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DETERMINATION OF THE NATURALLY OCCURRING MONOACETYL DERIVATIVES OF DI- AND POLYAMINES

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SUMMARY

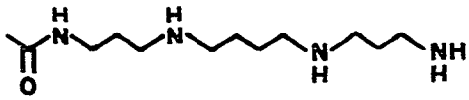
A method is described for the determination of pmol quantities of monoacetylputrescine, N^1-acetylspermidine, N^8-acetylspermidine and related compounds. The method is based on the derivatization of these compounds with 5-dimethylaminonaphthalene-1-sulphonyl-chloride, followed by thin-layer chromatographic separation. Cleanup steps allow the application of the method to urine analyses. From the repeated determination of acetylated polyamines in the urine of healthy individuals it can be concluded that these conjugates are the major excretory form of di- and polyamines.

The cleanup steps used in this procedure and the method described for the stabilization of 5-dimethylaminonaphthalene-1-sulphonyl derivatives on thin-layer plates are advantageous also for the analyses of total polyamines in urine hydrolysates, and in related applications of the dansylation method.

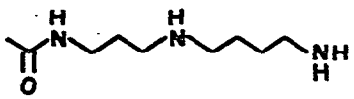
INTRODUCTION

Urinary polyamine analyses have been employed for the early detection of cancer, or for the evaluation of the efficacy of anticancer treatment [1—18]. In all these studies and in related work in which serum [19—21] or cerebrospinal fluid [22—24] polyamines were measured, the samples were hydrolysed with 6 *N* HCl prior to analysis, in order to liberate polyamines from their conjugates. It is known from previous work that acetylputrescine, acetylcadaverine and acetylspermidines are normal urinary constituents [25—31]. Structural formulae and chemical nomenclature of the acetylated polyamines are shown in Fig. 1.

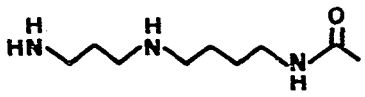
Abdel-Monem and Ohno [30—32] suggested the measurement of these acetylated polyamines as a more specific method than total polyamine determination for the detection of cancer. They were able to show significantly



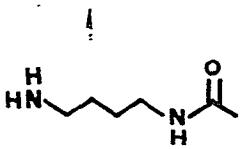
N^1-Monoacetylspermine
(N^1-(3-acetamidopropyl)-N^4-(3-aminopropyl) - 1,4-diaminobutane)



N^1-Monoacetylspermidine
(N^1-(3-acetamidopropyl) - 1,4 - diaminobutane)



N^8-Monoacetylspermidine
(N^1-(4-acetamidobutyl)-1,3-diaminopropane)



Monoacetylputrescine
(N^1-acetyl - 1,4 - diaminobutane)

Fig. 1. Structural formulae of acetylated di- and polyamines.

increased excretion of N^1-monoacetylspermidine in comparison with N^8-monoacetylspermidine in about half of fifteen cancer patients.

A great variety of methods are presently available for the determination of polyamines [33, 34]. In principle several approaches are feasible for the analysis of both free polyamines and their acetylation products. Ion-exchange column chromatography [25, 26, 35] and coupled gas—liquid chromatography—mass spectrometry [28, 36] can give adequate separations. However, the only method which has been actually used for the quantitative estimation of monoacetylputrescine and the acetylspermidines in urine consists of the following steps [37, 38]: (a) reaction of the urine samples with 5-dimethylaminonaphthalene-1-sulphonyl chloride (Dns-Cl) [39—41]; (b) thin-layer chromatographic (TLC) separation of the Dns derivatives; (c) extraction of the (partially) separated compounds from the thin layer; (d) separation and quantitation of the different compounds by high-performance liquid chromatography, using a silica gel column and a fluorescence flow detector.

In the present paper a method is presented which also relies on the dansylation reaction. Derivatization is followed by TLC and by in situ fluorescence measurement of the separated Dns derivatives. In order to obtain "clean" chromatograms, pre-separation and purification steps have been

worked out. These are applicable to the analysis of free polyamines in hydrolyzed urine samples as well.

MATERIALS AND METHODS

Chemicals

Laboratory chemicals were usually of A-grade. They were purchased from Baker Chemicals (Deventer, The Netherlands) or from Merck (Darmstadt, G.F.R.). Putrescine dihydrochloride, spermidine phosphate ($(C_7H_{19}N_3)_2 \cdot 3H_3PO_4 \cdot 6H_2O$) and spermine phosphate ($C_{10}H_{26}N_4 \cdot 2H_3PO_4 \cdot 6H_2O$) for standardization purposes as well as the corresponding (less pure) hydrochlorides of spermidine and spermine were from Fluka (Buchs, Switzerland).

