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REVERSED-PHASE LIQUID CHROMATOGRAPHIC SEPARATION AND SIMULTANEOUS FLUORIMETRIC DETECTION OF POLYAMINES AND THEIR MONOACETYL DERIVATIVES IN HUMAN AND ANIMAL URINE, SERUM AND TISSUE SAMPLES: AN IMPROVED, RAPID AND SENSITIVE METHOD FOR ROUTINE APPLICATION

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SUMMARY

A highly sensitive and precise method for the determination of the polyamines putrescine, cadaverine, spermidine and spermine and all their monoacetyl derivatives in a single analysis in human and animal urine, serum and tissue samples is described. For polyamine separation, an ion-pairing reversed-phase high-performance liquid chromatographic (HPLC) method is used, followed by post-column derivatization with *o*-phthalaldehyde and consecutive fluorescence detection. Urine and serum samples are purified with a Bond Elut silica cartridge. The detection limit for polyamines is 0.5–1.0 pmol and excellent linearity is achieved in the range from 3 pmol up to more than 10 nmol. The influence of some modifications of different analytical steps such as the temperature of the HPLC column and the derivatization reaction coil and the *o*-phthalaldehyde flow-rate is described. Quality control data and measurements of the reproducibility of the method are presented. In order to establish a rapid analytical method for easy routine use, all steps for preparation and quantitative analysis are minimized. This method was applied to the determination of total polyamines in human urine and serum hydrolysate and of free and acetylated polyamines in human urine and pancreatic tissue of the rat. Values for normal polyamine concentrations in the urine and serum of fifteen male and fifteen female healthy volunteers and in the pancreas of ten normal rats are presented.

INTRODUCTION

The naturally occurring polyamines are normal constituents of all human and animal cells and play an important role in cell proliferation and differentiation [1,2]. In physiological fluids and tissue extracts the polyamines putrescine, cadaverine, spermidine and spermine occur in free, acetylated, peptidic and protein- and complex-bound forms [3]. In 1971, Russell [4] described increased

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polyamine concentrations in the urine of human cancer patients. Since then, many reports have been published reporting elevated levels of various polyamines in the urine [5–11] and serum [12,13] of cancer patients, and it was also demonstrated that increased polyamine concentrations in urine and serum returned to normal after successful surgery, chemotherapy or radiotherapy [5–7,13]. Although the efficacy of polyamine determination in urine and serum as markers of early neoplastic processes is still under debate [14,15], their usefulness as markers of disease activity, indicators of the efficacy of specific therapy and early predictors of relapse is well established [14–16]. In this respect, particularly the acetylated polyamines play an important role [10,17,18].

Today, the most common methods for polyamine determination are ion-exchange chromatography [7], high-voltage paper chromatography [8,9], thin-layer chromatography [19], gas chromatography [20–22], high-performance liquid chromatography (HPLC) [23–26], enzymatic methods [27] and radioimmunoassay [28] (detailed information is given in reviews [29,30]). Most of these methods suffer from various disadvantages, such as reduced sensitivity, insufficient purification steps or time-consuming preparation. Further, most of them allow the determination of only the parent polyamines [7–9] or incomplete detection of the acetyl derivatives [10,11,18–20,25]. Another disadvantage of many methods is that they are time-consuming and not suitable for automation, thus rendering them inconvenient for routine use.

Recently, two elegant procedures for the determination of polyamines in urine have been published which use HPLC [26] or gas chromatography [22]. We have modified Seiler and Knödgen's method [26] for the determination of polyamines in urine, and describe here a highly sensitive and reproducible method for polyamines in tissue, urine and serum involving reversed-phase HPLC and post-column derivatization with *o*-phthalaldehyde (OPA). In view of the increasing interest in polyamines in clinical studies, our method is highly suitable for easy and precise routine use; all steps for preparation and quantitative analysis are minimized and the method is fully automated. This makes the simple and simultaneous determination of polyamines and all monoacetyl derivatives possible.

EXPERIMENTAL

Chemicals and materials

The polyamine standards for putrescine, acetylputrescine, cadaverine, acetylcadaverine, 1,6-diaminohexane, 1,7-diaminoheptane, spermidine, N^1-acetylspermidine, N^8-acetylspermidine, spermine and N^1-acetylspermine, and also sodium octanesulphonate, Brij 35, OPA and calf thymus DNA were purchased from Sigma (St. Louis, MO, U.S.A.). Perchloric acid (PCA), ammonia, trichloroacetic acid (TCA), diethyl ether, sodium acetate, acetonitrile, boric acid, mercaptoethanol, disodium phosphate, hydrochloric acid and picric acid were obtained from Merck (Darmstadt, F.R.G.) and bisbenzimidazole (Hoechst H 33258) from Hoechst (Frankfurt, F.R.G.). Millex-GS filters (pore size 0.22 μm) were purchased from Millipore (Molsheim, France) and Bond Elut silica cartridges from Analytichem International (Harbor City, CA, U.S.A.).

