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SEPARATION OF N^1- AND N^8-ACETYLSPERMIDINE ISOMERS BY REVERSED-PHASE COLUMN LIQUID CHROMATOGRAPHY AFTER DERIVATIZATION WITH DANSYL CHLORIDE

CLAUDIO STEFANELLI, DANIELA CARATI and CARMEN ROSSONI*

Istituto di Chimica Biologica e Centro Studi e Ricerche sul Metabolismo Cardiaco, Facoltà di Medicina e Chirurgia dell'Università di Bologna, Via Irnerio, 48-40126 Bologna (Italy)

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SUMMARY

The separation of dansyl derivatives of N^1- and N^8-acetylspermidine by reversed-phase column liquid chromatography is reported The influence of organic solvents on the retention of acetylspermidines was studied Best resolutions were achieved using a C_{18} column and a ternary mobile phase composed of water, methanol and acetonitrile The pre-column derivatization method permitted the detection of picomole quantities A method for the determination of acetylspermidines in rat tissues is described

INTRODUCTION

Polyamines are acetylated by distinct enzymes [1] In mammals, spermidine may be acetylated in the N^1 or N^8 position, the former is produced in the cytosol by a specific acetyltransferase, which is induced by toxic and hormonal stimuli, while the latter is synthesized in the nucleus [2] Generally, the acetyl-polyamines do not accumulate, however, a high N^1-acetylspermidine concentration was found in some neoplastic tissues [3]

Column liquid chromatography (LC) is widely used for polyamine analysis in tissues [4] Several methods are based on the derivatization of amines with dansyl chloride (Dns-Cl) and subsequent separation of the fluorescent derivatives on reversed-phase columns [5] The advantages of the precolumn derivatization procedure with Dns-Cl are discussed by Seiler [5] and by Bontemps et al [6] The method is considered to be efficient and highly sensitive, however, this technique was limited by the fact that the Dns derivatives of the isomeric monoacetylspermidines could not be separated by

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reversed-phase chromatography [5, 7] Abdel-Monem and Merdink [8] therefore developed a two-step procedure employing two normal-phase columns In the present work, we show that with a highly efficient stationary phase and a proper choice of eluents, the reversed-phase separation of Dns derivatives of N-acetylspermidine isomers is possible, and a method for their analysis in mammalian tissues is presented

EXPERIMENTAL

Chemicals

N-Acetylputrescine, N^1-acetylspermidine and N^8-acetylspermidine were generous gifts from Dr N Seiler (Merrel International, Strasbourg, F R G) Monoacetylcadaverine was synthesized by the procedure described by Tabor and Tabor [9] and N^1-acetylspermine as described by Dubin and Rosenthal [10], these compounds are now commercially available Dansyl chloride and polyamines were from Sigma (St Louis, MO, U.S.A.). Solvents for LC were purchased from Aldrich (Milwaukee, WI, U.S A) Laboratory chemicals and silica gel thin-layer plates were from Merck (Darmstadt, F R.G)

Apparatus

The LC equipment was composed of an M6000 and an M45 pump, a Model 660 solvent programmer, a U6K injector, an M420 fluorescence detector (excitation filter at 338 nm and emission cut-off filter at 425 nm) and an M730 data module (Waters Assoc. Milford, MA, U S A) Separations were achieved with an Ultrasphere ODS 5-μm column (150 × 4 6 mm I.D) (Beckman) protected by a 25 × 3 9 mm I D precolumn filled with pellicular ODS Care was taken to reduce death spaces.

Sample preparation

Standard solutions of the polyamines were prepared from $1 \cdot 10^{-2}$ *M* stock solutions in 0.3 *M* perchloric acid and stored at 2°C Tissues from sacrificed rats were washed in chilled 0 9% sodium chloride, blotted and homogenized in 4 vols of 0 3 *M* perchloric acid in a glass Potter The homogenates were centrifuged at 10 000 *g* for 30 min To 0 2-ml aliquots of the clear acid extract, the following solutions were added 20 μl of $2 \cdot 10^{-4}$ *M* 1,8-diaminooctane (internal standard), 15 μl of 3 *M* potassium hydroxide, 100 μl of 1 5 *M* sodium carbonate adjusted to pH 10 with saturated sodium bicarbonate and 0 5 ml of a solution of Dns-Cl in acetone (10 mg/ml) After vortexing, the tubes were stored overnight in the dark To each tube, 0 2 ml of 3 *M* potassium hydroxide were added and after vigorous vortexing the Dns polyamines were extracted with 1 0 ml of benzene The organic phase was evaporated to dryness at 40°C in vacuo. The residue was dissolved in 20 μl of isopropanol