The acetylated polyamine hydrochlorides and 5-dimethylaminonaphthalene-1-sulphonyl chloride were prepared in our laboratory according to published procedures [39, 42].

Thin-layer chromatography

20 × 20 cm silica gel plates (silica gel 60; Merck,) were used throughout. The plates were developed by ascending chromatography in filter paper lined tanks (Camag, Muttenz, Switzerland).

Quantitative evaluation of the plates

This was achieved by in situ fluorescence scanning, using the TLC scanner of Camag. Fluorescence was activated at 320 nm and total emission was measured, using a 400 nm cut-off filter for the elimination of the UV light. Slit: 6 × 0.8 mm.

Collection of urine samples

Urine samples were collected for 24-h periods from healthy adult drug-free males and females. 50 ml of acetic acid plus 1 g sodium metabisulphite, or 100 ml of 3 *N* HCl were placed in the collection flasks in order to avoid bacterial contamination. Immediately after the collection period, 25-ml aliquots of the urine samples were stored at —20° until they were analyzed.

Pre-separation of urine on Dowex 50W-X8 columns

Purification of the ion-exchange resin (Dowex 50W-X8, 200—400 mesh; Serva, Heidelberg, G.F.R.) was achieved by washings with large volumes of 1 *N* NaOH, water and 6 *N* HCl. It was stored at +3° suspended in 6 *N* HCl. Columns were prepared from 5-ml polypropylene pipettor tips (Gilson, Villier-Le-Bel, Arnouville-Les-Gouesse, France) by adjusting a cotton wool plug into the constricted end of the tip and application of a total of 5 ml of resin, suspended in 6 *N* HCl. The resin columns, usually 12 at a time, were connected by polyethylene tubes with the silicon pumping tubes (1 mm I.D.) of a peristaltic pump (mp 13 or mp 13 GJ-4; Ismatec, Zürich, Switzerland) and washed with distilled water to neutral pH. Aliquots (2 ml) of the urine samples were mixed with 2 ml of ethanol and allowed to stand for 1 h at 3° after which the precipitate was removed by centrifugation. The clear supernatants were applied to the columns by pumping at a rate of about 0.8 ml/min. Subsequently the

columns were washed at the same pumping rate with 25 ml of 2.0 *N* HCl. The polyamine fractions (containing both conjugated and non-conjugated derivatives) were eluted with 25 ml of 6 *N* HCl. They were evaporated to dryness at a pressure of about 15 mm Hg, using a Rotavapor (Büchi, Flawil, Switzerland).

Hydrolysis of urine samples

Mixtures of 5 ml of urine with 5 ml of 12 *N* HCl were sealed in Pyrex glass tubes (15 × 160 mm) and heated for 18 h at 120°. The content of the tubes was evaporated in vacuo, and the residues were dissolved in 5 ml of water. Two millilitres of these solutions were mixed with the same volume of ethanol and subjected to chromatography on Dowex 50 columns, as described for the non-hydrolyzed samples.

Derivatization with 5-dimethylaminonaphthalene-1-sulphonyl chloride (Dns-Cl)

The dried residues of the column eluates were dissolved in 0.4 ml of 0.2 *N* perchloric acid and 0.8 ml of acetone. (This amount of solvent was used to quantitatively transfer the residues of the eluates from a 100-ml round bottom flask into glass tubes (12 × 100 mm).) A solution of 20 mg of Dns-Cl in 0.4 ml of acetone was added; the solutions were saturated with sodium carbonate and reacted overnight at room temperature, as described previously [39–41]. After reaction of the excessive Dns-Cl with proline, the samples were extracted with 4 ml of toluene, and the toluene extracts were evaporated in a stream of air to dryness. Subsequently a solution of 150 µl of 5 *M* KOH in methanol was added, and the samples were heated for 30 min at 50° in a water bath in order to remove easily hydrolyzable Dns derivatives [43]. By addition of 1.5 ml of water and 100 mg of a 1:1 mixture of KH_2PO_4 and Na_2HPO_4, the Dns derivatives were extracted with 4 ml of toluene from the aqueous phase. Usually 20-µl aliquots of these extracts were separated by TLC.

