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SINGLE-STEP HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE ANALYSIS OF ACETYLATED POLYAMINES

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SUMMARY

A high-performance liquid chromatography method for the determination of urinary acetyl derivatives of the polyamines putrescine, cadaverine and spermidine is described. This procedure utilizes an ion-exchange column for the separation of acetyl derivatives and the compounds are quantitated by fluorescence after reaction with *o*-phthalaldehyde. The steps necessary for sample preparation are minimized, and the assay is both sensitive and reproducible. This chromatographic procedure was used for the determination of the urinary acetylated polyamines of seven normal volunteers and three cancer patients.

INTRODUCTION

The naturally occurring polyamines, putrescine, spermidine, and spermine, are essential for cell growth and viability. All living organisms and tissues studied to date contain polyamines, and polyamine biosynthesis and accumulation is increased in hypertrophy and hyperplasia [1—6]. Russell [7] in 1971 reported that patients with diagnosed metastatic cancer excreted elevated polyamines and that in one patient, the excision of a large solid tumor returned polyamine excretion to near normal values. Elevated urinary polyamine excretion in cancer patients has now been demonstrated by many groups [8—14]. Urinary polyamine analysis can be used for the rapid evaluation of the success or failure of cancer chemotherapy [5]. A two-fold or greater elevation of urinary spermidine within 48 h predicts a partial or complete response with a high degree of accuracy [5, 8, 11].

In a majority of studies reported of urinary polyamine concentrations, samples were hydrolyzed prior to analysis, liberating the parent polyamines from conjugated forms. Over 90% of the human urinary putrescine and spermidine have been identified as the monoacetyl derivatives while 10% or less of

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spermine is estimated to be present as conjugated products [15—17]. Although N^1-acetylspermine has been detected in tissues [18] and other conjugates are possible [19], the nature of possible spermine urinary conjugates is unknown.

Enzymes that acetylate polyamines forming the monoacetyl derivatives have been reported in the nucleus and the cytosol of many different rat tissues [20—24]. The enzymes are apparently distinct with differing substrates and products. The substrates for the nuclear N-acetyltransferases are histones and numerous polyamines including putrescine [20—22]. In the presence of spermidine, N^8-acetylspermidine is the only spermidine derivative formed by the nuclear enzyme(s) [25, 26]. The substrates of the cytosolic enzyme are spermine and spermidine forming the N^1-monoacetyl derivatives of these compounds [18, 24]. Putrescine and histones are not substrates for this enzyme. Pretreatment of a rat with carbon tetrachloride increases the cytosolic enzyme activity and the formation of N^1-acetylspermidine and N^1-acetylspermine [18, 23, 24, 27]. The nuclear N-acetyltransferase activity, apparently not induced by carbon tetrachloride, can be stimulated in the kidneys of rats after the administration of growth hormone and ACTH [28].

The acetylated polyamines formed by the N-acetyltransferases do not accumulate to high levels in stimulated tissues, probably due to rapid enzymatic degradation and excretion of the acetyl derivatives from tissues [26, 29—33]. N^1-Acetylspermidine and N^1-acetylspermine are better substrates than the parent compound for the enzyme polyamine oxidase, and the products are putrescine and spermidine, respectively [29]. N^8-Acetylspermidine is not a substrate for polyamine oxidase but is deacetylated by a cytosolic enzyme to form spermidine [30]. Acetylputrescine can also be deacetylated in the cytosol [31] and is a substrate for the enzyme monoamine oxidase forming γ-aminobutyric acid [32].

