Journal of Chromatography, 379 (1986) 157—176
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2984

REVIEW

POLYAMINES

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(First received October 2nd, 1985; revised manuscript received November 1st, 1985)

CONTENTS

1. INTRODUCTION

An essential role of the natural polyamines spermidine and spermine in normal growth and proliferation of eukaryotic cells has been firmly established [1—5]. Both these compounds and their precursor putrescine (1,4-butanediamine) are present in all tissues and body fluids. The major urinary excretion forms of putrescine and spermidine are, however, their monoacetyl derivatives [6].

Tissue levels of the acetyl derivatives are usually very low, but may be enhanced after intoxication, food deprivation and other pathophysiological conditions [7—9]. In addition to free and acetylated polyamines, some amino acids which are formed from the polyamines by oxidative deamination have

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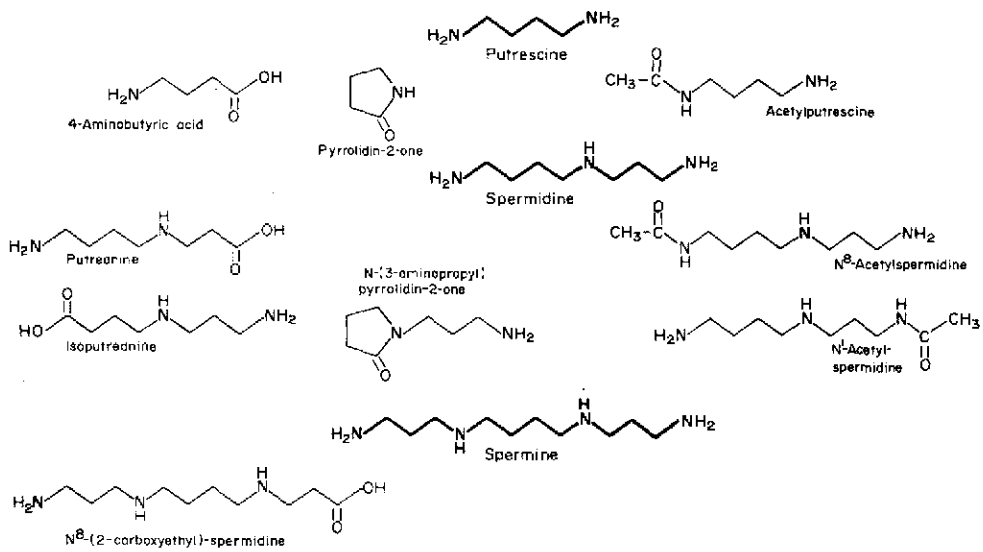


Fig. 1. Natural derivatives of putrescine (1,4-butanediamine).

been found in rat and human urine [10- 12]. One of these amino acids, putreanine, is a normal constituent of vertebrate brains [13].

Fig. 1 shows the structural formulae of these compounds. For the sake of simplicity we shall use the terms polyamines and polyamine derivatives for all compounds shown in Fig. 1.

The comparison of normal tissue with tumour tissue is an especially instructive example of the role of the polyamines in cellular growth. If hepatic tissue is dedifferentiating to a hepatoma, striking changes occur in ornithine metabolism: the activity of ornithine transcarbamylase, which normally channels ornithine into the urea cycle, nearly disappears. Similarly, the activity of the transaminase, which catalyses the first step of the formation of glutamate and proline from ornithine, decreases. In contrast, the activity of ornithine decarboxylase increases and with it the rate of putrescine formation [14]. With few exceptions, the putrescine concentration is elevated in proportion to tumour growth rate, and spermidine concentrations are also frequently elevated [15]. In other words, during dedifferentiation cells lose their ability for specialized functions and their enzymatic machinery is geared to rapid growth. Polyamine changes in tissues appear, therefore, to be indicative of changes in growth rate.

The enhanced polyamine metabolism in rapidly growing tissues prompted Russell to study the potential usefulness of polyamines as tumour markers [16]. Stimulated by this apparently successful approach, an enormous amount of work has been carried out in this area during the last 15 years. This work has recently been reviewed [17–23]. There is still no definitive answer to the question of how significant polyamine determinations in urine or body fluids are in the diagnosis of tumours or other diseases [24] and the scope and limitations of this approach need further clarification. However, it seems certain that longitudinal studies of individual patients can in many instances produce very important information on the efficacy of therapies and the recurrence of the disease [25, 26].

For diagnostic purposes putrescine, spermidine and spermine were initially determined in hydrolysates of urine and body fluids. When the excretion of the polyamines in the form of their monoacetyl derivatives had been demonstrated [27], more detailed information could be expected from the determination of the polyamine conjugates. This aspect is still under investigation. Very recently some of the polyamine-derived amino acids have been considered as potential markers of disease states and a suitable method has been proposed [28, 29]. Thus it appears that a demand exists for more complete and precise or more simple and rapid assay procedures for the polyamines and their derivatives. This is documented by the still growing number of methodological papers.

As appears from previous reviews [30–33], the whole arsenal of analytical methods has at one time or another been used for polyamine determinations. This tendency has continued and attempts have been made even in recent years in which new versions of virtually all chromatographic methods have been proposed for polyamine assay. The only exception seem to be electrophoretic methods, which have not been developed further during the last decade [34].

The concentration of polyamines in tissues, body fluids and urine is in the micromoles per litre range, comparable to that of many other biogenic amines and amino acids, with which they share the amino groups as the most conspicuous structural feature. Hence methods for their analysis require a high degree of specificity and sensitivity.