Thin-layer chromatography

Samples were applied manually, using a 20-µl "Pipetman" pipettor (Gilson). The rapid application of 20-µl volumes of the toluene solutions of Dns derivatives does not produce starting zones on silica gel thin layers with a diameter exceeding 2 mm. Only some rapidly migrating side products of the reaction tend to move further, but have no significant influence on the quality of the subsequent chromatographic separations. Normally the spots are applied at a distance of 20 mm from one plate edge and at a distance of 10 mm from each other, i.e. 20 samples can be separated on each plate. Three standards, prepared from authentic, pure compounds and run through the entire procedure, including chromatography on the Dowex columns were normally applied on each plate.

Separation of the acetylated polyamines. The non-polar Dns derivatives, including Dns-ammonia are moved near the solvent front by developing the plates with ethyl acetate (1 run). For the actual separation of the relatively non-polar acetyl derivatives the following solvent was used in a solvent-vapor-saturated tank: chloroform—tetrachloromethane—methanol (70:30:5) (2 runs). Between each run, the plates are dried at room temperature for 5 min. It

should be pointed out that the ethanol usually present in the commercial chloroform has to be removed by distillation.

Free polyamines and total polyamines. The previously described solvents [39—41] for the separation of Dns-polyamine derivatives were used for the separation of both free polyamines and total polyamines, as obtained by hydrolysis of the urine samples with 6 *N* HCl. Chromatography with cyclohexane–ethyl acetate (1:1) (2 runs) followed by cyclohexane—ethyl acetate (3:2) (1 run) was most appropriate in our hands. This solvent separates bis-Dns-cadaverine from bis-Dns-putrescine, however it does not allow one to determine the former compound because it is too close to the large spot of Dns-ammonia. If bis-Dns-cadaverine is to be determined, chloroform—triethylamine (5:1) is preferable. [39—41, 44, 45] In this case, however, the spermine spot is close to side products of the dansylation reaction.

Stabilization of the Dns derivatives

Spraying with a solution of triethanolamine in propanol-2 enhances fluorescence intensity and stabilizes the Dns derivatives on thin layers [39—41]. Recently, spraying with solutions of viscous organic solvents (paraffin oil in cyclohexane or toluene, among others) was suggested for the same purpose [46]. We have tried to avoid this technically difficult process. The following practical solution for this problem was found: the plates are immersed twice for 1 min into a 10% solution of Rhodorsil oil SI 710 (a silicon oil available from Prolabo, Paris, France) in cyclohexane. Between the immersions they are dried for 5 min at room temperature. Fluorescence is stable at least for 1 week if the plates are stored in the darkness. No significant diffusion of the Dns-polyamine spots is observed. One can imagine that paraffin oil similarly applied would be equally useful.

RESULTS

Separation of the monoacetyl polyamines

The solvent mixture consisting of chloroform—tetrachloromethane—methanol (70:30:5) is capable of separating the Dns derivatives of putrescine, cadaverine, spermidine, spermine, monoacetylputrescine, monoacetylcadaverine, N^1- and N^8-monoacetylspermidine and N^1-monoacetylspermine either on the usual 20 × 20 cm silica gel plate or on a 10 × 10 cm high-performance thin-layer plate. This solvent was therefore previously used for the separation of labelled polyamines in metabolic studies, with subsequent preparation of autoradiographs [47]. For the quantitative evaluation of the plates by fluorometry this separation has some disadvantages: (a) Spermine moves close to the solvent front and by-products of the dansylation reaction can interfere with its quantitative evaluation. In this respect the solvent is similar to chloroform–triethylamine (5:1) [39—41] which has been repeatedly used for the analysis of total polyamines in urine [44, 45]. (b) Since the thin-layer scanner has only a limited resolution capacity, one would like to achieve a better separation of the two monoacetylspermidines, and of monoacetylputrescine from monoacetylcadaverine. (c) The two monoacetyl-spermidines move closely behind Dns-ammonia. If the latter is present in large amounts, it interferes with the estimation of the acetylspermidines.

In the case of urine analyses there is normally no restriction in the availability of material. It was decided, therefore, to separate free polyamines (and total polyamines, as obtained by acid hydrolysis of urine) and acetylated polyamines on two plates using two different solvents. Chromatograms showing separations of various sample types in the two solvent systems are shown in Figs. 2 and 3. Typical fluorescence scans of acetylated polyamines and of the amines of a urine hydrolysate are shown in Figs. 4 and 5.