The concentration of acetylated polyamines excreted in urine will depend on the rate of formation and breakdown of these compounds in the tissues. Seiler et al. [26] studied the influence of cell proliferation and polyamine production on urinary excretion of the acetylated polyamines in rats. Rats pretreated with thioacetamide which markedly increased liver polyamine concentrations resulted in increased N^1-acetylspermidine excretion. Treatment of the rats with agents which cause considerable cell damage such as epidermal UV irradiation or cyclophosphamide injection caused both N^1- and N^8-acetylspermidine excretion to increase. However, rats pretreated with 7,12-dimethylbenzanthracene which caused the formation of mammary tumors did not increase excretion of the acetylpolyamines. Elevated acetylpolyamine excretion, therefore, has not been shown to be a reliable marker for the presence of tumors. In another study, Seiler et al. [33] measured urinary acetylspermidine excretion in hepatoma-bearing rats and found that urinary N^1-acetylspermidine excretion increased exponentially during the time of linear increase in tumor mass. The excretion of N^8-acetylspermidine increased when the tumor mass was 35 g, shortly before the period of observed necrosis.

Acetylpolyamine excretion in human pathology has been less well studied. Abdel-Monem and Ohno [16] found increased urinary acetylpolyamine excretion in all diagnosed cancer patients when compared to normals. In addition, thirteen out of fifteen patients had an elevated N^1- to N^8-acetylspermidine

ratio when compared to normals. Seiler et al. [26] studied urinary acetylpolyamine excretion in two male melanoma patients. In one patient, acetylputrescine excretion was normal, N^8-acetylspermidine excretion was slightly elevated, and N^1-acetylspermidine excretion was several-fold higher than in healthy male controls. In the other patient, monoacetylputrescine, N^1- and N^8-acetylspermidine were excreted in elevated amounts as compared to controls. It seems that extensive studies of intracellular and extracellular acetylpolyamine derivatives are required in order to ascertain the possible significance of nuclear and cytosolic acetylation of polyamines in tumor evolution and growth.

Many reported methods exist for the separation and quantitation of free polyamines [34—40]. However, only a few have been adapted for the separation and quantitation of acetylpolyamines [41—43], and these are rather complex, sometimes requiring two separation steps [41—43]. We report the development of a single-step procedure for the separation and quantitation of urinary acetylpolyamine derivatives which is reproducible and sensitive.

MATERIALS AND METHODS

Chemicals

Putrescine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride, *o*-phthalaldehyde, sulfosalicylic acid and mercaptoethanol were purchased from Sigma (St. Louis, MO, U.S.A.). Sodium citrate was purchased from Mallinckrodt (St. Louis, MO, U.S.A.), sodium chloride from Ashland (Columbus, OH, U.S.A.), sodium hydroxide from Matheson, Coleman and Bell (Norwood, OH, U.S.A.), boric acid from VWR (San Francisco, CA, U.S.A.), and thiodiglycol from Pierce (Rockford, IL, U.S.A.). Acetylpolyamines were prepared by the method of Tabor et al. [44].

Column packing

The Bio-Rad A-9 column was slurry-packed into a 250 × 3 mm column at room temperature with a 1:1 mixture of buffers B and C (see below). Buffer flow-rate was approximately 0.2 ml/min, and the pressure on the column was not allowed to exceed 100 bar during the 8-h packing procedure. Using this method, we have not encountered increased column packing after more than 150 runs, and the resulting peaks in the chromatograms remain sharp.

Elution buffers

The elution system consisted of four buffers. Buffer A was prepared by dissolving 19.6 g of sodium citrate in 1 l of double-distilled water (DDW). The pH was brought to 4.25 with concentrated hydrochloric acid; the final molarity was 0.2 *M* sodium. Buffer B was prepared by adding 2.0 g sodium hydroxide and 8.76 g of sodium chloride to 1 l of DDW. The pH was adjusted to 10.1 with boric acid; the final molarity was 0.2 *M* sodium. Buffer C was prepared by adding 2.0 g sodium hydroxide and 23 g sodium chloride to 1 l DDW. The pH was adjusted to 10.1 with boric acid; the final molarity was 0.44 *M* sodium. Buffer D was prepared by dissolving 12 g sodium hydroxide in 1 l DDW. All solutions were filtered through a 0.45-μm filter and preserved with pentachlorophenol.

o-Phthalaldehyde reagent

o-Phthalaldehyde (OPA) reagent was prepared by adding 25 g potassium hydroxide to 800 ml DDW. Boric acid was used to adjust the pH to 10.4. DDW was then added to bring the solution to 1 l. To the liter of borate buffer solution, 4.5 ml 2-mercaptoethanol, 3.0 ml Brij, 5.8 g potassium thiocyanate and 800 mg OPA dissolved in 20 ml methanol were added, and the solution was mixed. This solution was filtered through a 0.45-μm filter before use.

