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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC PROCEDURE FOR THE SIMULTANEOUS DETERMINATION OF THE NATURAL POLYAMINES AND THEIR MONOACETYL DERIVATIVES

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SUMMARY

The separation of the natural polyamines and their monoacetyl derivatives by high-performance reversed-phase liquid chromatography is reported. Octane sulfonate was used to form ion pairs with the polycations and the *o*-phthalaldehyde method for post-column derivatization. The method allows polyamine and acetylspermidine determinations directly from tissue extracts and body fluids without pre-purification.

INTRODUCTION

In a recent paper we reported a method for the determination of the naturally occurring monoacetyl derivatives of di- and polyamines [1]. This method and the earlier method of Abdel-Monem and Ohno [2] relied on the derivatization of the amines with dansyl chloride and subsequent thin-layer chromatographic separation. Although suitable for urine analyses from the point of view of specificity and rate, the method requires considerable manual work and experimental skill and is not suited for automation.

The *o*-phthalaldehyde—2-mercaptoethanol reagent [3] is well established now for post-column derivatization of the polyamines after ion-exchange column chromatographic separations [4,5]. Therefore, it was decided to work out a separation of the known natural monoacetyl derivatives and of the non-conjugated polyamines to an extent which would allow their direct assay in conjunction with the *o*-phthalaldehyde method in tissues and body fluids without sample pre-purification. Since even very sophisticated cation-exchange

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column chromatographic procedures seemed not to give fully convincing results [6—9], a reversed-phase system was chosen using *n*-octane sulfonate for ion pairing. Separation of ion pairs on reversed-phase columns is now well established [10], but the method has not been employed to our knowledge to polyamine separations.

The new method turned out to be efficient, rapid, reproducible and sufficiently simple as to allow its routine application.

MATERIALS AND METHODS

The chromatographic system

A Varian high-pressure liquid chromatograph (Model 8500) was used, to which a loop injector (Valco valve CV-6-UHPa-N60; Valco Instruments, Houston, TX, U.S.A.) (loop volume 250 μl) was attached. A pre-column (100 mm × 3 mm) was filled with a pellicular silica core with C_{18}-brushes (CO:PELL ™ODS, Catalog No. M018; Reeve Angel, Clifton, NJ, U.S.A.). The actual separations were performed with a μBondapak C_{18} column (3.9 mm × 300 mm; 10 μm particles) (Waters, Paris, France). Column effluent and *o*-phthalaldehyde reagent were mixed in a T-piece and after flowing through a coil of PTFE tubing (2 m × 0.5 mm I.D.) the effluent—reagent mixture passed through a flow-cell of 1.5 mm optical path length (Hellma, Düsseldorf, G.F.R.). A Perkin Elmer fluorescence spectrometer Model 204 A was used as detector. This was equipped with a 150 W xenon arc lamp. Fluorescence excitation was achieved at 345 nm, and emission was measured at 455 nm. Column, T-piece, and PTFE coil were kept at 35 ± 0.5°C by means of circulating water, which was temperature-controlled by a thermostat. The signal of the fluorescence detector was recorded at two sensitivities using a two channel recorder (Model 1200, W + W Electronic A.G., Basel, Switzerland). The reagent was pumped with a piston pump (Dosapro; Milton Roy, St. Pierre, France). The pulses of the pump were damped by a bulb trap between pump and mixing T-piece.

Chemicals

Sodium acetate ($CH_3COONa \cdot 3H_2O$) (DAB6-grade), boric acid, potassium hydroxide, 2-mercaptoethanol and the wetting agent Brij-35 (30% solution in water) were from E. Merck (Darmstadt, G.F.R.). *o*-Phthalaldehyde was from C. Roth (Karslruhe, G.F.R.); acetic acid, acetonitrile and other common chemicals were from Baker Chemicals (Deventer, The Netherlands). 1-Octanesulfonic acid (sodium salt) was a product of Eastman Kodak (Rochester, NY, U.S.A.). Putrescine dihydrochloride, cadaverine dihydrochloride, spermidine phosphate, spermine phosphate and carnosine were from Fluka (Buchs, Switzerland); anserine nitrate, homocarnosine sulfate, agmatine sulfate, histamine dihydrochloride, dopamine hydrochloride and serotonin creatinine sulfate were from Sigma (St. Louis, MO, U.S.A.); putreanine was from Cal-Biochem (San Diego, CA, U.S.A.). 1,7-Diaminoheptane (base) was from Aldrich Europe (Beerse, Belgium). The dihydrochloride was prepared by reaction with HCl and recrystallization from ethanol. The acetyl-di- and -polyamine

hydrochlorides were prepared in our laboratory according to published procedures [11].