The above-mentioned separation problems with acetylated polyamines were solved by moving all non-polar Dns derivatives, including those of the polyamines and ammonia near the solvent front, by developing the plate first with ethyl acetate. Two successive runs with chloroform—tetrachloromethane—methanol (70:30:5) then adequately separated the acetylated polyamines (Fig. 2). Samples separated by cyclohexane—ethyl acetate (1:1) result in separations shown in Fig. 3. It should be pointed out here that the low background fluorescence achieved in these chromatographic separations is due to the pre-separation of the urine samples on Dowex 50W-X8 columns, and especially due to the exposure of the Dns derivatives to alkaline conditions before separation, as is described in detail in the Methods section.

Urine contains a large number of compounds with primary amino groups. It is therefore difficult to establish the specificity of the method. The criteria for the uniformity of the spots of the Dns derivatives of monoacetylputrescine, and of N^1- and N^8-monoacetylspermidines were: (a) elution of the non-derivatized amines from Dowex 50W-X8 columns with 4 *N* HCl and 6 *N* HCl,

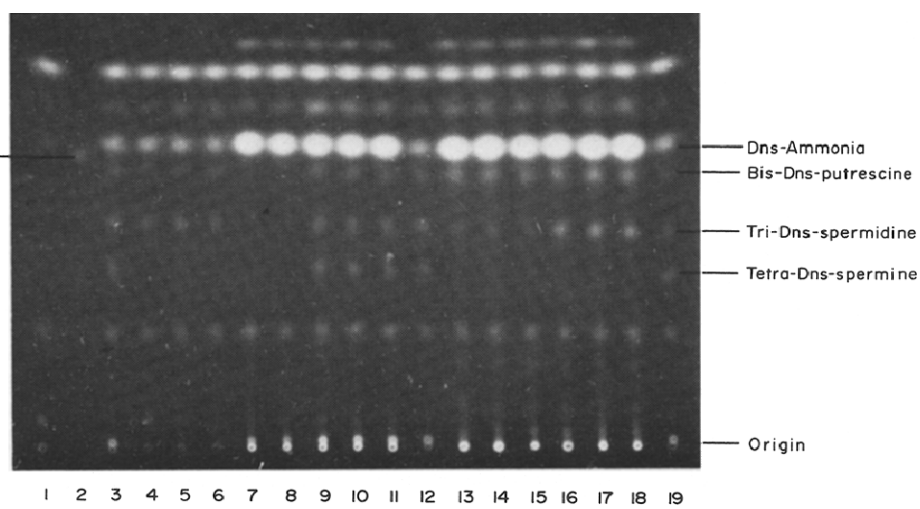


Fig. 2. Thin-layer chromatogram of Dns derivatives, standard samples and dansylated urine fractions. Solvent: 1st run: ethyl acetate; 2nd and 3rd run: chloroform—tetrachloromethane—methanol (70:30:5). 1 = Blank; 2 = tri-Dns-monoacetylspermine; 3—5, 10, 11, 19 = standard mixture containing 50 pmol of mono-Dns-monoacetylputrescine, bis-Dns-N^1-monoacetylspermidine, bis-Dns-N^8-monoacetylspermidine, bis-Dns-putrescine, tri-Dns-spermidine and tetra-Dns-spermine; 6—8 = Dns derivatives of urine samples (equivalent to 10 μl of urine); 12—15 = urine samples corresponding to 6—8, however, with added standard mixture (corresponding to 3—5); 16—18 = urine samples, hydrolyzed prior to derivatization with 6 *N* HCl. The Dns derivatives of the free polyamines move under these chromatographic conditions faster than Dns-ammonia. They cannot be detected on this chromatogram.