HPLC equipment

The HPLC equipment (Merck-Hitachi, Darmstadt, F.R.G.) consisted of a 655 A-12 pump, 655 A-40 autosampler, L-5000 LC controller, 655 A-13 reaction pump for the post-column derivatization with *o*-phthalaldehyde, an F 1000 fluorescence spectrophotometer, a D-2000 chromato-integrator and a 655 A-52 column oven. For the analytical procedure we used a NovaPak C_{18} (4 μm) column (150 mm $\times$ 3.9 mm I.D.), which was protected by a Guard-Pak μBondapak C_{18} (10 μm) column (0.4 cm $\times$ 0.6 cm I.D.), both from Millipore-Waters (Eschborn, F.R.G.).

Sample preparation

Tissue. Biopsies and tissue samples of different human and animal organs were quantified. Although in this paper normal polyamine concentrations are given for rat pancreas only, the identical preparation and analytical procedure allows precise polyamine determinations in many other human or animal tissues (data not shown).

The tissue sample was diluted 1:20 in 0.9% sodium chloride, homogenized on ice with an Ultra-Turrax homogenizer (Janke & Kunkel, Staufen, F.R.G.) at 20 000 rpm for 4 $\times$ 15 s, sonicated in a sonicator cell disruptor (Ultrasonics, Plainview, NY, U.S.A.) and 500 μl of the homogenate were mixed with 100 μl of an internal standard (3000 nmol/ml 1,7-diaminoheptane). A 1.4-ml volume of 0.2 *M* PCA was added to precipitate the proteins, followed by centrifugation for 5 min at 3200 *g*. The supernatant was filtered through a Millex-GS filter (pore size 0.22 μm). For routine analysis, 20 μl of this filtered homogenate were passed through the HPLC column. As a reference standard the DNA content in the homogenate was measured according to Labarca and Paigen [31] and polyamine concentrations in the tissue are expressed as nmol/mg of DNA.

Urine. Fresh morning urine from 30 healthy humans (age 30–68 years) was immediately frozen and stored at $-20\,^{\circ}C$ until analysis. Creatinine was measured using picric acid [32].

A 2-ml urine sample was mixed with 200 μl of internal standard (30 nmol/ml, 1,7-diaminoheptane) for the determination of free and acetylated polyamines. Further, 2 ml of the urine were mixed with 100 μl of internal standard (300 nmol/ml 1,7-diaminoheptane) for the determination of total polyamines after acid hydrolysis.

Serum. Blood samples from 30 healthy human volunteers (age 24–73 years) were taken in the morning, centrifuged immediately and the supernatant was stored at $-20\,^{\circ}C$ until analysis. A 200-μl volume of internal standard (30 nmol/ml 1,7-diaminoheptane) was added to 3 ml of the defrozen serum and this solution was diluted 1:1 with 10% TCA for precipitation of serum proteins. The mixture was stored for 30 min at $4\,^{\circ}C$, then centrifuged at 3000 *g* for 10 min. The supernatant was taken, mixed with 2 ml of diethyl ether and centrifuged for 5 min at 1000 *g*. The TCA-free phase was taken with a pipette and saved for acid hydrolysis.

Acid hydrolysis. Acid hydrolysis was performed for the analysis of total polyamines in urine and serum. The prepared urine and serum samples were mixed 1:1 with 1 *M* hydrochloric acid and hydrolysed at $100\,^{\circ}C$ for 14 h. The influence

of different acid concentrations (0.1–12 *M*) on the effectiveness of the hydrolysis was tested.

Sample purification. The pH of the hydrolysed urine and serum and of the unhydrolysed urine was adjusted to 9–10 by adding 25% ammonia solution and the solutions were shaken and passed through Bond Elut silica cartridges. Then the columns were washed with 10 ml of aqua bidest and the polyamines were eluted by washing the columns with 10 ml of 1 *M* hydrochloric acid. The eluate was evaporated to dryness in a Rotax Evaporator (Büchi, Eislingen, F.R.G.) and the residue was dissolved in 500 μl of 0.1 *M* hydrochloric acid. From each urine and serum solution 20 μl were applied by the autosampler to the HPLC column for polyamine determination.

Analytical procedure

Buffers. The two buffer solutions for the elution system were prepared as described previously [25]. Buffer A was 0.1 *M* sodium acetate adjusted to pH 4.5 with acetic acid plus 0.01 *M* sodium octanesulphonate. Buffer B was 0.2 *M* sodium acetate (adjusted to pH 4.5 with acetic acid) plus acetonitrile (10:3, v/v) plus 0.01 *M* sodium octanesulphonate.

Gradient. For the analytical procedure two different gradients were used (Table I). Gradient I, with an analysis time of only 17 min, was used for the separation of polyamines and their monoacetylated derivatives except for cadaverine and acetylcadaverine, particularly in tissue samples. Gradient II had an analysis time of 46 min and allowed the precise separation of all known polyamines and monoacetylated polyamine derivatives. Gradient II was mainly used for the determination of polyamines in urine and serum.