Elution buffer

The elution system consisted of a gradient which was prepared from two buffer solutions. Buffer A: 0.1 *M* sodium acetate adjusted to pH 4.50 with acetic acid and containing 10 m*M* octane sulfonate. Buffer B: 0.2 *M* sodium acetate (adjusted to pH 4.50 with acetic acid) plus acetonitrile (10: 3, v/v), containing 10 m*M* octane sulfonate. The acetonitrile was distilled over phosphorus pentoxide before use.

o-Phthalaldehyde reagent

This was prepared by dissolving 50 g of boric acid, 44 g of potassium hydroxide and 3 ml of the Brij-35 solution per litre of distilled water. To this solution 2 ml of 2-mercaptoethanol and 400 mg of *o*-phthalaldehyde dissolved in 5 ml of distilled methanol were added before use.

Sample preparation

Tissue samples were freshly prepared from decapitated rats and were immediately homogenized in 0.2 *N* perchloric acid which contained 0.5 μM 1,7-diaminoheptane dihydrochloride as internal standard. The extracts were filtered through a Millipore filter (Millex 0.22 μm) and were applied either directly or after appropriate dilution with 0.1 *M* sodium acetate buffer (pH 4.50) to the column.

In order to avoid bacterial contamination urine samples were collected in polyethylene flasks containing 4 *N* HCl. Samples of 100 μl were mixed with 100 μl of a solution of 500 pmoles of 1,7-diaminoheptane dihydrochloride in 0.2 *N* perchloric acid and were diluted with 800 μl of 0.1 *M* sodium acetate buffer (pH 4.50) immediately before analysis.

Samples and standard solutions were diluted with buffer only in polypropylene tubes, and dilutions were never stored over extended periods in order to avoid spermidine and spermine losses due to adsorption to surfaces.

Chromatographic separation

The buffer flow-rate was 90 ml/h. Before each run the column was equilibrated for 6 min with buffer A. Then the sample was applied to the column and separated as follows. A linear gradient was prepared from buffer A and buffer B with an increment of 2% per min of buffer B for 30 min. At this time the gradient contained 60% buffer B. For the remaining time the increment of buffer B was increased to 4% per min and elution was completed. Resetting to buffer A was usually done at 45 min after commencing the run, if no unusual impurities were eluted from the column. Accordingly one separation required 51 min.

Column eluent and *o*-phthalaldehyde were mixed in a 1:1 ratio and fluores-

cence intensity was continuously recorded at two sensitivities which usually differed by a factor of 10 in the case of urine samples, and by a factor of 20 in the case of tissue samples.

Quantitative evaluation of the chromatograms

Since the eluted zones gave Gaussian signal responses and since the peak width at half height was nearly identical for all compounds, it was possible to evaluate quantitatively simply by peak height measurements. Besides using an internal standard, 1,7-diaminoheptane dihydrochloride, a standard mixture was run after every third sample in order to control instrumental sensitivity, and reproducibility of the separations.

RESULTS AND DISCUSSION

Many reports are available now on various aspects of the *o*-phthalaldehyde procedure as a method of continuous post-column derivatization of polyamines [4—7, 11]. Therefore it seems not necessary to give a detailed account of the experiments which were performed to determine sensitivity, reproducibility and linearity of dose—response curves.

Linear relationships between amine concentration and fluorimeter signal were established with mixtures of the following compounds: monoacetylputrescine, putrescine, histamine, N^1-acetylspermidine, N^8-acetylspermidine, spermidine, monoacetylspermine and spermine, in the concentration range 50 n*M* to 5 μM. The smallest amount applied in these experiments was 125 pmoles. However, the method allows less than 50 pmoles to be measured. In order to establish reproducibility of the method, a perchloric acid liver extract was diluted 1 : 1; 1 : 4; 1 : 9 and 1 : 19. From each dilution three samples were run, and standard deviations were calculated from the twelve determinations. They were as follows: putrescine ± 8.1%; spermidine ± 3.9% and spermine ± 6.4%.