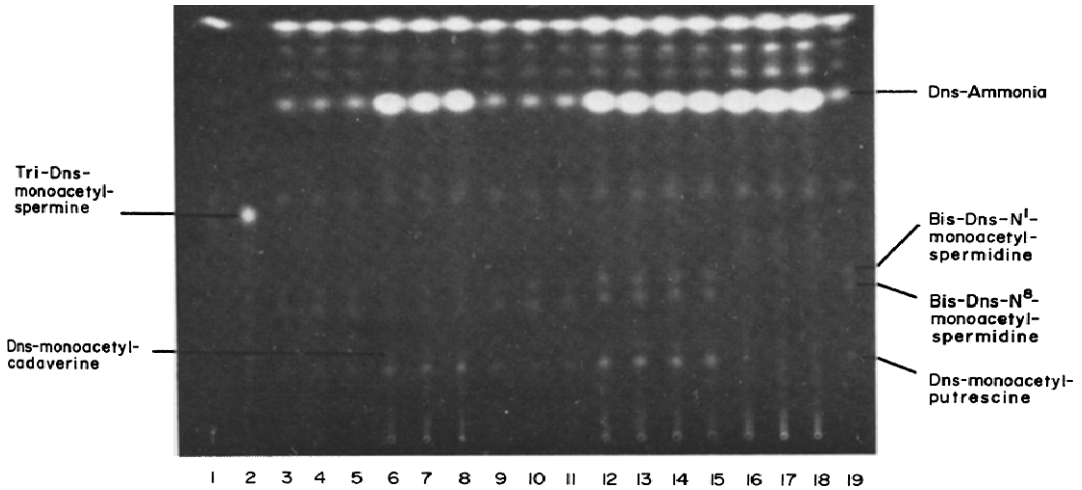


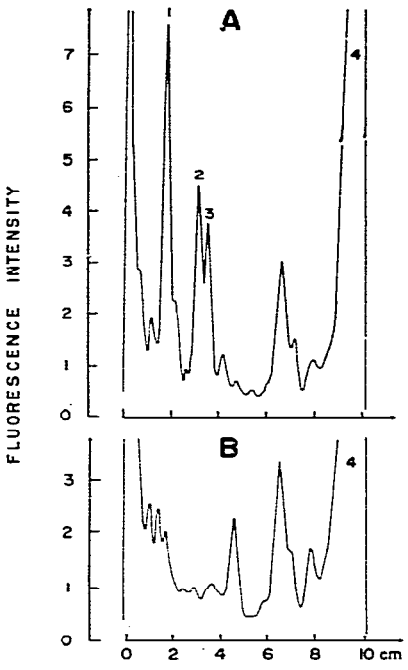
Fig. 3. Thin-layer chromatogram of Dns derivatives, standard samples and dansylated urine fractions. Solvent: cyclohexane—ethyl acetate (1:1) (2 runs) and cyclohexane—ethyl acetate (3:2) (1 run). 1 = Blank; 2 = bis-Dns-cadaverine; 3, 12, 19 = standard mixture (containing 50 pmol of mono-Dns-acetylputrescine, bis-Dns-N^1-acetylspermidine, bis-Dns-N^8-acetyl spermidine, bis-Dns-putrescine, tri-Dns-spermidine and tretra-Dns-spermine); 4—6 = standards with 50 pmol monoacetylputrescine, N^1-monoacetylspermidine and N^8-monoacetyl spermidine, hydrolyzed with 6 *N* HCl prior to derivatization; 7, 8 = non-hydrolyzed urine (corresponding to 10 μl); 9—11 = urine with added standard mixture; 13—15 = urine hydrolyzed with 6 *N* HCl; 16—18 = urine with added monoacetylputrescine, N^1-acetylspermidine and N^8-acetylspermidine, hydrolyzed with 6 *N* HCl prior to derivatization.

respectively; (b) identical mobility of the non-derivatized amines on thin-layer electrophoretograms using pyridine—acetic acid buffer pH 4.8 [47]; (c) identical mobility of the Dns derivatives of these compounds in various solvents, and (d) upon hydrolysis of the urine samples prior to ion-exchange column chromatography with 6 *N* HCl (120°; 12 h) and dansylation, spots did not appear on the chromatograms corresponding with the Dns-acetyl-polyamines (Fig. 2), but rather in zones corresponding to the Dns derivatives of the free polyamines (Fig. 3).

Sensitivity, recovery, reproducibility

As might be expected, the method has the usual sensitivity of procedures using dansylation. With the stabilization of the Dns derivatives by silicon oil, and using the Camag TLC-scanning equipment, it was possible to routinely measure picomole amounts of the various acetylated and free polyamines on normal (20 × 20 cm) thin-layer plates. For the measurement of urine samples the aliquot of the Dns derivatives normally applied on the plates, corresponded to 10 μl of urine and 50 pmol of polyamine.