Because they do not exhibit any structural feature that would allow their sensitive detection without derivatization, all chemical methods of polyamine analysis require at least two steps: separation from all other amino groups containing constituents of a tissue extract, body fluid or urine, and derivative formation. The order of these two steps can be reversed.

In all current methods the amino groups are used for derivative formation. As these reactions are not specific for the polyamines, the specificity of the chemical methods is exclusively based on separation procedures. Disregarding some gas–liquid chromatographic methods, the detection sensitivity depends on the derivatization reaction.

2. METHODS FOR SEPARATING NON-DERIVATIZED AMINES

2.1. Thin-layer and paper chromatography

Just over a decade ago an impressive amount of work had been performed in polyamine biochemistry based on planar chromatographic methods and paper electrophoresis [35]. These methods have now been abandoned by most laboratories, and find only very occasional application. Not even the recent development of reversed-phase thin-layer chromatography has found use in polyamine analysis.

The major difficulty in the practical applications of paper and thin-layer chromatography or electrophoresis is based on the high polarity of the polyamines, which makes their isolation from salt-containing solutions difficult. Disregarding exceptional cases, it is not possible to apply tissue extracts directly on paper or thin layers in amounts that allow the determination of

putrescine. Moreover, amino acids and polyamines move together, so that extensive separations are required that are not easily achieved by one-dimensional development. Although planar chromatographic methods are of little value in the profiling of polyamines in tissues and body fluids, they may still be advantageously used in in vitro enzyme tests and related applications. Some details of these methods can be found in a recent review [34].

2.2. Separation of polyamines by ion-exchange chromatography

The structural features of the polyamines obviously lend themselves to cation-exchange chromatographic separation. The use of cation-exchange resin paper was suggested 25 years ago [36]. Recently, overpressured thin-layer chromatography, using cation-exchange resin thin layers, staining with ninhydrin and video-densitometry were combined in a very rapid method for the determinaton of the usual polyamines and some basic amino acids (ornithine, arginine) in the nanomole range [37].

The application of ion-exchange column chromatography to the determination of the polyamines has a long tradition [38]. Elaborate separations were achieved on cellulose phosphate [39], a support still in use in laboratories without access to sophisticated equipment [40—42]. Mostly, however, cation exchangers were used. Initially separations were performed manually, but later almost all commercial automated amino acid analysers were employed for polyamine analysis [30] and continue to be applied [43—48]. By taking advantage of a high-performance cation-exchange column, a series of amino acids, amines and peptides can be separated in addition to the polyamines [49]; however, none of these recent developments show significant improvements over the method of Marton and Lee [50], which was the first to employ resin with a bead diameter of 10 μm (and consequently a higher pressure than was usual for the conventional amino acid analysers). With ninhydrin as detection reagent 25—100 pmol of the polyamines could be determined. Post-column derivatization with *o*-phthalaldehyde—2-mercaptoethanol [51] increased the sensitivity of the method. A 3—6 pmol concentration of putrescine and spermidine and, owing to peak broadening only, 12—15 pmol of spermine could be routinely determined with a relative standard deviation of less than 5%. This procedure was the one most widely used in the past for the screening of polyamines in tissue extracts and in hydrolysates of body fluids and urine.

Interestingly, fluorescamine, another reagent suitable for post-column derivatization of primary amino groups containing compounds, which increases the detection sensitivity similarly to *o*-phthalaldehyde, has not found much application in polyamine analysis, although its practical usefulness has been demonstrated [52].

With one exception [53, 54], all early ion-exchange chromatographic procedures were limited to the determination of non-conjugated polyamines. Therefore, polyamine determinations in human urine were restricted to hydrolysates. Using more recent amino acid analysers, elution programmes could be worked out that allowed the routine determination of monoacetylputrescine, and of the monoacetylspermidines in human urine [55, 56]. These

methods are currently used to study the significance of acetylpolyamine excretion in a variety of diseases. As shown in Fig. 2, for the establishment of a complete pattern of polyamines and acetylpolyamines in a sample of deproteinized urine, 53 min are required plus 25 min for the re-equilibration of the column [56]. Using *o*-phthalaldehyde—2-mercaptoethanol reagent and recording of fluorescence intensity at 455 nm (excitation of fluorescence at 340 nm), the sensitivity limit is 10—30 pmol and the relative standard deviation is 3.2%.

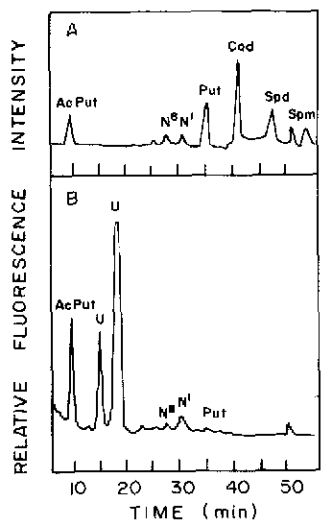


Fig. 2. Separation of a standard mixture of polyamines and acetylpolyamines (A) and of normal human urine (B) by cation-exchange column chromatography. Column (11.5 cm × 1.7 mm I.D.) packed with Aminex A-9 cation-exchange resin (bead diameter 11–12 μm); flow-rate, 19.2 ml/h; column temperature 54°C. Step-wise elution with five sodium citrate buffers containing increasing amounts of sodium chloride, and pH changing between 5.6 and 10.2 (for details see ref. 56). Detection by recording of fluorescence intensity after reaction of the column eluate with *o*-phthalaldehyde– 2-mercaptoethanol reagent (activation of fluorescence at 345 nm, emission wavelength 455 nm). The standard mixture contained 200 pmole of each amine. Peaks: AcPut = monoacetylputrescine; N^8 = N^8-acetylspermidine; N^1= N^1-acetylspermidine; Put = putrescine (1,4-butanediamine); Cad = cadaverine (1,5-pentanediamine); Spd = spermidine; Spm = spermine; U = unidentified compound. (After Russell et al. [56].)

