precoated silica-gel 60 plates for TLC were products of Merck (Darmstadt, G.F.R.) Acetonitrile, acetone, benzene, dioxane, chloroform, morpholine, triethylamine, perchloric acid, and L-proline were of special grade reagent from Wako Junyaku (Osaka, Japan). The ^{14}C-labelled polyamines, putrescine dihydrochloride (60 mCi/mmole), spermidine trihydrochlorine (122 mCi/mmole) and spermine tetrahydrochloride (115 mCi/mmole), were obtained from New England Nuclear (Boston, Mass., U.S.A.).

High-performance liquid chromatography

The high-performance liquid chromatograph was constructed in our laboratory. A high-pressure piston pump (Hitachi, Model 634) supplied the mobile phase at a rate of 1 ml/min to the column. The mobile phase for elution was a linear gradient between 20% acetonitrile in water (10 ml) and acetonitrile (10 ml), which was performed by using a pair of cylindrical glass vessels of the same size (1 × 20 cm). The column (25 cm × 2.2 mm I.D.) was Micropak CH-10, octadecylsilanized silica particles of 10 μm average diameter (Varian, Palo Alto, Calif., U.S.A.). The eluted fluorophores were detected with a Union Giken ultraviolet—fluorospectrophotomotor Model SM 303A, equipped with a Xenon lamp (Jobin Yvon), using wavelengths at 342 and 512 nm for activation and emission, respectively.

Procedure for determination of polyamines in human blood

Human blood, which was collected from the cubital vein with a heparinized syringe, was centrifuged at 550 *g* for 5 min. After the plasma and buffy coat

had been carefully removed by suction, the erythrocytes were suspended in 4 volumes of 0.9% NaCl, and the suspension was centrifuged at 2000 rpm for 5 min. After the sediment had been washed five times with 0.9% NaCl, packed erythrocytes were obtained. In this procedure, the packed erythrocytes had a haematocrit value of 90 ± 2%, and were contaminated with less than 5% of leucocytes.

To 0.25 ml of packed erythrocytes was added 0.75 ml of water and the cells hemolysed by mixing on a vortex mixer for 20 sec. To the resulting hemolysate was added 1 ml of 10% perchloric acid and extracted by mixing on the vortex mixer for 1 min; the precipitate was removed by centrifugation at 1200 *g* for 10 min. The, 0.5 ml of 5 *N* sodium carbonate and 2 ml of dansyl chloride (10 mg dansyl chloride/ml acetone) were added to the supernatant solution. Dansylation was allowed to proceed in the dark at room temperature for 16 h. Excess reagent was consumed by reaction with 0.1 ml of 1 *M* L-proline for 30 min in the dark at room temperature. Acetone was removed in vacuo on a rotary evaporator at 50°. The dansylated derivatives were extracted twice using 1.5 ml of benzene each time. The benzene was evaporated in vacuo at 50° on a rotary evaporator, and the residue was redissolved in 50 µl of benzene; 5 µl of the solution were subjected to HPLC.

Determination of recovery

The ^{14}C-labelled putrescine, spermidine and spermine were added to the haemolysate and processed as described in the determination procedure. Separated dansyl putrescine, dansyl spermidine and dansyl spermine were collected in counting vials from the outlet of the flow-cell of the high-pressure liquid chromatograph, monitoring with the fluorescence monitor, and the radioactivity was measured with a Beckman liquid scintillation spectrometer, Model DPM 100, using Bray's scintillator.

Preparation of dansyl polyamines

A 100-µl portion of 5 *N* sodium carbonate and 0.5 ml of dansyl chloride (10 mg dansyl chloride/ml acetone) were added to 0.4 ml of 0.25 m*M* spermine, and the reaction mixture was left to stand for 2 h at room temperature in the dark. After the excess dansyl chloride had been converted to dansyl proline by the addition of 50 µl of 1 *M* L-proline, acetone was evaporated in vacuo at 50°. Dansyl spermine was extracted twice with 1 ml of benzene each time. The benzene extract was evaporated to dryness in vacuo at 50° to give dansyl spermine.

Dansyl spermidine and dansyl putrescine were prepared in the same way as described above.