The long-term reproducibility was tested by comparing the peak heights obtained from standard solutions over a period of three months. Only monoacetylputrescine showed an S.D. > ± 10%. This is due to the relative instability of the separation system at the beginning of the run (see below). For the evaluation of the actual concentration measurements the standard samples run after every third tissue samples were used in order to exclude sensitivity changes of the detection system.

So far our results were entirely in agreement with previously published reports. The considerably higher concentration of the borate buffer used for the preparation of the *o*-phthalaldehyde reagent was necessary to ensure alkaline reaction conditions in our chromatographic system. This buffer had no obvious disadvantage over the more dilute buffer used in conjunction with ion-exchange column chromatographic separations [4,6,12].

The main emphasis of this work was on the improvement of polyamine separations. Since these polycations are highly polar, *n*-octane sulfonate was chosen for ion pairing. Preliminary results showed, however, that heptane sulfonate, and even *n*-hexane sulfonate, can give adequate separations in conjunction with a column with C_{18}-brushes.

In order to obtain single ion species even with the polycations spermidine and spermine, a high concentration (10 m*M*) of the pairing anion was required.

Fig. 1 shows a separation of a mixture of some amino acids, the basic dipeptides anserine, carnosine and homocarnosine, and of a number of amines. Fig. 1B shows a typical chromatogram of the standard mixture which was used for external standardization and was usually run after every third tissue or urine sample.

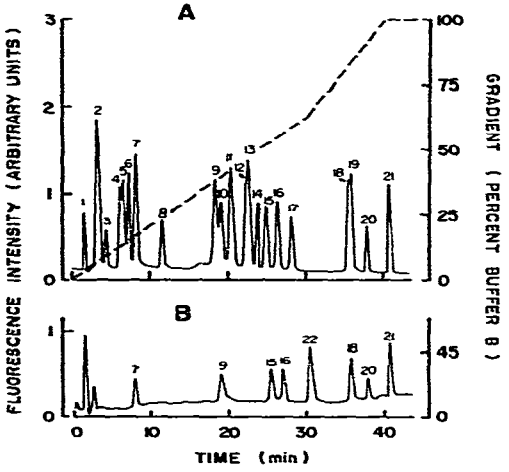

Fig. 1. Separations of mixtures of reference compounds. For details of the separation conditions see the Methods section. (A) Complete amino acid and amine mixture. (B) Standard mixture (5 nmoles/ml). This was normally used for external standardization of polyamine determinations in urine and tissues. Peaks: 1 = glutamic acid; 2 = methylamine and ammonia; 3 = histidine; 4 = anserine; 5 = carnosine; 6 = homocarnosine and putreanine (N-(4-aminobutyl)-3-aminopropionic acid); 7 = monoacetylputrescine; 8 = monoacetylcadaverine; 9 = 1,3-diaminopropane and 1,4-diaminobutane (putrescine); 10 = *p*-tyramine; 11 = 1,5-diaminopentane (cadaverine); 12 = histamine; 13 = serotonin; 14 = N^1-methylhistamine; 15 = N^1-acetylspermidine; 16 = N^8-acetylspermidine; 17 = agmatine; 18 = spermidine; 19 = N^1-(3-aminopropyl)-1,3-diaminopropane; 20 = N^1-acetylspermine; 21 = spermine; 22 = 1,7-diaminoheptane.

As Fig. 1 shows, the two isomeric monoacetylspermidines are completely separated from each other and from other known compounds. Monoacetylspermine is also well separated from both spermidine and spermine and the method should also allow the determination of agmatine.

From separations with a sophisticated cation-exchange method [6] it was presumed that the complete separation of the usual amino acids and the basic dipeptides carnosine and homocarnosine from monoacetylputrescine would allow the unambiguous determination of the latter compound. Indeed, there was only one major inadequacy in the separations of reference compounds, as appears from Fig. 1A: 1,3-diaminopropane co-migrated with 1,4-diaminobutane (putrescine). This seems to limit the usefulness of the method for polyamine analysis. However, 1,3-diaminopropane has not been demonstrated unambiguously in mammalian tissues or urine. The fact that spermidine and 3-aminopropyl-1,3-diaminopropane partially overlapped is also of little practical conse-

quence. The separations shown in Fig. 1 are more complete than any previously reported polyamine separation, and they are, moreover, somewhat faster than some of the ion-exchange column chromatographic procedures [4,7]. Spermine elutes from the column at 41 min and elution is usually stopped at 45 min. An additional 6 min are required for column equilibration with buffer A, before the following sample can be applied; i.e. one needs 51 min for each run.