Chromatographic conditions. The flow-rate was 1.5 ml/min. The temperature of the column oven was normally 35 °C. The influence of different temperatures on the polyamine analysis was studied. For the separation of the polyamines we used a NovaPak C_{18} column. After post-column derivatization with OPA, the

TABLE I

COMPOSITION OF GRADIENTS I AND II

	Gradient I			Gradient II		
	Elution time (min)	Percentage of buffer		Elution time (min)	Percentage of buffer	
		A	B		A	B
	0	50	50	0	90	10
	10	25	75	40	0	100
	12	0	100	45	0	100
	16	0	100	46	90	10
	17	50	50			
Equilibration time (min)	8			10		
Total time (min)	25			56		

fluorescence intensity was measured with the F 1000 fluorescence spectrophotometer (excitation at 345 nm, emission at 455 nm).

Post-column derivatization. The OPA–2-mercaptoethanol reagent for the post-column derivatization procedure was prepared according to the method of Seiler and Knödgen [25] with minor modifications. Boric acid (12.34 g) and 25 ml of 25% potassium hydroxide were dissolved in water in a final volume of 1 l and the pH was adjusted to 10.4 with 50% potassium hydroxide solution. A 100-ml volume of this solution was mixed with 3 ml of Brij 35 solution, 2 ml of 2-mercaptoethanol and 400 mg of OPA, dissolved in 5 ml of methanol. The OPA reagent was mixed with the solution of the HPLC system behind the HPLC column in the 655 A-13 reaction pump. The OPA reagent was added through three different valves by three different pumps, each with a flow-rate of 0.25 ml/min. For normal analysis the temperature of the reaction coil was 45 °C. The influence of the variation of the OPA reagent flow-rate and the temperature of the reaction coil was studied.

Quantification and quality control

Before and within each analytical series a standard mixture of known amounts of all polyamines was analysed as a reference standard. This polyamine standard mixture was prepared in the same way as all the samples before analysis, i.e., it was passed through the Bond Elut silica cartridges and was also hydrolysed. To all sample homogenates 1,7-diaminoheptane was added as an internal standard. The different polyamines were characterized on the basis of their retention times compared with the analytical standards and co-injection with a known amount of standard polyamines.

For quality control of the different preparative procedures, such as purification by Bond Elut silica cartridges and hydrolysis with hydrochloric acid, we compared the determination of polyamines in a pooled sample of urine or serum without and after adding different known amounts of standard polyamines.

The polyamine concentrations in tissues are expressed as nmol/mg of DNA, in urine as nmol/mg of creatinine and in serum as nmol/ml.

RESULTS AND DISCUSSION

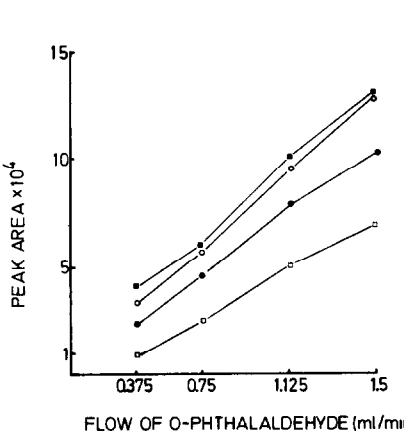
Chromatographic methodology

The liquid chromatographic method presented allows the precise and simultaneous determination of the polyamines acetylputrescine, putrescine, acetylcadaverine, cadaverine, N^1-acetylspermidine, N^8-acetylspermidine, spermidine, N^1-acetylspermine and spermine. The method is based on an HPLC separation described by Seiler and Knödgen [25,26]. As will be pointed out in detail, we modified and improved the above method, particularly in the following respects: improved sensitivity by different modifications of analytical steps such as the temperature of the HPLC column and the post-column derivatization reaction coil, the detection limit was decreased several-fold up to less than 1 pmol, more effective purification of urine samples by using Bond Elut silica cartridges, hydrolysis with 1 *M* instead of 12 *M* hydrochloric acid and extension of the method to polyamines in serum samples.

Table I shows the composition of the two gradients used for HPLC separation of polyamines. While gradient II allows the determination of all polyamines in tissues, serum and urine within 56 min, gradient I is used for the rapid (25 min) determination of polyamines in samples containing no cadaverine or acetylcadaverine, such as in several tissue samples.

The effects of variations of different chromatographic conditions such as pH, concentration of the buffers or concentration of sodium octanesulphonate are well known [23,24]. We investigated the influence of the OPA flow-rate, the temperature of the HPLC column and the temperature of the post-column derivatization reaction coil on the chromatographic analysis. As shown in Fig. 1, the peak area increases linearly with increasing flow-rate of OPA in the post-column derivatization procedure. Further, it is possible to increase the peak area exponentially by increasing the temperature of the reaction coil at which the post-column derivatization with OPA takes place (Fig. 2). For the routine determination of polyamines we use an OPA flow-rate of 0.75 ml/min and a reaction coil temperature of 45 °C. Moreover, the retention time of the polyamines decreases moderately with increasing temperature of the HPLC column, therefore shortening the analytical procedure. For normal analysis the temperature of the HPLC column is kept at 35 °C.