RESULTS

Resolution and quantitation

The best separation was accomplished by using a reversed-phase column, Micropak CH-10, with linear gradient elution from 20% acetonitrile in water to acetonitrile as a mobile phase. As shown in Fig. 1, the mixture of dansyl deriv-

TABLE I

CONCENTRATION OF SPERMIDINE AND SPERMINE IN BLOOD

The procedure for whole blood was the same as for erythrocytes as described in Experimental but using 0.25 ml of whole blood instead of packed erythrocytes. Leucocytes were isolated by the method using 6% dextran. Isolated leucosytes (10^6 cells) in water (0.4 ml) were subjected three times to freezing and thawing, and the resulting supernatant was dansylated. Spd, = spermidine; Spm,= spermine; n.d., not determined.

Subject	Polyamine	Polyamine concentration in:			Hematocrit (%) (C)	BxC 100 (D)	Dx100/A (%)
		Whole blood (nmoles/ml) (A)	Erythrocytes (nmoles/ml) (B)	Leucocytes (nmoles/10^9 cells)			
Normal	Spd	7.30	14.0	135	47	6.58	90
	Spm	4.50	8.62	338		4.05	90
Normal	Spd	6.84	12.43	77	44	5.47	80
	Spm	4.85	8.27	305		3.64	75
Gastric cancer	Spd	14.5	33.6	n.d.	38	12.8	88
	Spm	14.6	33.8			12.8	88
Gastric cancer	Spd	8.70	24.2	n.d.	31	7.50	86
	Spm	15.3	36.8			11.4	75

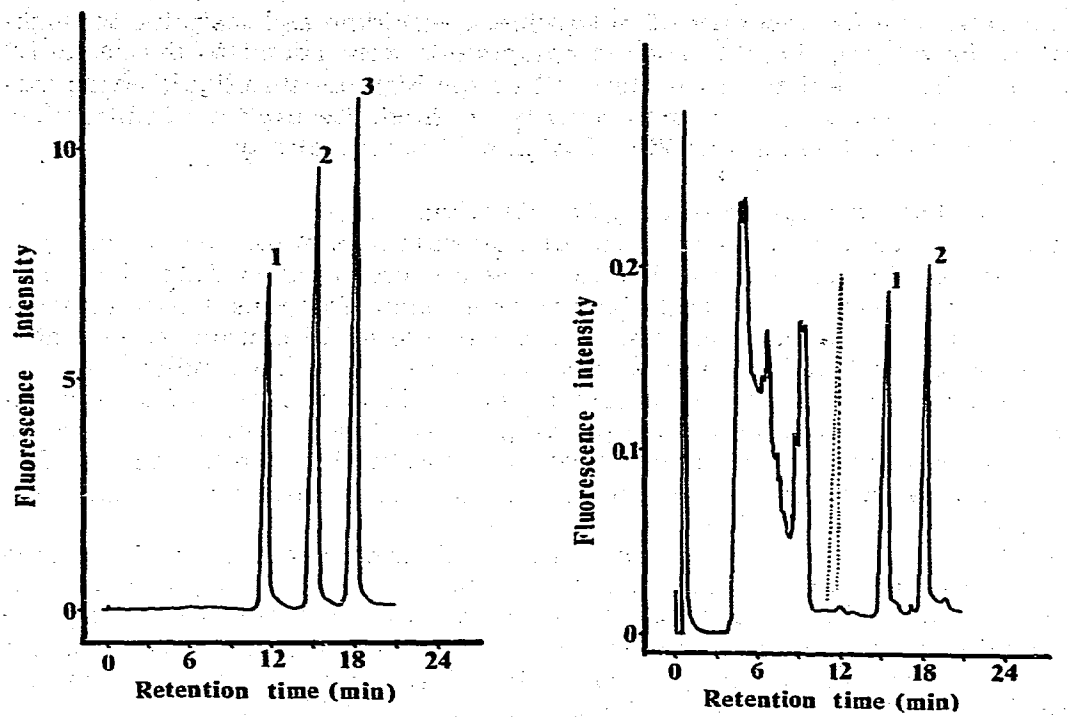

Fig. 1. Chromatogram of dansyl derivatives of putrescine, spermidine, and spermine. Standard samples were prepared as described under Experimental. Dansylated polyamines in benzene were injected into the high-performance liquid chromatograph. Conditions: column, Micropak CH-10 (25 cm × 2.2 mm I.D.); mobile phase, linear gradient between 10 ml of 20% acetonitrile in water and 10 ml of acetonitrile; flow-rate, 1 ml/min, 1, Putrescine; 2, spermidine; 3, spermine.