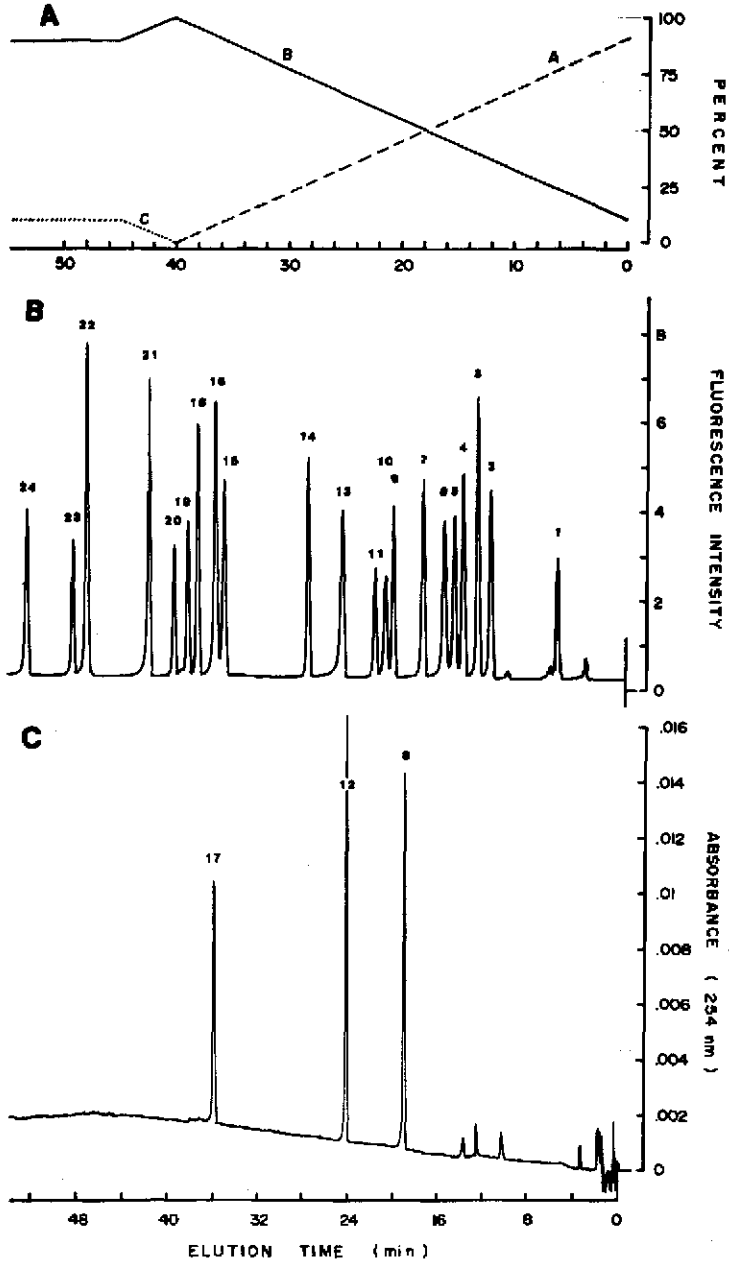
Owing to the high capacity of the ion-exchange resins it is possible to apply samples without prepurification or concentration of the polyamines. Hence it appears that contemporary ion-exchange column chromatographic procedures are reliable, fully automated methods that are very well suited to routine polyamine assay.

2.3. Separation of ion pairs on reversed-phase high-performance columns

The natural polyamines have structural features to form ion pairs with readily ionizable organic acids, e.g., *n*-hexane-, *n*-heptane- and *n*-octanesulphonic acid. For the separation of ion pairs of the polyamines the usual reversed-phase columns with C_{12}–C_{18} aliphatic chains or phenyl residues bound to a silica core are suitable.

The precise mechanism underlying the method is not known. One has to assume that within the mobile phase the cations (polyamines) are in dynamic equilibrium with their respective ion pairs. The ion pairs in solution are in equilibrium with the ion pairs which are hydrophobically bound to the column matrix. In addition, the column can bind the organic acid by hydrophobic interaction and thus a kind of cation exchanger is formed on the matrix surface. The amines move along the column with the mobile phase in the form of ion pairs rather than as free cations.

In order to avoid peak broadening it is important to increase the concentra-



tion of the ion pair-forming acid to an extent that the equilibrium is shifted to the right, so that the polycations form single species of ion pairs, i.e., one has to ensure that putrescine binds two, spermidine three and spermine four molecules of the organic acid.

The parameters (pH, temperature, dielectric constant and ionic strength of the solvent) that affect the chromatographic behaviour of the ion pairs on reversed-phase columns have been studied [57] and resemble in many ways those which are important in cation-exchange column chromatography. Only the hydrophobic interaction with the column matrix of the compounds to be separated is stronger than with the matrix of ion-exchange resins. This may cause interferences with some peptides or long-chain amino groups containing lipids.