If the method is to be used for histamine determinations in tissue or urine there are two possibilities to circumvent the problem caused by the overlapping of the histamine and serotonin zones:

(A) Sample pre-separation on small Dowex 50W-X8 columns (H^+) as described previously [1] removes serotonin (and *p*-tyramine) and recovers histamine quantitatively.

(B) An *o*-phthalaldehyde reagent of basically the same composition, however, without 2-mercaptoethanol reacts with histamine and a few other primary amines such as spermidine [13,14] to give fluorescent derivatives. Serotonin and most other amines react to give compounds with negligibly low fluorescence quantum yields.

The method has been used extensively for the determination of free and acetylated polyamines in rat and human urine, and for polyamine determinations in rat liver. Fig. 2 shows the separation of a urine sample of a normal rat. The retention times of the various amines are very reproducible (0.5 min), with the exception of the compounds with retention times of less than 12 min. Among these compounds monoacetylputrescine is the most significant. Its retention time was not only somewhat dependent of the time of equilibration with buffer A, but was also influenced by the size of the urine sample. Moreover, it turned out that urine contains a number of constituents which overlap with the monoacetylputrescine zone under our chromatographic conditions; this is a serious but the only major practical limitation so far known to the present method. Pre-separation of samples on Dowex 50W-X8 columns [1] removes quite a number of obviously lipophilic urinary constituents, as can be seen in Fig. 3. This figure shows the separations of the urine of a melanoma patient (A) without any pre-purification, and (B) after chromatography through the above-mentioned cation-exchange column.

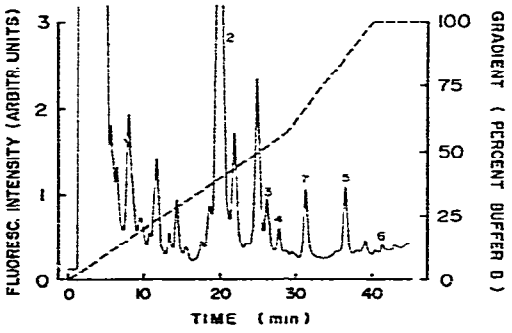

Fig. 2. Separation of a 1:9 dilution of the urine of a normal rat. Peaks: 2 = putrescine; 3 = N^1-acetylspermidine; 4 = N^8-acetylspermidine; 5 = spermidine; 6 = spermine; 7 = 1,7-diaminoheptane (internal standard). For details of the separation conditions see the Methods section.

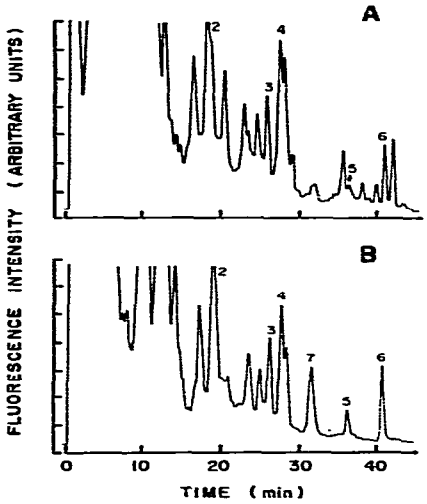

Fig. 3. (A) Separation of a 1:9 dilution of the urine of a male melanoma patient. (B) Separation of the same urine sample after pre-chromatography through a Dowex 50W-X8 column (H^+) [1], addition of internal standard, and appropriate dilution. Peaks: 2 = putrescine; 3 = N^1-acetylspermidine; 4 = N^8-acetylspermidine; 5 = spermidine; 6 = spermine; 7 = 1,7-diaminoheptane (internal standard). For details of the separation conditions see the Methods section.

The recovery of the acetylspermidines from the Dowex columns was quantitative, in agreement with our previous finding [1]. The amounts of free spermidine and spermine seem, however, somewhat increased after column chromatography of human urine. The most likely explanation of this finding is that acid-labile conjugates exist from which the polyamines are liberated. The recently detected polyamine—pyridoxal Schiff bases [15] are likely candidates. Unfortunately, even pre-purification by ion-exchange column chromatography did not remove all interfering compounds, and was therefore not an adequate procedure for monoacetylputrescine determinations in urine. But, disregarding this compound, all other di- and polyamines of biological interest could be determined quantitatively in urine, even without sample pre-purification.