Altogether the modifications of the above-mentioned parameters in combination with pre-purification with Bond Elut silica cartridges improve the sensitivity of the method and decreases the detection limit for polyamines to within the range 0.5–1 pmol. Excellent linearity is achieved from about 3 pmol up to more than 10 nmol. The reproducibility of this method in terms of retention times is similar to that of other methods [13,16], the relative standard deviation being about 1%.



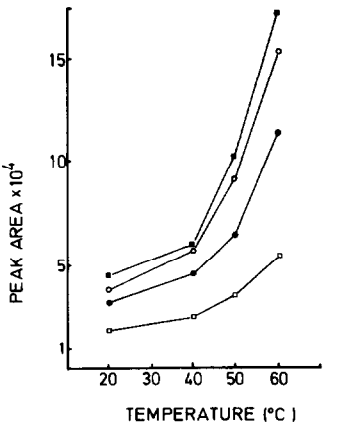


Fig. 1. Relationship between the variation of *o*-phthalaldehyde flow-rate (ml/min) in the post-column derivatization reaction and the peak area of the chromatogram after fluorescence detection. (■) Spermidine; (○) spermine; (●) putrescine; (□) 1,6-diaminohexane.

Fig. 2. Relationship between the temperature of the post-column derivatization reaction coil and the peak area of the chromatogram after fluorescence detection. (■) Spermidine; (○) spermine; (●) putrescine; (□) 1,6-diaminohexane.

Sample preparation

Different purification procedures using different agents such as silica gel [13,20–22], Cellex P [33] or Dowex [5,19] have been described, whereas some workers do not prepurify their samples at all [25,26]. In our opinion, a prepurification is essential to eliminate interfering compounds and to concentrate the substances of interest. The quality and precision of the analysis are much improved after effective prepurification with Bond Elut silica cartridges, although not all interfering substances could be removed completely. After testing different purification procedures, Bond Elut silica cartridges proved to be the most effective and also simple and selective purification column.

In order to measure the recovery of the preparation and purification with Bond Elut silica cartridges, we determined the polyamine concentrations in a standard mixture containing 1, 5 or 10 nmol of each polyamine, with and without the prepurification procedure. The recovery of free and acetylated polyamines from the silica column ranged between 92 and 98%.

One experiment was carried out to evaluate the long-term stability of the polyamine concentrations in animal tissue and human urine samples depending on the temperature. Different samples were stored for several months at $-20°C$, $+4°C$ or room temperature and the polyamine concentrations were determined every ten days. It was clearly shown that all polyamine concentrations remained stable for more than three months at all the storage temperatures tested (data not shown).

Determination of polyamines in tissue samples

For the determination of polyamines in human and animal tissues we used gradient I (Table I). Fig. 3 shows a typical chromatographic separation of polyamines in the rat pancreas. The concentrations amounted to 7.95 ± 1.18 nmol/mg of DNA for putrescine, 1561 ± 132 nmol/mg of DNA for spermidine and 169.2 ± 24.7 nmol/mg of DNA for spermine (mean $\pm$ S.D., $n=10$). As has been pointed out earlier [30], tissue levels of acetyl derivatives are usually very low. We could not detect a quantifiable amount of any acetyl derivative or cadaverine in unstimulated normal rat pancreas.

With the method described, the precise and reproducible determination of polyamines in tissue samples weighing < 1 mg is possible. We were able to measure polyamines in several human tissue samples which were obtained by endoscopic biopsies, indicating that this technique could be applied for extensive use in clinical research and practice.

Determination of polyamines in urine

After efficient prepurification with Bond Elut silica columns, our HPLC method allows the precise simultaneous determination of polyamines and all monoacetyl derivatives in a single run using gradient II (Table I). A representative chromatogram of a hydrolysed and non-hydrolysed human urine sample is shown in Fig. 4, and Table II depicts the normal concentrations of free, acetylated and total polyamines in the urine from 30 healthy humans. Our data confirm previous findings that the acetyl derivatives are the major excreted conjugates of polyamines