The first method that allowed the separation and determination of natural polyamines and their monoacetyl derivatives (except monoacetylputrescine and monoacetylcadaverine) in the picomole range was published in 1980 [58]. Subsequently, several versions of this method have appeared [57, 59–64]. Using a more efficient column, it is now possible to determine in a single run the complete polyamine pattern (including monoacetylputrescine) of urine or tissue extracts, and in hydrolysates one can measure isoputreanine in addition to the polyamines and histamine [64]. Isoputreanine is formed by hydrolysis from its lactam and conjugates thereof, which are natural degradation products of spermidine [65].

One advantage of the method is the high quality of the columns, which allows excellent separations within a short time. They do not have the disadvantage of variations in the bed volume during elution with a pH gradient, as is the case with cation-exchange resins.

Similarly to ion-exchange column chromatographic procedures, ion-pair separations require minimum sample preparation. Usually one separates up to 200 μl of tissue extracts in 0.2 *M* perchloric acid, or comparable preparations from urine or blood. The method can be fully automated, and as the organic acids used for ion pair formation do not interfere with the *o*-phthalaldehyde reaction, the same reagent can be used as for post-column derivatization after ion-exchange column chromatography.

Wagner et al. [63] demonstrated that S-adenosylmethionine, methylthio-

Fig. 3. Separation of polyamines and related compounds by reversed-phase ion-pair liquid chromatography. Column, Beckman Ultrasphere I.P.; solvents (A) 0.1 *M* sodium acetate (pH 4.50) containing 10 m*M* sodium octanesulphonate, (B) 0.2 *M* sodium acetate (pH 4.50)–acetonitrile (10:3) with 10 m*M* sodium octanesulphonate, (C) methanol, room temperature; flow-rate, 1 ml/min; gradient composition as shown in (A). Detection: adenosyl derivatives, absorptimetry (254 nm) (C); polyamines, reaction with *o*-phthalaldehyde–2-mercaptoethanol reagent and recording of fluorescence intensity (activation of fluorescence at 345 nm, emission wavelength 455 nm) (B). Peaks: 1 = tyramine; 2 = anserine; 3 = phenylalanine; 4 = carnosine; 5 = homocarnosine; 6 = acetylputrescine; 7 = putreanine; 8 = S-adenosylmethionine; 9 = arginine; 10 = acetylcadaverine; 11 = tryptamine, 12 = 5′-methylthioadenosine; 13 = isoputreanine lactam; 14 = isoputreanine; 15 = putrescine; 16 = cadaverine; 17 = decarboxylated S-adenosylmethionine; 18 = histamine; 19 = N^1-acetylspermidine; 20 = N^8-acetylspermidine; 21 = 1,7-diaminoheptane (internal standard), 22 = spermidine; 23 = N^1-acetylspermine; 24 = spermine. Amounts: polyamines, 100 pmol; S-adenosylmethionine derivatives, 200 pmol. For more details, other binary and ternary gradients and examples of polyamine determinations in urine, see ref. 64.

adenosine and decarboxylated S-adenosylmethionine can be determined together with the polyamines if the column eluent passes the flow cell of a spectrophotometer before it is mixed with *o*-phthalaldehyde—2-mercaptoethanol reagent and the absorbance is monitored at 254 nm. Fig. 3 shows the separation of polyamines and related compounds. This method is suitable for their determination in tissues and body fluids.

It appears that ion partition chromatography is at present the most versatile and efficient routine method for polyamine analysis.

3. METHODS FOR SEPARATING AMINE DERIVATIVES

3.1. Liquid—liquid chromatographic methods

Pre-chromatographic derivatization is used in order to allow the sensitive determination of polyamines. The following absorbing (UV or visible light) reagents have been suggested for polyamine determinations: benzoyl chloride [66], *p*-toluenesulphonyl chloride [67], 2,4-dinitrofluorobenzene [68], 4-fluoro-3-nitrobenzotrifluoride [69], quinoline-8-sulphonyl chloride [70] and 4-dimethylaminoazobenzene-4'-sulphonyl chloride [71]. Among the many available fluorogenic reagents [72, 73], the following have been used: 5-dimethylaminonaphthalene-1-sulphonyl chloride (dansyl chloride) [74], *o*-phthalaldehyde—2-mercaptoethanol [75,76] and fluorescamine [77,78].

In contrast to the acid chlorides, which form derivatives not only with primary and secondary amino groups but also with imidazole nitrogen and phenolic hydroxy groups, and even with some alcohols, the last two reagents react only with primary amino groups and are therefore more selective reagents. Nevertheless, their practical application has been limited by the instability of the reaction products. A fixed time schedule of sample preparation and sample separation is required. The introduction of fully automated procedures may increase the number of applications of these methods in cases where a limited number of compounds have to be determined at high sensitivity, e.g., in hydrolysates of cerebrospinal fluid.

The acid chlorides do not differ greatly in their reactivity and the same principles of derivative formation are valid for all these reagents. Reaction is normally achieved in alkaline buffered (pH 8—10) aqueous solutions containing an organic solvent in order to ensure a homogeneous reaction medium. Similar conditions are also used for derivatization with fluorescamine [77,78] and *o*-phthalaldehyde—2-mercaptoethanol [75]. The reagents must be applied in excess in order to obtain quantitative reaction with all amino groups of the polyamines. Insufficient amounts of reagent are a frequent source for unsatisfactory results.

