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DETERMINATION OF DI-AND POLYAMINES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF THEIR 5-DIMETHYLAMINONAPHTHALENE-1-SULFONYL DERIVATIVES

NIKOLAUS SEILER*, BERND KNÖDGEN

Max-Planck-Institute for Brain Research, Frankfurt/M (G.F.R.)

and

FRIEDHELM EISENBEISS

E. Merck, Darmstadt (G.F.R.)

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SUMMARY

Using a Lichrosorb RP-8 reversed-phase column and a methanol—water gradient elution program, it is possible to separate within 40 min and to determine routinely in picomole quantities the natural di- and polyamines. The precision of the method is comparable to the thin-layer chromatographic procedures, the separations are most efficient, and the method can be fully automated. A modified gradient enables the repeated assay of spermidine and spermine within 20 min. The method is suited for polyamine analyses in tissues and body fluids.

INTRODUCTION

Widespread interest in the natural di- and polyamines [1, 2] has led to the development of a number of methods for their rapid and sensitive assay. Most of these methods have been summarized recently [3—6]. Separation of the amines by ion-exchange column chromatography with automated instruments [7—17] is the favored method for routine assay of polyamines in urine and body fluids. Reaction of amines with fluorescamine either before or after their separation by thin-layer or high-performance liquid chromatography (HPLC) has been published [18—21]. However, no practical application of these methods has been reported. Radio-immunological methods [22, 23] for polyamine determinations in clinical screening programs seem to be most

*Correspondence should be addressed to: N. Seiler, Centre de Recherche Merrell International, 16 rue d'Ankara, 67084 Strasbourg, France.

promising. If the radio-immunoassay be disregarded, the most sensitive method is still the estimation of polyamines as their 5-dimethylaminonaphthalene-1-sulfonyl (Dns) derivatives [3, 24—29], especially if high-performance thin-layer chromatography (HPTLC) is used for the separation [30]. To combine the sensitivity of the dansylation procedure with a method suited for automation, HPLC was suggested for the separation of the Dns derivatives of polyamines. Two systems have been described in the literature, using mixtures of organic solvents for elution [31, 32]. During recent years our laboratory has made several attempts to develop a routine procedure of polyamine determination on the basis of the dansylation reaction, using reversed-phase columns with lipophil molecules bound to silica cores. Separations were adequately rapid and reproducible in successive runs with reference samples. However, difficulties were encountered with putrescine determinations in tissues and body fluids containing only low concentrations (10—20 nmoles/g) of putrescine. These difficulties were overcome by a purification step between the dansylation reaction and the HPLC separation. This procedure and a routine HPLC system are described in the present work.

MATERIALS

Chemicals

Chemicals were of A grade (E. Merck, Darmstadt, G.F.R.). The methanol for the preparation of the gradient was distilled before use. 5-Dimethylaminonaphthalene-1-sulfonylchloride (Dns-Cl) and reference samples of the Dns derivatives were prepared in our laboratory according to published procedures [25]. 1,4-Diamino-2-butyne was a gift of Dr. H. Fischer, Max-Planck-Institute for Brain Research, Frankfurt/M., G.F.R.

Equipment

A Varian 8500 high-pressure liquid chromatograph was used in combination with a Perkin-Elmer fluorescence spectrophotometer 204, equipped with an 8-μl flow cell and a Varian two-channel recorder. To minimize the influence of scattered light, a Woods filter was adjusted in the path of the activating, and a 420-nm cut-off filter in the emitted light. (Activation of fluorescence at 360 nm, fluorescence measurement at 510 nm.) Separations were achieved on a 250 × 3 mm Hibar pre-packed column, Lichrosorb RP-8, 7 μm, Merck; based on its capacity, mass-transfer properties and selectivity this support is especially suited for the separation of compounds of medium polarity.

METHODS

Sample preparation

Tissues and cells were homogenized with 0.2 *N* perchloric acid. A known amount of 1,6-diaminohexane · 2 HCl was added to the homogenates as internal standard. The perchloric acid extracts were reacted with Dns-Cl by addition of 3 volumes of a solution of Dns-Cl in acetone (10 mg/ml), saturation with sodium carbonate, and reaction at room temperature overnight. Excess of reagent was removed by the addition of a concentrated solution of proline in water, as described previously [25]. The Dns derivatives were extracted

by shaking with 6 ml of toluene. Five milliliters of the toluene phase were evaporated to dryness. The residue was redissolved in 3 ml of toluene.

Pre-separation of the polyamine derivatives

Disposable 5-ml polypropylene pipettor tips are closed on the constricted end with a cotton-wool plug and filled with 2 g of silica gel 60 (0.06—0.2 mm). The toluene solutions of the Dns derivatives are applied to these silica-gel columns and the columns are first washed twice with 3 ml of toluene, and then with one 5-ml portion of toluene—triethylamine (10 : 1). The eluates are discarded. The Dns-di- and polyamines are eluted together with other Dns-amine derivatives with 4 ml of ethyl acetate. Dns-amino acid derivatives are not eluted under these conditions. The ethyl acetate solutions are evaporated to dryness. The residues are dissolved in 3 volumes of methanol and then 1 volume of water is added. Dns-amine derivatives of liver extracts equivalent to 100 mg of tissue are dissolved in 0.6 ml of methanol—water (3 : 1), cell extracts in appropriately smaller volumes.