Fig. 2. Chromatogram of dansylated polyamines from human erythrocytes. Running conditions were the same as described in the legend of Fig. 1. All procedures are described in the text. Injected amount (5 μl) is equivalent to 25 μl of packed erythrocytes. 1, Spermidine; 2, spermine. The dotted line indicates the elution position of putrescine.

atives of putrescine, spermidine and spermine were well separated in a total analysis time of 20 min. The limit of detection was 30 pmoles. Peak heights of the fluorescence intensity trace on the recorder were plotted against the amount of each polyamine added, and a satisfactory linearity was obtained.

Determination of polyamines in human blood

Fig. 2 represents a chromatogram of dansylated derivatives obtained from erythrocytes. The injected amount of dansylated derivatives in benzene (5 μl) was equivalent to 25 μl of packed erythrocytes. As can be seen from the figure, determination of the polyamines can be achieved without any purification step such as cation-exchange column chromatography. This is achieved by using ^{14}C-labelled polyamines. As shown in this figure, erythrocytes of normal human blood contain both spermidine and spermine, but the content of putrescine is negligible. In this method, putrescine is eluted in the position indicated in the figure by the dotted line.

To determine the recovery of putrescine, spermidine and spermine throughout the procedure, the ^{14}C-labelled compounds were added to the haemolysate, and the effluent from the flow-cell of the high-pressure liquid chromatograph was collected and the radioactivity counted. Recoveries of putrescine, spermidine and spermine were 95%, 94% and 91%, respectively.

Polyamine concentration in healthy human blood

We examined the concentrations of polyamines in whole blood, erythrocytes, leucocytes and plasma. Several cases are presented in Table I. Putrescine was not detected in whole blood nor in blood fractions. Concentrations of polyamines in plasma were less than 0.1 nmole/ml of plasma. As indicated in the last column of Table I, more than about 80% of the spermidine and spermine in circulating blood was present in the erythrocytes. Most of the remaining amount of polyamines, corresponding to less than about 20% of spermidine and spermine in circulating blood, was localized in the leucocytes. The same situation was also found in the blood from a patient with malignant neoplasm, as shown in Table I.

As shown in Table II, the average concentrations of spermidine and spermine in erythrocytes were, respectively, 14.1 ± 3.1 and 8.4 ± 2.8 (mean ± S.D.) nmoles/ml packed erythrocytes. It is of interest that erythrocytes contain more spermidine than spermine and, on the other hand, that leucocytes contain more spermine than spermidine. The ratio of spermidine to spermine in erythrocytes was found to be 1.7 and that in leucocytes 0.25.

TABLE II

POLYAMINE CONTENT OF ERYTHROCYTES AND LEUCOCYTES

	Spermidine (mean ± S.D.)	Spermine (mean ± S.D.)	Spermidine/Spermine
Erythrocytes (nmoles/ml; n = 27)	14.1±3.1	8.4±2.8	1.7
Leucocytes (nmoles/10^9 cells, n = 7)	95±26	387±61	0.25

Polyamine content of erythrocytes from patients with malignant neoplasm

In order to know whether it is possible to use the polyamine level in erythrocytes as a marker of malignant neoplasm, the concentrations of polyamines in erythrocytes from patients with malignant neoplasm were determined.

The concentrations of polyamines were found to be significantly elevated in the erythrocytes as shown in Table III. As shown in the last column of Table III, the values of the spermidine/spermine ratio were widely distributed, and the relation between these values and the activity of malignant neoplasm is uncertain at the present. From these findings we consider it useful to determine the polyamine level in erythrocytes as a clinical test in the diagnosis and follow-up of patients with malignant neoplasm.

TABLE III

POLYAMINE CONTENT OF ERYTHROCYTES FROM PATIENTS WITH MALIGNANT NEOPLASM

F, female; M, male; IBL, immunoblastic lymphadenopathy.