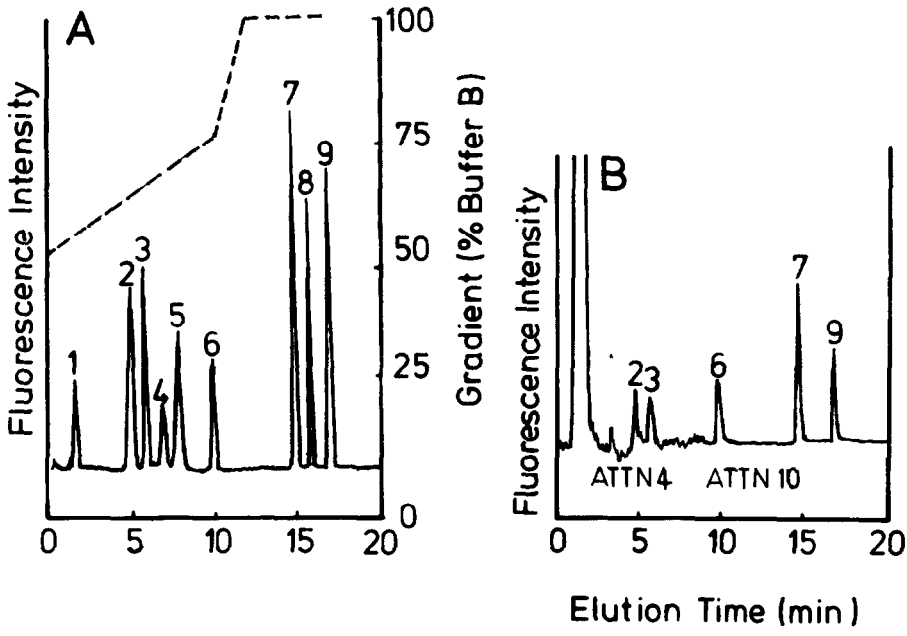


Fig. 3. Separation of polyamines in the pancreas of normal rats by reversed-phase HPLC using gradient I. Chromatogram of (A) a standard mixture of polyamines (50 nmol/ml) and (B) polyamines in normal rat pancreas. Attn = attenuation. Peaks: 1 = acetylputrescine; 2 = putrescine; 3 = histamine; 4 = N^1-acetylspermidine; 5 = N^8-acetylspermidine; 6 = 1,7-diaminoheptane (internal standard); 7 = spermidine; 8 = N^1-acetylspermine; 9 = spermine.

[17–19,22,25,26]. Although several workers reported sex differences and age-dependent variations of urinary polyamine excretion [17,19,21,22], these differences could not be confirmed by others [8,18,20] or in the present investigation.

In contrast to most of the previous studies, we were able to detect spermine in all the urines, although quantification of these small amounts was sometimes not possible. Recently, Van den Berg et al. [22] pointed out that N^1-acetylspermine is a normal constituent of urine, although it was not detectable in all urine samples. This finding could be confirmed in this study, as our method allows the sensitive detection of N^1-acetylspermine in most human urines and in only a few instances was it not detectable.

The different origins of cadaverine and acetylcadaverine that are detectable in human serum and urine are still a matter of discussion [13,20–22]. Initially, it was suspected to be produced solely by bacterial degradation, but it was also found to be synthesized in mammalian cells [13]. Most of the published methods cannot detect cadaverine or acetylcadaverine at all [5,6,8,9,18,19], whereas others gave varying amounts, especially of acetylcadaverine, which in some instances was highly elevated [20], possibly owing to bacterial contamination, and in others not even detectable [11,13,22]. Using the present method we could detect and quantify cadaverine precisely in all instances, but as far as acetylcadaverine is concerned this compound could not be detected in about 30% of all cases, mainly owing to the interference of another substance which eluted almost in the same area as acetylcadaverine.

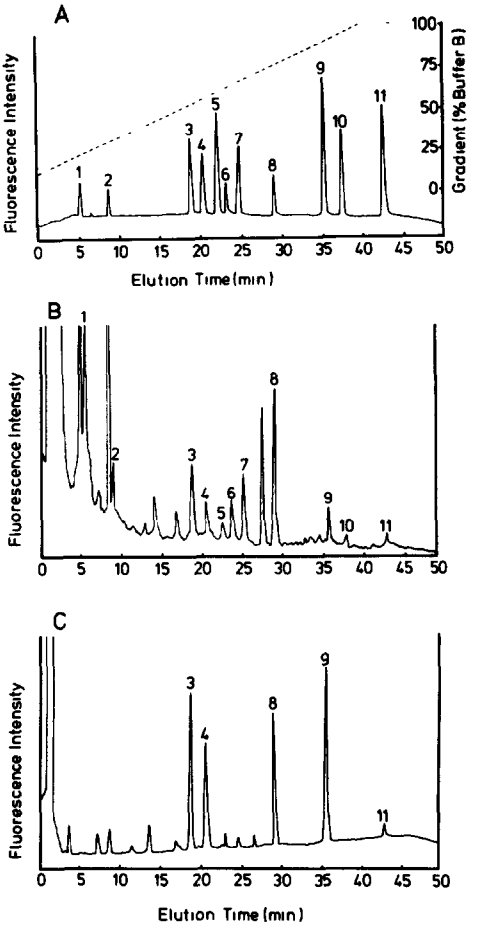


Fig. 4. Separation of polyamines in normal human urine by reversed-phase HPLC using gradient II. (A) Chromatogram of a standard mixture of polyamines (5 nmol/ml), (B) chromatogram of free and acetylated polyamines in normal human urine and (C) chromatogram of total polyamines in normal human urine after hydrolysis (1 *M* hydrochloric acid, 120°C, 14 h). Peaks: 1 = acetylputrescine; 2 = acetylcadaverine; 3 = putrescine; 4 = cadaverine; 5 = histamine; 6 = N^1-acetylspermidine; 7 = N^8-acetylspermidine; 8 = 1,7-diaminoheptane (internal standard); 9 = spermidine; 10 = N^1-acetylspermine; 11 = spermine.