Column liquid chromatography

Aliquots of 2—10 μl of the final isopropanol solution were injected in the chromatographic system The Dns derivatives of acetylspermidines and other polyamines were separated with a solvent composed of water–acetonitrile–methanol (5.3 2) as solvent A and acetonitrile–methanol (3 2) as solvent B

The sample was eluted with a concave solvent gradient using the gradient curve No 8 of the Waters M660 solvent programmer The gradient changed from 28 to 90% solvent B in 19 min at a flow-rate of 1 0 ml/min Resetting to initial conditions was done after 25 min with a reverse program in 1 min, and the column was allowed to re-equilibrate for 4 min before a second sample was injected

RESULTS AND DISCUSSION

It was previously reported that Dns N^1- and N^8-acetylspermidine could not be separated by reversed-phase LC [7], however, we found that by means of improved stationary phases, the acetylspermidine isomers may be separated using commercial columns filled with 5-μm C_{18} particles and a simple elution system composed of water and an organic solvent. Fig. 1 shows the influence of different organic solvents in binary mixtures with water on the retention of bis(Dns) acetylspermidines High capacity factors (k') with increased selectivity are obtained by lowering the concentration of the organic solvent. Acetone is not suitable for acetylpolyamine separation, in contrast, both methanol and acetonitrile allow adequate resolutions Methanol is more effective than acetonitrile, but the latter produces sharper peaks.

The relative retention of acetylspermidines can also be modulated by using an eluent composed of water and a binary organic phase in which the proportion of the organic components varies. Fig 2 shows the results obtained by isocratic elution with 40% water and 60% methanol– acetonitrile The use of a binary organic phase was useful because better separations of N^1- and N^8-acetylspermidine were obtained than by using single organic modifiers, and adequate resolutions (R_s) [11] were achieved in a relatively short time. Furthermore, in rat tissue samples it was more effective to change the relative proportion of methanol and acetonitrile in order to separate acetylspermidines from interfering peaks than to vary the composition of an eluent containing

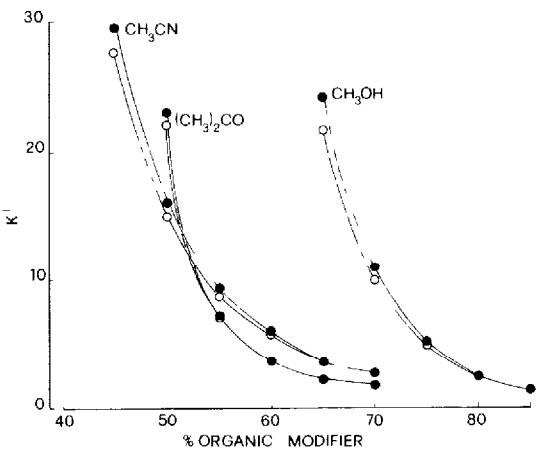

Fig 1 Dependence of the capacity factors of N^1-acetylspermidine (•) and N^8-acetylspermidine (○) on the mobile phase composition using water–organic solvent binary mixtures Stationary phase 5 μm Ultrasphere ODS 150 × 4 6 mm I D , flow-rate 1 0 ml/min

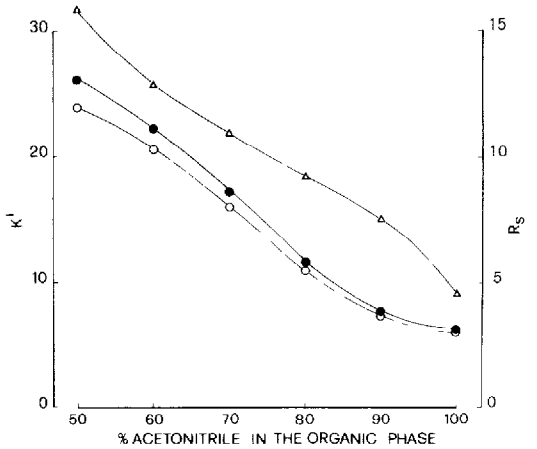

Fig 2 Dependence of capacity factors of N^1-acetylspermidine (•) and N^8-acetylspermidine (○) and of their resolution (R_s, △) on the mobile phase composition using a ternary eluent (water—methanol—acetonitrile) Chromatography was performed isocratically with 40% water and 60% organic phase in which the relative contents of methanol and acetonitrile varied, further details as in Fig 1

a single organic component For example, with water—methanol mixtures, N^1-acetylspermidine was not resolved from putrescine even after prolonged retention