Case	Age (Sex)	Diagnosis	Spermidine (nmoles/ml packed erythrocytes)	Spermine (nmoles/ml packed erythrocytes)	Spermidine/ Spermine
S.Y.	81 (F)	Gastric cancer	49.2	23.6	2.08
E.S.	51 (F)	Gastric cancer	164.0	101.0	1.62
T.T.	37 (F)	Duodenal cancer	42.4	111.0	0.38
H.N.	78 (M)	Rectal cancer	68.4	27.6	2.08
T.H.	74 (F)	Hepatoma	62.4	19.2	3.25
H.S.	64 (M)	Pancreatic cancer	48.8	16.7	2.92
F.F.	72 (M)	Pancreatic cancer	36.0	10.4	3.46
A.K.	37 (F)	Ovarian cancer	18.9	35.4	0.53
F.O.	72 (F)	Lymphosarcoma	34.8	11.1	3.14
M.T.	40 (M)	IBL	32.8	12.4	2.65

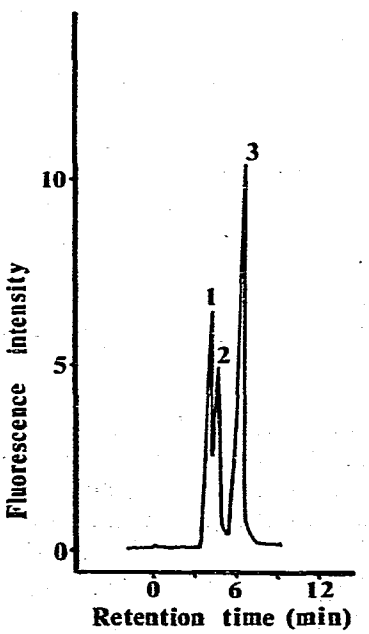

Fig. 3. Chromatogram of dansyl derivatives of putrescine, spermidine, and spermine. Running conditions: column, Micropak Si-10 (30 cm × 2.2 mm I.D.); mobile phase, chloroform—dioxane—triethylamine (100:10:1); flow-rate, 0.5 ml/min. 1, Spermine; 2, spermidine; 3, putrescine.

DISCUSSION

Several systems for the separation of dansylated polyamines were examined before a suitable one was selected. For example, at the beginning of the present work we tried TLC and found that dansyl derivatives of polyamines were well separated on a silica gel G 60 plate using chloroform—dioxane—N-ethyl morpholine (40:4:1) as a solvent system. On the basis on this finding a Micropak Si-10 (silica gel of 10 μm average diameter, Varian associates) column and a mobile phase consisting of chloroform—dioxane—triethylamine (100:10:1) was tested. As shown in Fig. 3 this system gave a fairly good resolution. We decided to abandon this system for the following reasons: (1) a good separation was not constantly obtained and depending on the lot number of the column overlap of the peaks of dansyl spermidine and dansyl spermine was observed: (2) irreversible contamination of the packing material occurred after about twenty runs.

Owing to the hydrophobic nature of the dansyl derivatives, it was apparent that a reversed-phase column would give satisfactory results. Finally, we found that Micropak CH-10 and a mobile phase consisting of a linear gradient between 20% acetonitrile in water and acetonitrile gave a good, reproducible resolution as shown in Fig. 1. The total analysis time is about 20 min. The limit of detection of this method is 30 pmoles. Only 0.25 ml of packed erythrocytes was needed for the analysis and the recoveries of putrescine, spermidine and spermine were 95%, 94% and 91%, respectively.

One of the most interesting findings of the present work is that most of the polyamines in circulating human blood are compartmentalized in the erythrocytes. It may be speculated that the erythrocytes function as polyamine carriers in the circulation.

The concentrations of spermidine and spermine in healthy human blood were, respectively, 14.1 ± 3.1 and 8.4 ± 2.8 (mean ± S.D.). nmoles/ml packed erythrocytes and the ratio of spermidine to spermine was about 1.7. Putrescine was not detected in the erythrocytes. In plasma the concentrations of polyamines was less than 0.1 nmole/ml. Leucocytes were found to contain more spermine than spermidine and the ratio of spermidine to spermine was about 0.25 (Table II). Very recently Cohen et al. [26] reported the same results in their extensive studies on the distribution of polyamines in blood from patients with cystic fibrosis and normal subjects.

Elevated levels of polyamines in urine, cerebrospinal fluid and bone narrow cells from cancer patients have been reported since 1971. In the present experiments we determined the concentrations of polyamines in the erythrocytes from patients with malignant neoplasms, and found that the polyamine levels in the erythrocytes from these patients were significantly elevated (Table III). Extensive work on the determination of polyamines in blood from patients with malignant neoplasm is in progress and will be published elsewhere.

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