Urine preparation

Urine samples were collected in polyethylene specimen cups. Ten ml of urine were removed for creatinine analysis and the rest of the urine was acidified to 0.1 *N* hydrochloric acid with concentrated hydrochloric acid. An aliquot (0.8 ml) of acidified urine was added to 0.2 ml of 10% sulfosalicylic acid in a 1.5-ml microcentrifuge tube and vortexed. This solution was then spun on a Beckman B microfuge for 5 min. The supernatant was removed and the pH was brought to 2—3 with 0.25 ml of 0.3 *M* sodium hydroxide. The samples were then frozen until analyzed. Creatinines were determined by the direct (heat clot) procedure (Hycel, Houston, TX, U.S.A.).

Chromatographic separation

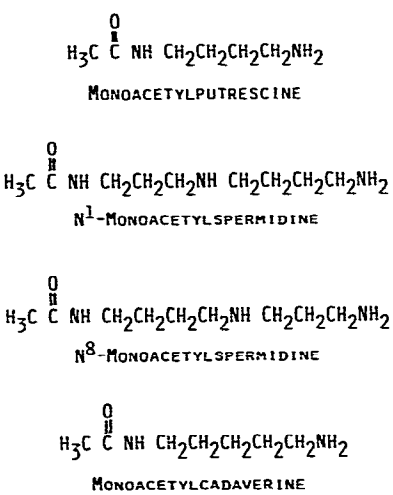
High-performance liquid chromatography (HPLC) was accomplished with a component system. The four buffers were contained in 1-l bottles and kept at room temperature. Due to the high pH, buffers B, C, and D were fitted with carbon dioxide traps of sodium hydroxide pellets. All buffers were connected by 0.3-cm tubing to a 4-part distribution timing assembly (Hamilton, Reno, NV, U.S.A.). Buffers were pumped onto the column by a Milton Roy Minipump (Applied Science, State College, PA, U.S.A.), equipped with a pulse dampener. The sample was injected onto the column by a 7010 sample injection valve fitted with a 200-μl sample loop (Rheodyne, Berkeley, CA, U.S.A.). A 250 × 4 mm stainless-steel column (Pierce), packed with approximately 7 g of Bio-Rad A-9 resin (11.5 ± 0.5 μm; Bio-Rad Labs., Richmond, CA, U.S.A.), was used for chromatographic separation. The column was jacketed with a 250-mm microbore column jacket (Altex, Fullerton, CA, U.S.A.) and kept at 60°C by a constant temperature circulating bath (Haake, Saddle Brook, NJ, U.S.A.). All stainless-steel tubing was 0.16 × 0.05 cm. The column outflow was connected to 60 cm of 0.6 × 0.3 mm microbore PTFE-tubing into a 3-way manifold tee (Pierce) where it was mixed with OPA. OPA was pumped by a Model 650 pressure pump (Rainin, Woburn, MA, U.S.A.) equipped with a pulse dampener and carried by 0.16-cm PTFE-tubing (Alltech, Deerfield, IL, U.S.A.) into the 3-way manifold tee. OPA and column effluent were mixed in 185 cm of 0.6 × 0.3 cm PTFE-tubing before entering the detector and were then connected to a waste container by a coiled 9.1-m section of 0.6 × 0.3 cm PTFE-tubing. Fluorescence was measured on a Spectro/Glo Fluorometer (Gilson, Middleton, WI, U.S.A.) equipped with a 5-μl microflow cell and OPA excitation and emission filters. The 100- and 10-mV output of the fluorometer was connected to a 2-channel Omniscribe strip chart recorder (Houston Instruments, Austin, TX, U.S.A.). Pen Y1 of the recorder was connected to the 100-mB output and was run at 0.1 V full-scale,

pen Y2 to the 19-mV output and was run at 1 V full-scale.