A summary of the results of different methods of measuring polyamines in human urine is shown in Table III, which clearly demonstrates the large discrepancies for the normal values given in the different studies and the fact that nearly all methods that detect the different polyamines and their acetyl derivatives are incomplete. So far, only two methods have been described that give a complex analysis of all known polyamines [22,26].

Acid hydrolysis of the urine sample is performed to release the protein- or complex-bound polyamines and to determine the total polyamine content. Many workers use 6 or 12 *M* hydrochloric acid for acid hydrolysis [5,13,19,21,26]. We used 1 *M* hydrochloric acid, because it was observed in several experiments that

TABLE II

FREE, ACETYLATED AND TOTAL POLYAMINE CONCENTRATIONS IN THE URINE OF HEALTHY MALE AND FEMALE VOLUNTEERS

AcPut = acetylputrescine; AcCad = acetylcadaverine; Put = putrescine; Cad = cadaverine; Histam = histamine; N^1-AcSpd = N^1-acetylspermidine; N^8-AcSpd = N^8-acetylspermidine; Spd = spermidine; N^1-AcSpm = N^1-acetylspermine; Spm = spermine.

		Age (years)	Concentration (nmol/mg of creatinine)									
			AcPut	AcCad	Put	Cad	Histam	N^1-AcSpd	N^8-AcSpd	Spd	N^1-AcSpm	Spm
Free and acetylated polyamines												
Male	Mean	53.5	9.2	2.6*	0.52	0.35	0.39	3.2	1.8	0.23	0.096*	0.39*
(n=15)	S.D.	10.2	3.4	1.6	0.34	0.14	0.25	1.3	0.8	0.12	0.04	0.23
Female	Mean	50.0	12.3	3.1*	0.87	0.71	0.56	3.7	2.3	0.24	0.096*	0.37*
(n=15)	S.D.	9.0	3.1	1.6	0.51	0.29	0.25	1.7	0.98	0.08	0.03	0.19
Total	Mean	51.6	10.7	2.8*	0.68	0.52	0.48	3.4	2.0	0.24	0.096*	0.38*
(n=30)	S.D.	9.5	3.5	1.6	0.45	0.29	0.26	1.5	0.9	0.1	0.03	0.21
Total polyamines												
Male	Mean	53.5			12.4	4.30				5.44		0.62
(n=15)	S.D.	10.2			5.1	2.9				2.1		0.44
Female	Mean	50.0			15.1	2.67				6.50		0.47
(n=15)	S.D.	9.0			4.7	1.6				3.1		0.31
Total	Mean	51.6			13.9	3.40				6.00		0.54
(n=30)	S.D.	9.5			5.0	2.4				2.6		0.37

* Acetylcadaverine and N^1-acetylspermine are not detectable in a few instances (for details, see text); spermine is detectable in all instances but sometimes not exactly quantifiable.

TABLE III

COMPARISON OF NORMAL CONCENTRATIONS OF TOTAL, FREE AND ACETYLATED POLYAMINES IN THE URINE OF HEALTHY ADULTS AS PUBLISHED USING VARIOUS TECHNIQUES

Abbreviations as in Table II; Ref. = Reference; creat. = creatinine; HPLC = high-performance liquid chromatography; OPA = *o*-phthalaldehyde; Dns-Cl = dansyl chloride; HPVE = high-voltage paper electrophoresis; AAA = amino acid analysis; TLC = thin-layer chromatography; GC = gas chromatography; IEC = ion-exchange chromatography.

Ref.	Year	Method	Hydrolysis	*n*	AcPut	AcCad	Put	Cad	N^1-AcSpd	N^8-AcSpd	Spd	N^1-AcSpm	Spm	Units
4	1971	HVPE	14 h, 110°C, 6 *M* HCl	11			2.9				3.6		3.5	mg/24 h
8	1975	HVPE	None	21			3.15				1.75		6.45	nmol/mg creat.
5	1976	HVPE	3 h, 100°C, 2 *M* HCl	56			125.3				20.3		46.4	nmol/mg creat.
6	1977	AAA	15 h, 110°C, 6 *M* HCl	16			2.1				1.2		0.04	μg/mg creat.
10	1978	HPLC, Dns-Cl	None	9	11.7	1.9	1.6		2.91	2.84	0.2		2.1	μmol/24 h
19	1979	TLC	None	10	18.8				5.3	4.4				μmol/24 h
20	1982	GC	None	15	12.9	4.5			3.4	3.0				μmol/24 h
11	1982	HPLC, OPA	None	6	2.3	0.32			0.83	0.99				μg/mg creat.
7	1983	IEC	None	13			1.8	0.09			0.58		0.2	mmol/mol creat.
9	1983	HVPE	None	40			1.4				1.32			μg/mg creat.
13	1983	HPLC, Dns-Cl	None	12			1.49	0.015			0.87		0.46	mmol/mg creat.
			12 h, 110°C, 6 *M* HCl	12			4.86	0.54			3.31		0.99	mmol/mg creat.
18	1983	AAA	None	8	15.6		1.0		3.2	1.5	<0.2		<0.2	nmol/mg creat.
21	1984	GC	18 h, 120°C, 12 *M* HCl	52			1.44	0.50			0.58		0.13	mmol/mol creat.
22	1986	GC	None	31	1.18	0.16	0.35	0.40	0.39	0.30	0.05	0.02	0.08	mmol/mol creat.
This work		HPLC, OPA	14 h, 100°C, 1 *M* HCl	30			13.9	3.4			6.0		0.54	nmol/mg creat.
This work		HPLC, OPA	None	30	10.7	2.8	0.68	0.52	3.4	2.0	0.24	0.096	0.38	nmol/mg creat.