For the analysis of the acetylspermidines and the non-conjugated polyamines a concave gradient (No 8) was used, which changed within 19 min from 64 to

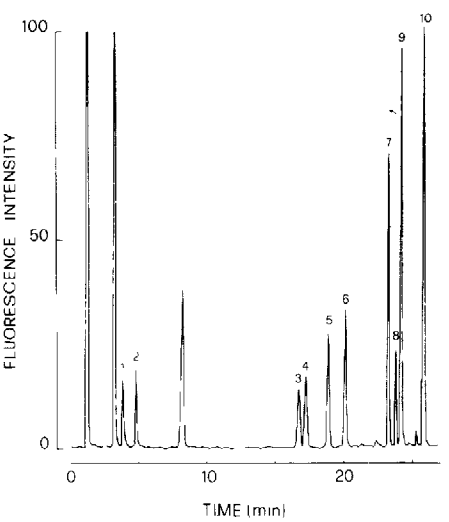

Fig 3 Separation of Dns derivatives of a standard solution of polyamines and acetylpolyamines Column 5 μm Ultrasphere ODS 150 × 4 6 mm I D Solvent A water—acetonitrile—methanol (5 3 2), solvent B acetonitrile—methanol (3 2) Concave gradient from 28 to 90% solvent B in 19 min Flow-rate 1 0 ml/min Peaks 1 = N-acetylputrescine (50 pmol), 2 = N-acetylcadaverine (50 pmol), 3 = N^8-acetylspermidine (50 pmol), 4 = N^1-acetylspermidine (50 pmol), 5 = putrescine (50 pmol), 6 = cadaverine (50 pmol), 7 = 1,8-diaminooctane (80 pmol, internal standard), 8 = N^1-acetylspermine (25 pmol), 9 = spermidine (80 pmol), 10 = spermine (80 pmol)

95% organic phase, composed of acetonitrile–methanol (3 2) Good separations of the polyamines and their acetyl derivatives were achieved within ca 26 min (Fig 3) The detection limit (detector response five times the noise level) was ca 3 pmol for the acetylspermidines, 1 pmol for acetylspermine, putrescine and cadaverine and somewhat lower for the other non-conjugated polyamines Retention times varied below ± 1 5% and the detector response was linear in the range 5–2000 pmol for all polyamines Quantitation was based on the ratio between the peak areas of the compounds to be determined and the peak area of the internal standard Repeated injections of analytical standards for several days gave a mean relative standard deviation below ± 4 5% (n = 15) for all compounds mentioned in Fig 3

The method was applied to the analysis of acetylspermidines in rat tissues Samples were prepared and analysed as described under Experimental In agreement with Abdel-Monem and Merdink [12], we found compounds in the lung and spleen with the retention times of the two acetylspermidines (Fig 4). These peaks disappeared when samples were hydrolysed in 6 *M* hydrochloric acid for 16 h at 110°C before dansylation The peaks were collected separately from large volumes of extract and analysed by thin-layer chromatography (TLC) on silica gel plates The compounds co-chromatographed with authentic standards of N^1- and N^8-acetylspermidine using chloroform–isopropanol (10.1), ethyl acetate followed by chloroform–triethylamine (10·1) and in a two-dimensional system composed of methyl acetate in one dimension and chloroform–carbon tetrachloride–methanol (14 6 1) in the other [5] In both lung and spleen, TLC analysis revealed a single spot for N^1-acetylspermidine while in the case of the N^8 isomer ca 75% of the fluorescence of the collected peak chromatographed in the same position as the authentic acetylpolyamine The contents (nmol/g wet wt) of N^1- and N^8-acetylspermidine were 2.7 and