The buffer flow-rate was 30 ml/h. Before each run, the column was equilibrated with Buffer A for 15 min. Immediately after sample injection, Buffer B was started and run for 38.5 min. Buffer C was then started and run for 20 min after which the column was washed with Buffer D for 15 min. Total analysis time was 63 min and total run time was 74.5 min. *o*-Phthalaldehyde was also run at 30 ml/h. The presence of the acetylpolyamines was confirmed by collection of the column eluate corresponding to the acetyl derivatives. The acetyl derivatives were then acid hydrolyzed (6 *N* hydrochloric acid, 103°C, 16 h) to form the parent polyamines which were quantitated as previously described [45].

RESULTS

Fig. 1 shows the structures of the naturally occurring acetylpolyamines found in urine. All acetyl derivatives detected which regenerate the parent polyamines are monoacetyl derivatives. Fig. 2 represents a standard separation of the known urinary acetylpolyamines using this method. Arginine, the final basic amino acid to elute from the column, the basic dipeptides anserine, carnosine and homocarnosine, and ammonia elute between 9 and 12 min. There is a large separation of these compounds from acetylputrescine (AcPut) which elutes at 23 min and from acetylcadaverine (AcCad) which elutes at 39 min. The two isomers of acetylspermidine are completely separated with N^8 (N^8AcSpd) eluting at 46.5 min and N^1 (N^1AcSpd) at 60 min. The reproducibility of this method in terms of retention time and response is demonstrated in Table I. In all cases, the relative standard deviation was 1.1% or less.



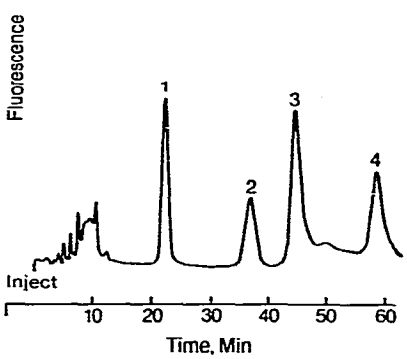


Fig. 1. Structures of naturally occurring acetyl derivatives of diamines and polyamines.

Fig. 2. A standard run of the acetylated polyamines. A 200-µl sample containing 1 nmol of each polyamine was chromatographed as described in Materials and Methods. Peaks: 1 = acetylputrescine; 2 = acetylcadaverine; 3 = N^8-acetylspermidine; 4 = N^1-acetylspermidine.

TABLE I

PRECISION OF ANALYSIS OF RETENTION TIME AND RESPONSE

Retention times and response were determined by measuring standard runs (1 nmol per 200 μl) over a 3-week period of time (n = 6). Amounts as low as 100 pmol can be accurately assayed. During this time, over 100 urine samples were run. All peaks were hand-integrated. R.S.D. (%) = relative standard deviation (%) = $\frac{\text{S.D.}}{\text{mean}} \times 100$.

	Retention time (min)				Response (nmol/200 μ)			
	AcPut	AcCad	N^1 AcSpd	N^8 AcSpd	AcPut	AcCad	N^1 AcSpd	N^8 AcSpd
Average	23.2	38.7	59.8	46.5	1.1	1.0	1.0	0.95
S.D.	0.26	0.43	0.18	0.29	0.05	0.09	0.02	0.03
R.S.D. (%)	1.1	1.1	0.30	0.62	0.05	0.09	0.02	0.03

Typical chromatograms of urine samples from normal volunteers are demonstrated in Fig. 3A and B showing the chromatograms from a dilute urine (30 mg creatinine/dl) and from a concentrated urine (240 mg creatinine/dl), respectively. Fig. 4 shows the separation of the acetylpolyamine derivatives from the urine of a patient with cancer. The separation of the acetylpolyamine derivatives was not affected by urine concentration and no compounds comigrated with the acetylpolyamine derivatives.