One advantage of the pre-chromatographic derivatization method is the solubility of the reaction products in organic solvents, which allows their extraction from aqueous phases and thus the convenient accumulation of small amounts from relatively large volumes of tissue extracts and body fluids. Another advantage of forming chemically defined, stable derivatives, such as the dansyl derivatives, is the possibility of their chromatographic isolation and unambiguous identification and even quantitation by mass spectrometry [79].

By far the greatest amount of work has been carried out with dansyl chloride. Because of the high fluorescence quantum yield of the derivatives, and their favourable chromatographic properties [80, 81], this reagent has found wide application for the determination of polyamines during the last 18 years, and new separation procedures have been published recently.

Benzoyl chloride and related reagents have the advantage of not forming side reaction products, as is the case with dansyl chloride, and therefore they are in use in some laboratories [82, 83] and may be advantageously used for the assay of certain enzymes [84]. Quantitation by UV absorptimetry at 254 or 230 nm allows the measurement of 2—50 μg of toluenesulphonamides [67] and 20—200 ng of quinoline-8-sulphonamides [70]. As dansyl derivatives absorb strongly, their determination by photometry is also feasible [85—87]; however, photometric methods cannot compete in sensitivity with fluorimetric determinations, which allow the measurement of polyamines in the picomole or even femtomole range.

The derivatives of the acid chlorides and also of fluorescamine are suitable for both thin-layer chromatographic (TLC) and reversed-phase column chromatographic separations. A number of TLC procedures have been reported for the determination of polyamines in tissue extracts and urine hydrolysates [79—81, 88—94]. Table 1 summarizes the solvents and thin layers used in these methods. TLC is also suitable for the determination of acetylated polyamines [95]. Separations of standard mixtures and urine samples are shown in Fig. 4.

Thin-layer plates are quantitatively evaluated by direct scanning, or by eluting the dansyl derivative with an organic solvent and measurement of the fluorescence intensity [79—81].

During the last 10 years, many groups have developed systems for the separation of dansyl polyamines, mostly using reversed-phase columns, with or without addition of ion-pairing agents [87, 96—105]. These methods mirror the development of reversed-phase columns and high-performance liquid chromatographic (HPLC) equipment in the last decade. All these procedures have in common that N^1-acetylspermidine and N^8-acetylspermidine could not be

TABLE 1

THIN-LAYER CHROMATOGRAPHIC METHODS FOR THE DETERMINATION OF POLYAMINES AS THEIR DANSYL DERIVATIVES

Ascending development in solvent vapour-saturated tanks. Silica gel G plates (250 μm layer thickness) and pre-coated plates (silica gel 60) give similar separations.

Thin layer	Solvent	Ref.
Silica gel G	Cyclohexane—ethyl acetate (3:2)	140
Silica gel G	(a) Cyclohexane—ethyl acetate (1:1) (b) Cyclohexane—ethyl acetate (7:5) (two runs)	90
Silica gel 60	Chloroform—triethylamine (5:1)	91, 92
Silica gel 60	Chloroform—toluene—triethylamine (15:7:3)	94
Silica gel 60, HPTLC plate	Cyclohexane—ethyl acetate (1:1) (two runs)	93
Kieselguhr G	Cyclohexane—ethyl acetate (13:4)	89
Alumina	Dioxane—glacial acetic acid—chloroform (2:1:97)	88