It is known from previous work [41] that recovery of Dns-polyamines during the derivatization reaction is high. No attempt was made, therefore, to determine the total recovery. However, the recovery of the free and acetylated polyamines from the ion-exchange column chromatographic procedure has been determined. Standard samples, containing 2, 5, 7.5 and 10 nmol



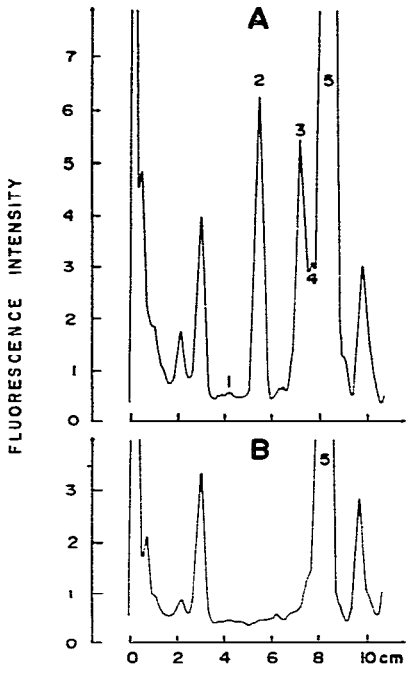


Fig. 4. In situ fluorescence scans of the Dns derivatives of urine constituents. (A) Scan of track 12; (B) scan of track 16 of Fig. 2. 1 = Dns-monoacetylputrescine; 2 = bis-Dns-N^8-acetyl-spermidine; 3 = bis-Dns-N^1-acetylspermidine; 4 = Dns-ammonia. For details see legend to Fig. 2.

Fig. 5. In situ fluorescence scans of the Dns derivatives of urine constituents. (A) Scan of track 13; (B) scan of track 7 of Fig. 3. 1 = Tetra-Dns-spermine; 2= tri-Dns-spermidine; 3 = bis-Dns-putrescine; 4 = bis-Dns-cadaverine; 5 = Dns-ammonia. For details see legend to Fig. 3.

respectively of each amine were applied on the columns; the columns were washed with 25 ml of 2.0 *N* HCl and the amines were then eluted with 25 ml of 6 *N* HCl. After evaporation the samples were dansylated, and similar samples not run through the ion-exchange columns were dansylated in parallel. From the 4 ml of toluene extracts, containing the mixture of Dns-derivatives, 20-μl aliquots were separated by TLC and measured by fluorescence scanning. The standard deviation of the determinations was ± 7% within the range of 10 to 50 pmol. There was no significant difference between the amounts of amines recovered from the ion-exchange columns and those subjected to dansylation directly. This shows that recovery of the acetylated amines from the ion-exchange column was over 90%. The same is true for the free polyamines.

The high recovery of the acetylated polyamines indicates their stability against acid hydrolysis. Indeed, free polyamines were never detected on the chromatograms when the acetylated products were run through the procedure. This can also be seen from Fig. 3 and Fig. 5B which show separations and a scan of a non-hydrolyzed urine sample, respectively. Virtually no free polyamines are detectable in this sample, prior to acidic hydrolysis. In order to

test the capacity of the 5-ml Dowex 50W-X8 columns, increasing volumes of the same urine sample were separated, and polyamines were determined subsequently. Fig. 6 shows the results. It appears from this figure that a linear relationship exists between the volume of the urine sample and the amount of the monoacetylspermidines, at least up to 10 ml of urine. In other words, the 5-ml Dowex 50W-X8 columns quantitatively retain the monoacetylspermidines even of 10 ml of urine. In contrast, monoacetylputrescine is lost from the column if more than 2 ml of urine are applied. This finding is in agreement with the fact that monoacetylputrescine and putrescine are eluted from Dowex 50W-X8 columns with 4 *N* HCl already whereas monoacetylspermidines and the free polyamines (spermidine and spermine) come off the column only with 6 *N* HCl [47].

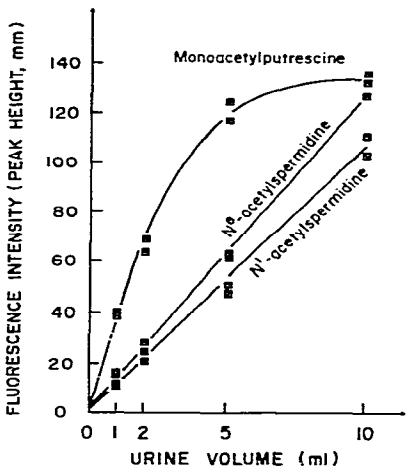


Fig. 6. Relationship between urine sample volume and recovery of monoacetylputrescine, N^1-acetylspermidine and N^8-acetylspermidine, from a 5-ml Dowex 50W-X8 column. In contrast with the acetylspermidines, significant losses of monoacetylputrescine are observed if the sample volume exceeds 2 ml. For details of the washing and elution procedure see Methods section.