The chromatographic procedure was used to determine the acetylpolyamine content of urine of normal volunteers and of cancer patients. The results are shown in Table II. The acetylated polyamines were found in all urines tested with the exception of acetylcadaverine which was sometimes nondetectable. The presence of the acetylpolyamines was confirmed as described in Materials and Methods. Acid hydrolysis of the column eluate corresponding to the acetyl derivatives yielded only the parent polyamine, with the complete disappearance of the acetyl derivative.

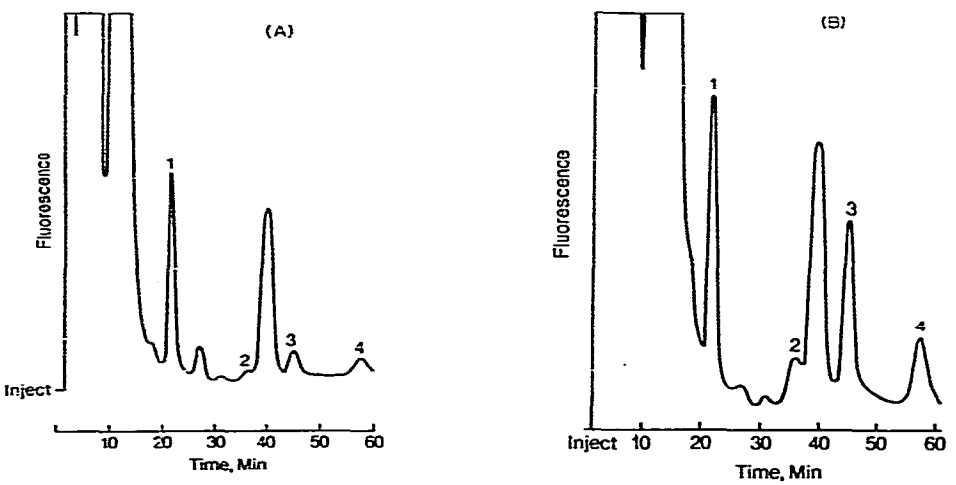


Fig. 3. Separation of acetylpolyamines in normal humans from (A) dilute urine (30 mg creatinine/dl) and (B) concentrated urine (240 mg creatinine/dl). Urine samples (200 μl) were prepared and chromatographed as described in Methods with no sample dilution necessary. Peaks as in Fig. 2.

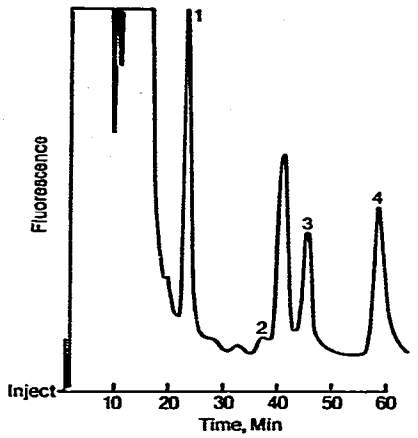


Fig. 4. Separation of a 200-μl urine sample from a male melanoma patient. Acetylpolyamine concentrations are reported in Table II. Peaks as in Fig. 2.

TABLE II

ACETYLPOLYAMINE CONTENT OF NORMAL HUMAN URINE AND URINE OF CANCER PATIENTS

The acetylpolyamine content of urine from six normal volunteers and three cancer patients. All values were standardized by basing acetylpolyamine excretion on urine creatinine as described in Materials and Methods.