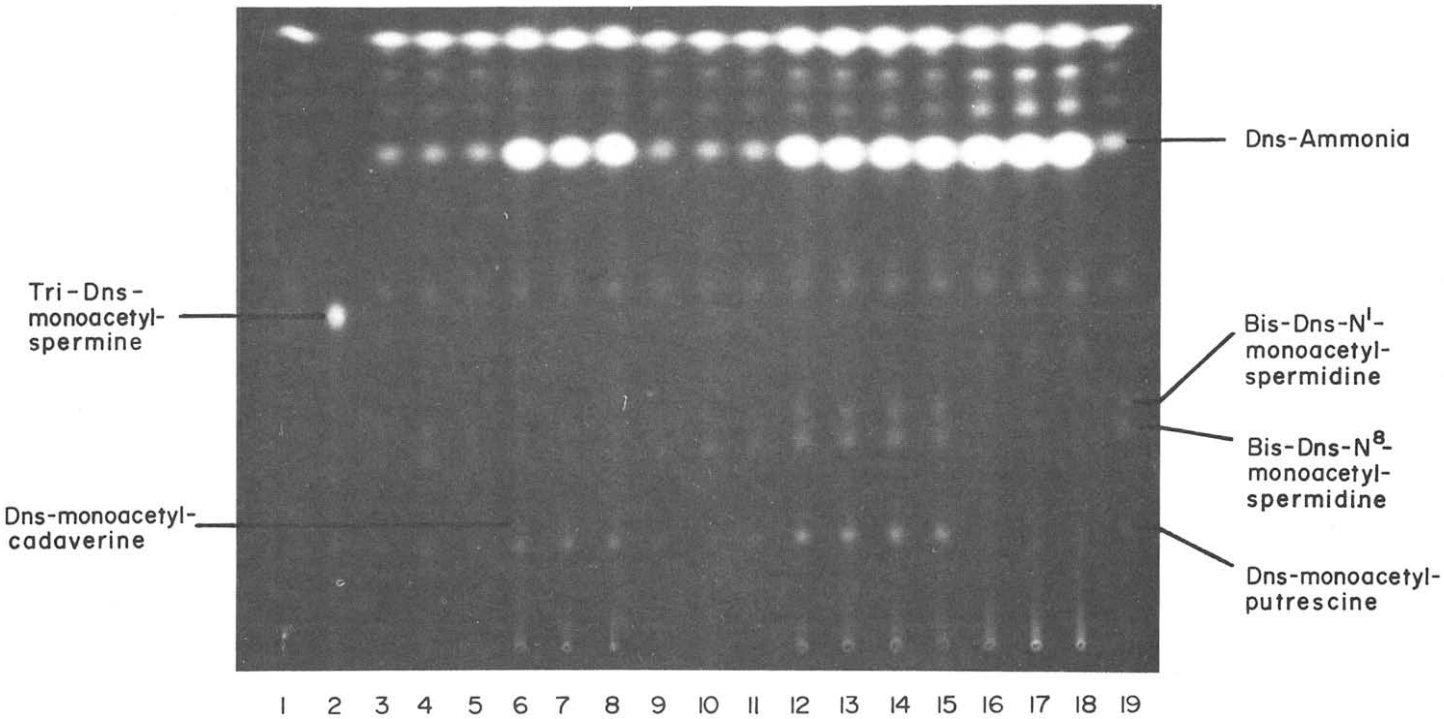


Fig. 4. Thin-layer chromatographic separation of the natural acetylpolyamines as their dansyl (Dns) derivatives. TLC plate, silica gel 60 (Merck); solvent, (a) ethyl acetate, (b) chloroform—tetrachloromethane—methanol (14:6:1) (two runs) (ascending development in solvent vapour-saturated tank). 1 = Blank; 2 = tridansylmonoacetylspermine; 3—5, 10, 11, 19 = standard mixture containing 50 pmol of monodansylmonoacetylputrescine, bisdansyl-N^1-acetylspermidine and bisdansyl-N^8-acetylspermidine; 6—8 = dansyl derivatives of human urine samples (equivalent to 10 µl of urine); 12—15, urine samples corresponding to 6—8, but with added standard mixture; 16—18, urine samples hydrolysed with 6 *M* hydrochloric acid prior to reaction with dansyl chloride. The dansyl derivatives of the non-conjugated polyamines move faster than ammonia under these chromatographic conditions and cannot be detected on this chromatogram. For further details, see ref. 95.

separated. This is true for all derivatives, not only for the dansyl derivatives*. Most of the published reversed-phase HPLC methods are, therefore, restricted to the sensitive determination of putrescine, cadaverine, spermidine and spermine. Kneifel and co-workers [97, 106] have shown, however, that dansylation in combination with reversed-phase HPLC can be extremely useful for the detection and determination of unusual polyamines in arachaebacteria (Fig. 5). In order to permit the determination of the monoacetyl derivatives after dansylation, Abdel-Monem and co-workers [107, 108] suggested two-step separation procedures. The separation of bis-dansyl-N^1-acetylspermidine and bis-dansyl-N^8-acetylspermidine was achieved either on silica gel plates or on a silica gel column after the initial separation.

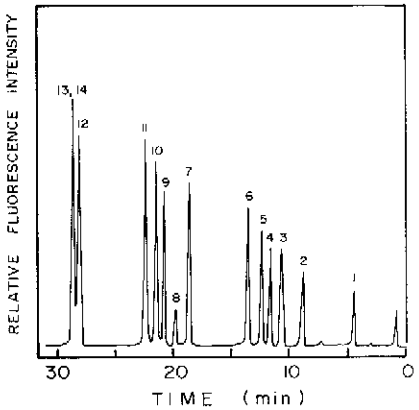


Fig. 5. Separation of dansyl derivatives of natural amines by reversed-phase HPLC. Column, Spherisorb ODS-1 (5-μm particles); solvent, linear gradient changing from water—acetonitrile (1:1) to water—acetonitrile (3:17) within 30 min; flow-rate, 1 ml/min. Detection by recording of fluorescence intensity (activation of fluorescence at 360 nm, emission wavelength 520 nm). Peaks: 1 = monodansylmethylamine; 2 = monodansylisobutylamine; 3 = monodansylisoamylamine; 4 = bisdansyl-1,3-diaminopropane; 5 = bisdansylputrescine; 6 = bisdansylcadaverine; 7 = bisdansyl-1,8-diaminooctane (internal standard); 8 = bisdansyltyramine; 9 = tridansyl-*sym*-norspermidine; 10 = tridansylspermidine; 11 = tridansyl-*sym*-homospermidine; 12 = tetradansyl-*sym*-norspermine; 13 = tetradansylspermine; 14 = tetradansylthermospermine. (After Kneifel et al. [141].)

Derivative formation opens up the possibility of another approach to sensitive polyamine assay, viz., the double isotope derivative assay. Samples to which [^{14}C]polyamines have been added in known amounts are derivatized with [^{3}H]dansyl chloride. The dansyl derivatives are then isolated by TLC and the ratio of the radioactivity of ^{14}C and ^{3}H is determined. The amount of polyamine in the sample is calculated from the relative dilution of the specific radioactivity of the [^{14}C]polyamine [109]. The advantage of this approach is that it is not necessary either to achieve quantitative derivative formation or to isolate quantitatively the dansyl derivatives. Moreover, fluorescence intensity measurements are replaced by the more convenient fully automated β-scintillation spectrophotometry.