The situation is somewhat more favourable in the case of tissue samples. These can be directly applied on the column as extracts in 0.2 *N* perchloric acid. Fig. 4 shows an example. In normal liver, the monoacetylputrescine concentration is very low. Its concentration is considerably increased, however, together with that of putrescine, after intoxication with thioacetamide. This increase can be clearly demonstrated, although the monoacetylputrescine zone was only incompletely separated from an unidentified compound which is present in liver. From the same figure it appears that neither N^1-acetylspermidine nor N^8-acetylspermidine are present in normal liver in amounts directly detectable with the method under standard separation conditions, i.e. the concentration of these compounds is lower than 5 nmoles/g. In the liver of thioacetamide-treated rats, the N^1-acetylspermidine concentration was markedly enhanced, and could be determined quantitatively. The N^8-acetylspermidine concentration was not increased to a detectable concentration by treatment with thioacetamide.

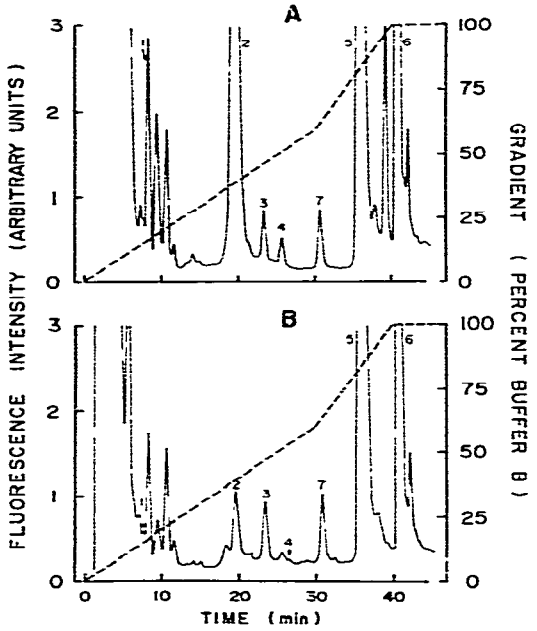

Fig. 4. Separations of perchloric acid extracts of rat liver (1:15 homogenate). (A) Liver of a rat treated with 150 mg/kg thioacetamide 16 h prior to isolation of the liver. (B) Liver of an untreated control animal. Peaks: 1 = monoacetylputrescine; 2 = putrescine; 3 = histamine; 4 = N^1-acetylspermidine; 5 = spermidine; 6 = spermine; 7 = 1,7-diaminoheptane (internal standard). For details of the separation conditions see the Methods section.

Since the concentrations of spermine and spermidine are very high compared with those of putrescine and the acetylated polyamines, fluorescence was recorded at two sensitivities (not shown in the figures), which usually differed by a factor of 20. The data obtained for rat liver putrescine, spermidine and spermine concentrations were identical which previously reported values [16].

It was mentioned above that retention times were constant in repeated runs, and were exactly the same for samples and standards, with the limitation that compounds eluting before 12 min showed slight changes in retention times which depended on various factors. However, after several hundred tissue and urine samples (i.e. after about three months' use of the same column) the average retention time had gradually decreased by 1 to 2 min, depending on the compound. This change, however, did not significantly influence the quality of the separations. Refilling the pre-column had no influence on the changed separation characteristics.

The method described here is still not fully satisfactory, because its application to monoacetylputrescine determination is limited. However, it is the first column chromatographic procedure which allows the direct determination of acetylspermidines and of non-conjugated polyamines in a single run. No method of comparable sensitivity for polyamine derivatives is presently available, disregarding the two methods relying on derivatization with dansyl chloride prior to separation [1,2].

Dansyl derivatives of the non-conjugated polyamines are well separated on

reversed-phase columns [17—20]. However, it was not possible to separate the dansyl derivatives of N^1-acetylspermidine and N^8-acetylspermidine from each other on this type of column [21]. It would be interesting to know, whether other derivatives which have been suggested for polyamine analyses, such as the *o*-phthalaldehyde [22], fluorescamine [23,24], tosyl [25] or benzoyl derivatives [26], are more suitable for reversed-phase liquid chromatography than the dansyl derivatives.

The combination of gas—liquid chromatography and mass spectrometry is in principle an alternative method for the determination of acetyl derivatives of spermidine [27,28]. The usual derivatization with trifluoroacetic anhydride and related reagents is, however, hampered by the possible displacement of acetyl groups.

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