Subject	Acetylpolyamine concentration (μg/mg creatinine)			
	AcPut	AcCad	N^1 AcSpd	N^8 AcSpd
Normal volunteers				
1. MM	2.6	0.33	0.66	0.58
2. JE	2.6	0.51	0.77	1.1
3. JR	2.0	TR	0.86	1.6
4. ND	3.0	0.38	0.96	1.0
5. AV	2.6	0.09	0.85	1.3
6. CP	1.3	n.d.*	0.92	0.4
Cancer patients				
1. RR (malignant mesothelioma)	10.7	0.46	2.2	0.45
2. RP (leukemia)	11.1	n.d.*	0.92	0.79
3. DC (malignant melanoma)	7.6	0.54	7.1	2.5

*n.d. = not detectable.

DISCUSSION

This is the first published report of acetylpolyamine excretion determinations as related to creatinine, although creatinine has been shown previously to be a reliable normalizer for urine concentration as related to polyamine

excretion [36, 46]. The ratios of the acetylpolyamine derivatives excreted by normals are in agreement with previously published studies [15, 17, 26, 41]. Cancer patients had greatly elevated urinary acetylputrescine as compared to normals. In two out of three patients, acetylspermidine excretion was elevated with N^1 excretion higher than N^8 excretion. In the patient with leukemia, the two acetylspermidine isomers were not excreted in elevated concentrations (Table II). These results are in agreement with the previous report of Abdel-Monem and Ohno [16], who found that certain cancer patients excrete primarily N^1-acetylspermidine. We are currently performing a large-scale study to examine the urinary excretion of the acetylpolyamines in cancer patients.

The first separation of acetylpolyamine standards was reported in 1960 by Dubin and Rosenthal [47] utilizing Dowex (50 H^+), a polystyrene-based cation-exchange resin. This method totally separated the polyamines from amino acids but the acetylated polyamines were not completely separated from the parent compounds. The eluate from this column was subjected to thin-layer chromatography for confirmation of the presence of acetylated polyamines. Tabor et al. [48] in 1973 described an automated HPLC method for the determination of di- and polyamines. They were able to obtain adequate separation of acetyl derivatives from parent polyamines. However, the run time was 5 h and the method was only sensitive to nanomolar concentrations.

Acetylpolyamine concentrations were initially measured by formation of the dansyl derivatives of the amines, thin-layer chromatography, and measurement of fluorescence [17, 49]. This method is sufficiently sensitive for the detection of acetylated polyamines, although the method has not been automated. Recently, three HPLC methods for the separation and quantitation of acetylpolyamines have been developed [41—43]. The method of Abdel-Monem and Merdink [41] utilizes a three-column system, the first two columns to clean and separate the amines, and the third to quantitate the acetylpolyamines. The HPLC method of Seiler and Knödgen [42] utilizes a single reversed-phase column for separation and detection after ion-pairing the amine-containing sample with octanesulfonate. This method has the advantage of measuring both the parent and acetylpolyamine derivatives. The accurate measurement of urinary acetylputrescine is not possible by this method because it is not separated from unknown interfering compounds. Mach et al. [43] separated the acetylpolyamines by classical ion-exchange techniques on an amino acid analyzer. The measurement of the acetylated and free polyamines using this method requires two separate runs. The first run separates and measures the acetylputrescine concentration and the second, the combined acetylspermidine derivatives and the free polyamines. This method does not separate the two isomers of acetylspermidine with N^1- and N^8-acetylspermidine cochromatographing as a single peak.

The chromatographic system described in this paper uses a single cation-exchange column which allows for the rapid, sensitive and reproducible analysis of all urinary acetylpolyamines, but not the parent compounds. Extensive sample preparation is not necessary and the large capacity of the cation-exchange column permits acetylpolyamine determinations in concentrated urines without sample dilution. We are currently using this method in our

laboratory for the routine measurement of acetylpolyamine excretion in cancer patients and to assess the effects of therapy on acetylpolyamine excretory patterns.

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