hydrolysis with acid of lower concentration does not increase the fractions of total polyamines, but rather the amounts of total putrescine, cadaverine and the internal standard 1,7-diaminoheptane decrease with increasing acid concentrations (data not shown). The acid used (1 *M*) is strong enough to convert all acetylated polyamines completely into their parent compounds. A similar observation has already been described [5].

In order to evaluate the recovery of the polyamines in urine with and without acid hydrolysis, 2.5, 5.0, 7.5 and 10.0 nmol of each standard polyamine were added to 1 ml of urine. The recovery of all polyamines and monoacetyl derivatives in non-hydrolysed urine was above 93%. Whereas the recovery after acid hydrolysis for spermine (resulting from parent spermine and N^1-acetylspermine) was about 70%, that of all other polyamines from their parent or monoacetyl compounds after acid hydrolysis was above 90%.

Determination of polyamines in serum

The method was also extended to the determination of total polyamines in hydrolysed serum. As already pointed out, the effective prepurification of the serum samples with Bond Elut silica columns and the hydrolysis with 1 *M* hydrochloric acid for 14 h at 100°C made the rapid and precise determination of polyamines in serum possible. A typical chromatographic separation of total polyamines in normal human serum is shown in Fig. 5. Table IV depicts the mean values of total polyamine concentrations in healthy male and female volunteers. No significant differences in polyamine concentrations in serum due to sex or age could be found.

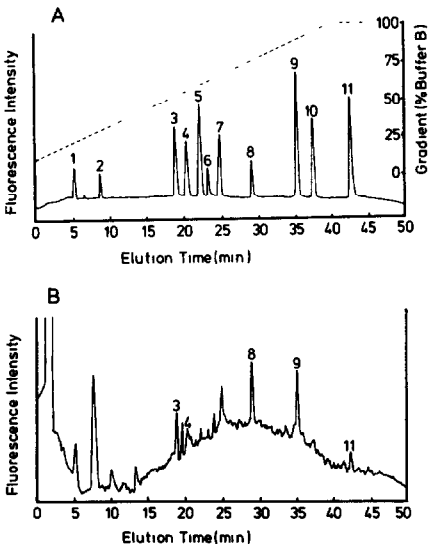


Fig. 5. Separation of polyamines in normal human serum by reversed-phase HPLC using gradient II. Chromatogram of (A) a standard mixture of polyamines (5 nmol/ml) and (B) total polyamines in a normal human serum sample after hydrolysis (1 *M* hydrochloric acid, 120°C, 14 h). Peaks as in Fig. 4.

TABLE IV

TOTAL POLYAMINE CONCENTRATIONS IN THE SERUM OF HEALTHY MALE AND FEMALE VOLUNTEERS

		Age (years)	Concentration (nmol/ml)			
			Putrescine	Cadaverine	Spermidine	Spermine
Male	Mean	52.4	0.464	0.048*	0.286	0.172*
(n=15)	S.D.	12.3	0.15	0.02	0.07	0.05
Female	Mean	45.4	0.513	0.055*	0.285	0.178*
(n=15)	S.D.	18.0	0.11	0.02	0.03	0.07
Total	Mean	49.3	0.488	0.052*	0.286	0.175*
(n=30)	S.D.	15.4	0.13	0.02	0.05	0.06

* Cadaverine is not detectable in a few instances; spermine is detectable in all instances but sometimes not exactly quantifiable (for details, see text).

It is well known that only a minor part of the polyamines in blood are detectable in serum and plasma, the majority of polyamines being bound to erythrocytes and leukocytes [14]. Whereas most workers were not able to detect spermine in all or some of their human plasma samples [13,33], our method enables spermine to be detected in all samples although sometimes the minute amount present in a few instances is not exactly quantifiable. On the other hand, it was not possible to detect cadaverine in some human serum samples, confirming previous studies [34].

The recovery of the different polyamines in serum samples was measured in the same way as described for urine. An excellent recovery of more than 92% for all polyamines and monoacetyl derivatives was achieved. Recoveries published for other methods [13,33] are below our values.