In practice the urine sample volume was kept constant at 2 ml. When only the monoacetylspermidines (or non-conjugated spermidine and spermine) are to be analyzed, the column volume can be decreased to 1 ml, with a concomitant decrease of the eluent volumes (10 ml of 2.0 *N* HCl for washing, and 6 ml of 6 *N* HCl for elution).

Recovery of polyamines after hydrolysis with 6 *N* HCl (120°, 18 h) of their acetyl derivatives was estimated by hydrolysis of standard mixtures and by hydrolysis of urine samples to which known amounts of the acetyl derivatives were added. Recoveries in these two types of samples were identical within the precision of the method. The mean recovery for putrescine from acetylputrescine was 94%, for spermidine from N^1- and N^8-monoacetylspermidine, 63%.

Acetylated polyamines in human urine

Five adult healthy males and five adult healthy females participated in this exploratory study. No restrictions were imposed on food or fluid intake. Urine was collected for 24-h periods at two different times, and the urine samples were analyzed as described above.

The results of the acetylpolyamine measurements are shown in Table I. From a further male, 24-h urine was collected on six consecutive days. Again there was no restriction with regard to food or fluid intake. The results of the analyses of this experiment are shown in Table II. It appears from the values of the tables that the daily variation of excretion of acetylated polyamines is of the order of 10—20% of the mean, and that the inter-individual variation is much greater. High excretors may excrete as much as 12—16 μmol/day of the acetylated spermidines, and low excretors only about 50% of this value. In

TABLE I

EXCRETION OF ACETYLATED POLYAMINES IN THE URINE (μmol/24 h) OF MALE AND FEMALE ADULT VOLUNTEERS

Monoacetylputrescine: AcPut; N^1-monoacetylspermidine: N^1-AcSpd; N^8-monoacetylspermidine: N^8-AcSpd. (a) urine collection January 25; (b) urine collection February 13. Mean values of duplicate determinations.

Subject no.		AcPut	N^1-AcSpd	N^8-AcSpd	N^1-AcSpd + N^8-AcSPd	$\frac{N^1\text{-AcSpd}}{N^8\text{-AcSPd}}$
Male						
1	a	20.28	6.91	5.75	12.66	1.20
	b	19.05	6.61	5.72	12.33	1.16
2	a	16.70	3.99	3.45	7.34	1.16
	b	13.79	5.50	4.54	10.04	1.21
3	a	27.38	6.35	6.10	12.45	1.04
	b	18.20	7.66	5.22	12.88	1.47
4	a	23.11	10.35	6.30	16.65	1.64
	b	13.23	6.26	6.08	12.34	1.03
5	a	22.65	5.13	4.99	10.12	1.03
	b	16.38	4.57	4.62	9.19	0.99
mean ± S.D.	a	22.0 ± 3.9	6.6 ± 2.4	5.3 ± 1.2	11.8 ± 3.4	1.21 ± 0.25
mean ± S.D.	b	16.1 ± 2.6	6.1 ± 1.2	5.2 ± 0.7	11.4 ± 1.6	1.17 ± 0.19
Female						
6	a	14.49	4.45	2.59	7.04	1.72
	b	12.54	3.34	2.11	5.45	1.58
7	a	22.09	4.52	3.48	8.00	1.30
	b	20.24	3.88	3.27	7.15	1.19
8	a	22.39	3.00	5.93	8.93	0.51
	b	16.45	2.60	4.41	7.01	0.59
9	a	28.97	6.77	4.95	11.72	1.37
	b	18.46	5.31	3.44	8.75	1.54
10	a	17.00	4.71	3.76	8.47	1.25
	b	13.04	4.81	4.23	9.04	1.14
mean ± S.D.	a	21.0 ± 5.6	4.7 ± 1.4	4.1 ± 1.3	8.8 ± 1.8	1.23 ± 0.4
mean ± S.D.	b	16.2 ± 3.4	4.0 ± 1.1	3.5 ± 0.9	7.5 ± 1.5	1.21 ± 0.4
mean of all measurements ± S.D.		18.8 ± 4.6	5.3 ± 1.8	4.4 ± 1.2	9.9 ± 2.8	1.21 ± 0.3

TABLE II

EXCRETION OF ACETYLATED POLYAMINES IN THE URINE (μmol/24 h) OF AN ADULT MALE VOLUNTEER* DURING SIX CONSECUTIVE DAYS

Monoacetylputrescine: AcPut; N^1-monoacetylspermidine: N^1-AcSpd; N^8-monoacetylspermidine: N^8-AcSpd. Mean values of duplicate determinations.