*Very recently Stefanelli et al. [142] succeeded in separating N^1-acetylspermidine and N^8-acetylspermidine as dansyl derivatives using an Ultrasphere ODS column.

TABLE 2

GAS—LIQUID CHROMATOGRAPHIC METHODS FOR THE DETERMINATION OF POLYAMINES AND THEIR DERIVATIVES

Type of derivative	Column dimensions (length × I.D.)	Stationary phase		Detection*	Sensitivity	Method applicable to polyamine derivatives	Ref.
Free bases	180 cm × 3 mm	Glass beads	Carbowax 20M (0.4%)—KOH (1%)	FID	1 nmol	No	114
		Chromosorb W (80—100 mesh)	Versamid 900 (5%)—KOH (5%)			No	115
Permethylated amines		Chromosorb W (80—100 mesh)	Versamid 900 (5%)—KOH (5%)	FID		No	115
		Chromosorb W (80—100 mesh)	OV-17 (3%)	MS		No	115
Trifluoroacetyl	100 cm × 2 mm	Gas-Chrom Q (100—120 mesh)	OV-17 (2%)—SP-1401 (1%)	FID	10 ng	No	110
	100 cm × 6 mm	Chromosorb W HP (100—120 mesh)	OV-17 (5%)	FID		No	111
	100 cm × 2 mm	Chromosorb W HP (80—100 mesh)	OV-17 (2%)	MS		Yes	113
	200 cm × 2 mm	Gas-Chrom Q (100—200 mesh)	OV-17 (3%)	MS			117, 119
		Chromosorb W HP (80—100 mesh)	OV-17 (3%)	SIM	1 pmol	Yes	117, 118
Pentafluoro-propionyl	200 cm × 2 mm		OV-1 (3)	MS		No	120

Heptafluoro-butyryl	150 cm × 3 mm	Chromosorb W HP (80—100 mesh)	OV-17 (3%)	ECD	0.1 pmol	No	121
	30 m × 0.32 mm	—	CP-Sil-5 (0.2 μm film thickness)	FID		Yes	28
	35 m × 0.2 mm	—	Methylsilicone (0.11 μm film thickness)	NPD		Yes	12
				SIM		Yes	29
Pentafluoro-benzoyl			Carbowax 20 M—TPA	ECD	0.1 ng	No	122
			OV-1 (1%)—SP-1000 (0.25%)	ECD	0.1 ng	No	122
Isobutyloxy-carbonyl		Gas-Chrom P (80—100 mesh)	SE-30 (1.5%)—SP-1000 (0.3%)	FID	1 nmol	No	123
			SE-30 (0.5%)—SP-1000 (0.5%)				
	50 cm × 2 mm	Chromosorb W HP (80—100 mesh)	SE-30 (1.5%)—FFAP (0.15%)	FID	1 nmol	No	127
	7 m × 0.2 mm	—	SE-30 (1.5%)—FFAP (0.15%)	FID	10—20 pmol	No	128
Ethyloxy-carbonyl	50 cm × 3 mm	Uniport HP (100—120 mesh)	KT-300 (0.5%)	MS		Yes	124
	100 cm × 3 mm	Uniport HP (100—120 mesh)	SP-1000 (0.5%)	FID, MS	1 nmol	Yes	125
	100 cm × 3 mm	Uniport HP (100—120 mesh)	SP-1000 (0.5%)	NPD	0.2 nmol	Yes	126

*FID = flame ionization detection; ECD = electron-capture detection; NPD = nitrogen—phosphorus sensitive detection; MS = mass spectrometry; SIM = selective ion monitoring (mass fragmentography).

3.2. Gas—liquid chromatography and gas chromatography—mass spectrometry

Gas—liquid chromatography (GLC) and gas chromatography—mass spectrometry (GC—MS) proved their usefulness immediately after a clinical diagnostic interest in the polyamines had been developed [110—112], and it was by means of GC—MS that the occurrence of N-acetylspermidine in human urine was detected [113].

In subsequent years GLC methods fell into disuse, presumably owing to the relatively laborious sample cleanup and derivatization procedures required [110], and were replaced by more convenient automated liquid chromatographic procedures. However, recently new derivatization methods have been developed, and a number of clinically relevant polyamine analyses have been carried out by GLC techniques in some instances also by mass fragmentography (see Table 2).

Because of inherent tailing problems, separations of free amines could not be satisfactorily achieved. Therefore, the suggestion of Beninati et al. [114] of separating non-derivatized polyamines or permethylation of the polyamines [115] was not taken up. Trifluoroacetyl [110—112, 116—119], pentafluoropropionyl [120] and heptafluorobutyryl derivatives [121] were used by most workers. The advantage of perfluoroacyl derivatives is that all the usual detectors (flame ionization, electron-capture and nitrogen-sensitive detectors) can be used. These derivatives are also suitable for mass spectrometric identification of unknown compounds [117, 119] and for mass fragmentographic determinations of the polyamines [118].

Without specific precautions, trifluoroacetylation leads to partial hydrolysis of acetyl derivatives and formation of the corresponding pertrifluoroacetyl derivatives. Suitable derivatization conditions [117], however, avoid the catalytic removal of acetyl residues and the formation of N,N-bistrifluoroacetyl derivatives.