CONCLUSIONS

A highly sensitive and reproducible reversed-phase HPLC method has been developed for the simultaneous and rapid determination of polyamines in tissues, urine and serum. Although various characteristic data of this method have been published earlier [26], various modifications of the preparative and analytical procedure have been made and quality control data were presented to establish an analytical method for polyamines that fulfils the following criteria: simultaneous determination of all polyamines and all monoacetyl derivatives in a single analytical run, improved sensitivity and detection limits, rapid preparative and analytical procedures, precise reproducibility, improved polyamine recovery and applicability of the same easy method to polyamine analysis in tissues, urine and serum. Therefore, this method could be applied for tests in clinical research and practice.

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REFERENCES

1 A.E. Pegg and P.P. McCann, Am. J. Physiol., 243 (1982) C212.
2 C.W. Tabor and H. Tabor, Annu. Rev. Biochem., 53 (1984) 749.
3 R.A. Campbell, D.P. Gretti, D. Bartos and F. Bartos, Adv. Polyamine Res., 3 (1981) 409.
4 D.H. Russell, Nature New Biol., 233 (1971) 144.
5 K. Fujita, T. Nagatsu, K. Maruta, M. Ito, H. Senba and K. Mihi, Cancer Res., 36 (1976) 1320.
6 B. Durie, S. Salmon and D.H. Russell, Cancer Res., 37 (1977) 214.
7 D. Depierre and A. Jung, J. Clin. Chem. Clin. Biochem., 21 (1983) 35.
8 M. Tsuji, T. Nakajima and I. Sano, Clin. Chim. Acta, 59 (1975) 161.
9 K. Shinpo, K. Fujita, K. Maruta, R. Teradaira and T. Nagatsu, Clin. Chim. Acta, 131 (1983) 143.
10 M.M. Abdel-Monem and K. Ohno, J. Pharm. Sci., 67 (1978) 1671.
11 C.E. Prussak and D.H. Russell, J. Chromatogr., 229 (1982) 47.
12 H. Takami, M.M. Romsdahl and K. Nishioka, Lancet, ii (1979) 912.
13 B. Brossat, J. Straczek, F. Belleville, P. Nabet and R. Metz, J. Chromatogr., 277 (1983) 87.
14 D.H. Russell, CRC Crit. Rev. Clin. Lab. Sci., 18 (1983) 261.
15 S.E. Bates and D.L. Longo, Cancer Treat. Rev., 12 (1985) 163.
16 D.H. Russell, B.G.M. Durie and S.E. Salmon, Lancet, ii (1975) 797.
17 N. Seiler, J. Koch-Weser, B. Knödgen, W. Richards, C. Tardif, F.N. Bolkenius, P. Schechter, G. Tell, P. Mamont, J. Fozard, V. Bachrach and E. Grosshans, Adv. Polyamine Res., 3 (1981) 197.
18 D.H. Russell, J.D. Ellingson and T.P. Davis, J. Chromatogr., 273 (1983) 263.
19 N. Seiler and B. Knödgen, J. Chromatogr., 164 (1979) 155.
20 S. Yamamoto, M. Yokogawa, K. Wakamatsu, H. Kataoka and M. Makita, J. Chromatogr., 233 (1982) 29.
21 F.A.J. Muskiet, G.A. van den Berg, A.W. Kingma, D.C. Fremouw-Ottevangers and M.R. Halie, Clin. Chem., 30 (1984) 687.
22 G.A. van den Berg, F.A.J. Muskiet, A.W. Kingma, W. van der Slik and M.R. Halie, Clin. Chem., 32 (1986) 1930.
23 J. Wagner, C. Danzin and P. Mamont, J. Chromatogr., 227 (1982) 349.
24 R.C. Simpson, H.Y. Mohammed and H. Veening, J. Liq. Chromatogr., 5 (1982) 245.
25 N. Seiler and B. Knödgen, J. Chromatogr., 211 (1980) 227.
26 N. Seiler and B. Knödgen, J. Chromatogr., 339 (1985) 45.
27 S. Kubota, M. Okada, K. Imahori and N. Ohsawa, Cancer Res., 43 (1983) 2363.
28 D. Bartos, R.A. Campbell, F. Bartos and D.P. Grettie, Cancer Res., 35 (1975) 2056.
29 U. Bachrach, Adv. Polyamine Res., 2 (1978) 5.
30 N. Seiler, J. Chromatogr., 379 (1986) 157.
31 C. Labarca and K. Paigen, Anal. Biochem., 102 (1980) 344.
32 H. Bartels, M. Böhmer and C. Heierli, Clin. Chim. Acta, 37 (1972) 193.
33 M. Kai, T. Ogata, K. Haraguchi and Y. Ohkura, J. Chromatogr., 163 (1979) 151.
34 J. Bontemps, J. Laschet, G. Dandrifosse, J.-L. van Cutsem and P.-P. Forget, J. Chromatogr., 311 (1984) 59.