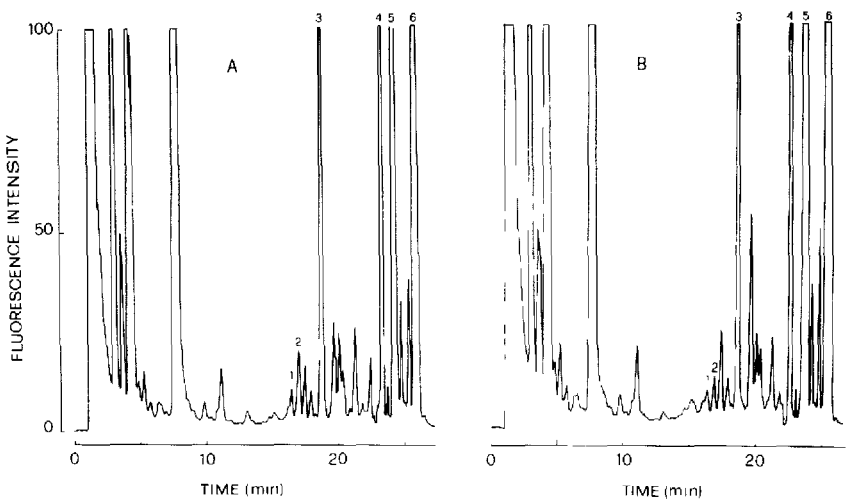

Fig 4 Separation of Dns derivatives obtained from rat lung (A) and spleen (B) extracts For sample preparation and chromatographic conditions, see Experimental The detector was set to 50% maximum sensitivity, between 22 and 22 5 min the sensitivity was reduced four times Peaks 1 = N^8-acetylspermidine, 2 = N^1-acetylspermidine, 3 = putrescine, 4 = 1,8-diaminooctane, 5 = spermidine, 6 = spermine

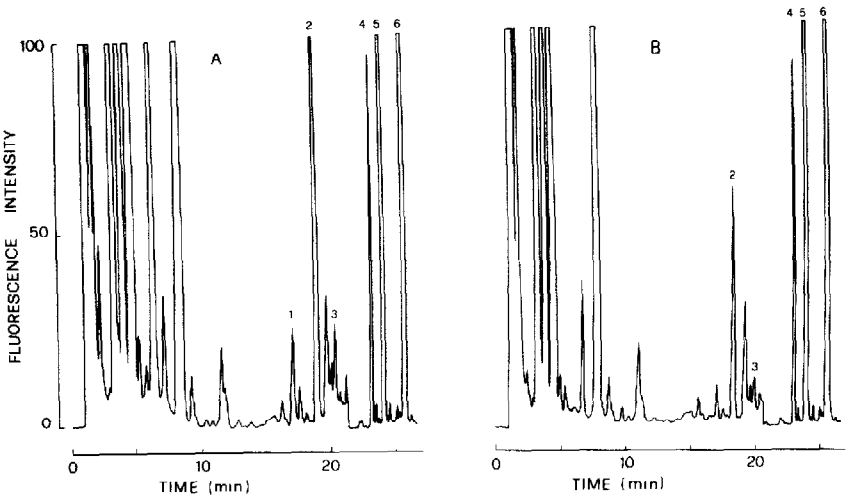

Fig 5 Chromatograms of Dns derivatives from rat liver extracts (A) Liver of a rat 8 h after treatment with 150 mg/kg diethylnitrosamine, (B) liver of an untreated control animal The detector was set to 50% maximum sensitivity, which was reduced eight times between 21 and 22 min Peaks 1 = N^1-acetylspermidine, 2 = putrescine, 3 = cadaverine, 4 = 1,8-diaminooctane, 5 = spermidine, 6 = spermine

1 7, respectively, in spleen and 4 2 and 1 2, respectively, in lung N^8-Acetylspermidine was not fully separated from a minor preceding peak, but the resolution was sufficient for an adequate quantitation

In liver, kidney and heart only very small peaks were observed in the position of the acetylspermidines, but the method was suitable for the determination of N^1-acetylspermidine in stimulated tissues The lack of interferences is evident from Fig 5, which shows chromatograms of liver extracts from a control rat and from a rat 8 h after treatment with 150 mg/kg of diethylnitrosamine, which induces spermidine N^1-acetyltransferase activity [13] The N^1-acetylspermidine peak appears in a zone of the chromatogram, which is free from other peaks

The proposed elution procedure is a compromise between resolution and analysis time and is suitable for the analysis of acetylspermidines and nonconjugated polyamines However, lowering the final concentration of the organic phase to 85% allows the determination of N^1-acetylspermine without interferences, while the resolution of acetylputrescine requires a very long run

The LC methods now generally used for acetylspermidine determination are based on ion pairing [7, 14] or ion-exchange column chromatography [15—17] with postcolumn derivatization, while for the analysis of Dns derivatives only a two-column procedure is currently available [8] The precolumn derivatization permits the determination of very low concentrations of acetylspermidines in tissues because the Dns polyamines are extracted from the derivatization mixture and thus may be concentrated even from large sample volumes [4] Furthermore, precolumn derivatization is compatible with chromatograhy on microbore columns, which allows a further lowering of the detection limits

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