For the determination of subnanogram levels of polyamines, derivatization with pentafluorobenzoyl chloride and the use of electron-capture detectors was introduced by Makita et al. [122]. Derivative formation with this reagent avoids the above-mentioned side reactions. Nevertheless, the method found very limited application in practice. The same group, however, introduced alkyl chloroformates as new reagents, mainly ethyl and isobutyl chloroformate [123]. The resulting N-ethyloxycarbonyl and N-isobutyloxycarbonyl derivatives of the polyamines not only turned out to be useful for the mass spectrometric identification of unusual polyamines (*sym*-homospermidine, *sym*-norspermine) in mosses and ferns [124], but also for the determination of urinary acetylpolyamines [125, 126]. Presumably owing to the convenient method of derivative formation, the procedure was adopted by several laboratories and, using nitrogen-sensitive detectors, clinical diagnostic studies of urinary polyamines were carried out [127, 128].

As an example for the efficiency and sensitivity of modern capillary columns and detectors, the work of Muskiet and co-workers can be considered [12, 28, 29]. They not only determined polyamines, but also the amino acids derived from the polyamines by oxidative deamination. Suitable derivatives in this instance are the methyl esters of the N-heptafluorobutyryl derivatives of iso-

putreanine, putreanine and spermine monocarboxylic acid [N^8-(2-carboxyethyl)spermidine]. The polyamines are determined in the same run together with the amino acids. Even from the limited data at present available, one can predict that the determination of isoputreanine in urine hydrolysates may add a new facet to the use of polyamines and their derivatives as tumour markers. It is well known that some tumours have increased diamine oxidase-like activity [129]. Diamine oxidase or closely related enzymes are capable of transforming spermidine into isoputreanine lactam, which may be excreted as such or in the form of conjugates [65, 130]. Isoputreanine is then isolated from these conjugates by acid hydrolysis.

It has been mentioned above that a liquid chromatographic method is now available for the assay of isoputreanine [64]. Nevertheless, GLC methods have again been shown to be powerful tools even in routine clinical trials. GC—MS, although a highly specific quantitative method and nearly indispensable for the unambiguous identification of unknown compounds, will remain an exception in clinically oriented trials, as it can nearly always be replaced by cheaper and less sophisticated methods.

4. BRIEF ACCOUNT ON NON-CHROMATOGRAPHIC METHODS

Two major approaches have been made in the past which avoid the chromatographic separation of the polyamines prior to their determination

(a) Specific antibodies against putrescine, spermidine and spermine were prepared and used in radioimmunoassays [131—133]. The method allows the determination of polyamines in serum samples. Direct comparison with a liquid—liquid chromatographic and a GC—MS method produced identical results [134], demonstrating the validity of the approach.

(b) The use of enzymes for polyamine determinations is not new [33], but the earlier methods were not sensitive enough owing to a high background. Recently, the use of purified oxidases, which are capable of degrading the diamines or the polyamines, has been revived in several laboratories. By employing simple cleanup procedures (mostly ion-exchange methods) and sensitive assays for the hydrogen peroxide formed during the reaction, the diamines (putrescine plus cadaverine) and the polyamines (spermidine plus spermine) were determined in the picomole or nanomole range [135—137]. The methods are simple and suitable for routine application. However, the usefulness of determining total polyamines or the two mentioned groups of amines has yet to be established. In particular, the fact that all these methods do not allow one to distinguish between putrescine and cadaverine is a serious handicap, as cadaverine is mainly a bacterial metabolite. Cadaverine does not follow the changes of putrescine concentrations in urine if its formation is enhanced or slowed [138]. Therefore, the diagnostic value of determining the sum of putrescine and cadaverine excretion is doubtful.

A very interesting development has recently been reported, namely the preparation of a specific enzyme electrode for spermine and spermidine [139]. The practical usefulness of this device has still to be proved, but it may turn out to make a major contribution to routine polyamine assay.

5. CONCLUSIONS

Assay procedures are now available that are suitable for the determination of the usual polyamines and their most common conjugates and derivatives in the picomole range.

Although methods may still be improved and extended to uncommon derivatives, there is no immediate need for an extensive expansion of our methodological arsenal. This is not only true of basic research, but also of clinical applications of polyamine analyses. The available methods allow one to obtain a relatively complete pattern of polyamines in urine and body fluids. There is, however, still a need for well controlled clinical studies and investigations of animal models that would allow us to clarify the effects of pathophysiological changes and of drugs on the distribution, metabolism and excretion of polyamines. As long as it has not been firmly established which factors are most important in the control of polyamine levels in blood, plasma, serum, cerebrospinal fluid and urine, it will be difficult to interpret polyamine patterns or changes efficiently. In this respect the contribution of polyamines of alimentary and bacterial origin to tissue and body fluid polyamines is one of the still open questions of considerable significance. The tools that seem necessary for addressing the pertinent questions are available, and it is up to us to make good use of them.

6. SUMMARY

Putrescine, spermidine, spermine and their derivatives are considered to be potential markers of certain diseases. Routine methods have, therefore, been developed in the course of the last decade. Several automated liquid chromatographic and gas chromatographic methods are presently at our disposal, which meet the practical requirements of sensitivity and specificity. They make the routine application of gas chromatographic—mass spectrometric methods dispensable. Very recently the amino acids deriving from polyamines have also been taken into consideration as tumour markers. Their separation by capillary gas chromatography has been reported. Separation of ion pairs on reversed-phase columns may also prove useful in the establishment of complete concentration profiles of polyamines and their derivatives in tissues and body fluids.

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