Day	AcPut	N^1-AcSpd	N^8-AcSpd	N^1-AcSpd + N^8-AcSpd	N^1-AcSpd / N^8-AcSpd
1	15.14	3.65	2.84	6.49	1.29
2	15.97	3.96	3.18	7.14	1.25
3	14.39	4.08	3.54	7.62	1.15
4	15.33	4.76	3.82	8.58	1.25
5	15.22	5.70	3.74	9.44	1.52
6	15.48	5.69	3.91	9.60	1.46
Mean ± S.D.	15.26 ± 0.52	4.64 ± 0.89	3.51 ± 0.42	8.15 ± 1.3	1.32 ± 0.14

*Subject No. 11.

repeated urine collections the pattern of excreted polyamine conjugates remaines practically the same, indicating that the method gives consistent results.

Free and total polyamines in human urine

The amounts of non-conjugated putrescine and spermidine were less than 10% of the total amines. In contrast, most of the spermine excreted in human urine seems to be non-conjugated. Total spermine found in the two urine collections from five males was: 2.2 ± 0.9 μmol/24 h and 1.3 ± 0.6 μmol/24 h. The free amine was only 10% less than the total. Interestingly, females excreted significantly less spermine. 0.7 ± 0.4 μmol/24 h were found in the average in the two collections, with only one excretor above 1 μml/24 h.

The values for total spermidine and putrescine (means of five males and five females) after correction for losses during hydrolysis were: putrescine 18.7 ± 6; spermidine 10.2 ± 2. These values are in excellent agreement with the average amounts of acetylputrescine and acetylspermidines, found in the same urine samples (Table I). It can be concluded from this finding that acetylputrescine and the two acetylspermidines are the major excretory forms of putrescine and spermidine, respectively.

DISCUSSION

The method described in this paper is sufficiently rapid and accurate to be routinely applied to the analysis of acetylputrescine, the acetylspermidines and free polyamines in human urine. Monoacetylspermine and monoacetylcadaverine are also sufficiently separated from the other spots to be detected, if present in significant amounts.

The cleanup procedure, using Dowex X8 columns was based on the work of Shimizu et al. [48], who suggested a comparable procedure for the determination of free polyamines from nervous tissue. Another procedure also employing

6 *N* HCl for the elution of free polyamines from Dowex 50W columns has been devised by Fujita et al. [49]. As was shown in the present work, the thin-layer chromatograms of dansylated polyamines can be considerably improved by the cleanup steps. Other methods of polyamine analyses might also profit from this procedure. The impregnation of the plates with a viscous oil by dipping is technically easier than the hitherto used spraying.

The analyses of acetylated polyamines in the urine of only ten adult healthy volunteers (five males and five females) can only be considered as being preliminary. However, these analyses together with the repeated determinations of the 24-h output of a single individual show already that the inter-individual variation is only about ±30% of the mean. They also show that the individual pattern of urinary acetylpolyamines is relatively constant, and not influenced too greatly by food and fluid intake. Abdel-Monem and Ohno [32] reported the following "normal" values for acetylputrescine and the acetylspermidines, based on the analyses of urines from nine individuals: monoacetylputrescine: 11.7 ± 1.5 μmol/24 h; N^1-monoacetylspermidine: 2.9 ± 0.6 μmol/24 h; N^8-monoacetylspermidine: 2.8 ± 0.5 μmol/24 h. These values are at the lower end of the range of reported total urinary excretion of putrescine and spermidine [16]. The values found in this work (Table I) are somewhat higher than these reported values [32], but are in agreement with them, in so far as the ratio of N^1- and N^8-monoacetylspermidine is close to one in both cases.

Hydrolysis of the urine samples showed that total putrescine and spermidine values are close to the value of acetylputrescine and the sum of the acetylspermidines. If more extended experience confirms the impression of the present finding, that virtually all conjugated putrescine and spermidine is represented by the monoacetylderivatives, hydrolysis of the urine samples may become dispensable. This step is one of the major sources for the limited precision of the method. Since detailed information of the polyamine pattern of urine can be obtained by the separation of the dansyl derivatives of non-hydrolyzed samples, if run in two different solvents, our method should be a useful tool in the exploration of the diagnostic value of urinary polyamine analysis in diseased states.

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