HPLC separation

After equilibration of the column at a rate of 60 ml/h with methanol—water (57.5:42.5) 10—50-μl aliquots of the Dns derivative solutions are applied to the Hibar pre-packed column via the stopped-flow injection system. Gradient elution is started with the same methanol concentration, which is then increased linearly by an increment of 0.5% per minute. From 20 to 30 min the methanol increment is 1.5% per minute and it is further increased to 3% per minute after 30 min. Elution with pure methanol is continued for 4 min to elute impurities (see Fig. 1). At 40 min the gradient is switched to the initial methanol—water mixture. Before the next run the column is equilibrated again with 57.5% methanol for 10 min.

When only spermidine and spermine are to be determined, a two-step methanol—water gradient can be used, as indicated in Fig. 2. This allows the repetitive separation of tissue samples within 20 min.

RESULTS

Fig. 1 shows the separation of equivalent amounts of mono-, bis-, tri- and tetra-Dns derivatives of amines under conditions described in the Methods section and in the legend to this figure. Clearly the retention time increased with the number of Dns residues. Only β-phenylethylamine eluted together with the group of bis-Dns derivatives, owing to the unsubstituted benzene ring. Within the groups of mono-, bis-, tri- and tetra-substituted amines, the length of the hydrocarbon chain dictated the retention time — as is to be expected for reversed-phase elution. This elution pattern is favorable for the analysis of di- and polyamines, since ammonia, which occurs at high concentration in tissues, and Dns-dimethylamine, the most abundant side-product of the dansylation reaction [25], were eluted well before the bis-Dns derivatives of the aliphatic diamines.

Another aspect of the method is also visible in Fig. 1: as pointed out pre-

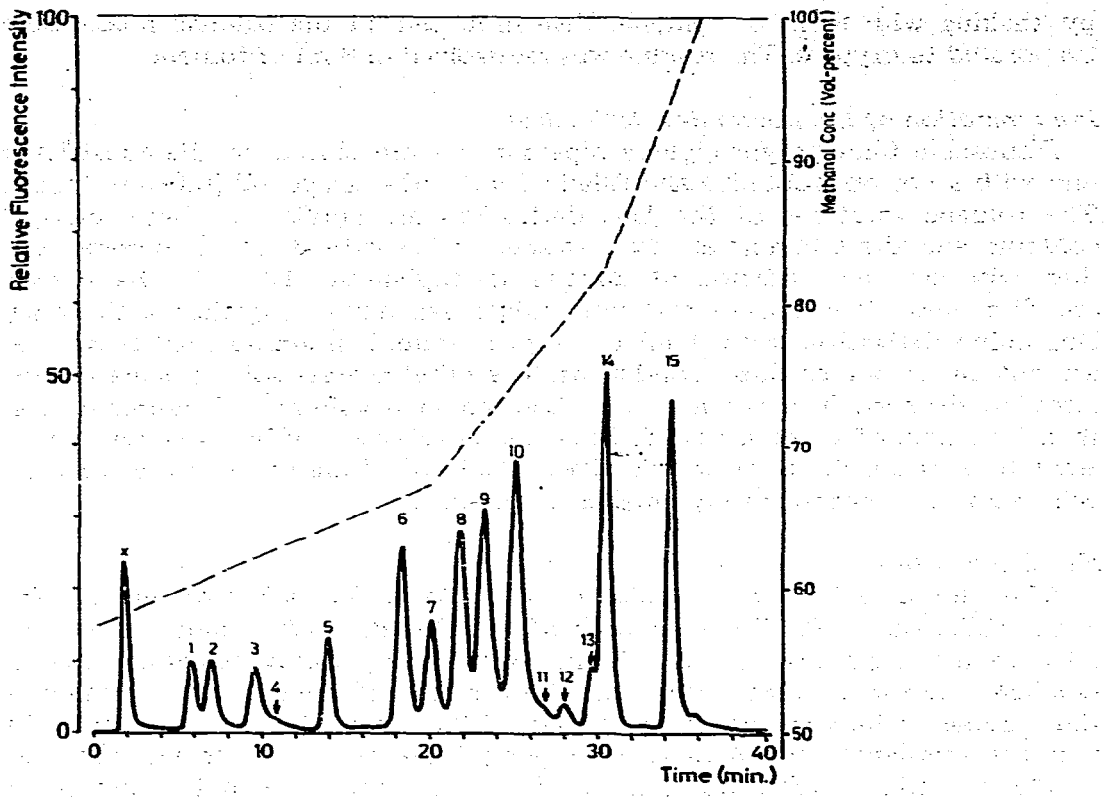

Fig. 1. Separation of Dns-amine derivatives by reversed-phase HPLC. Column: Hibar prepacked column (250 × 3 mm) Lichrosorb RP-8 (7 μm). Chromatograph: Varian 8500. Detector: Perkin-Elmer fluorescence spectrophotometer Model 204, with 8-μl flow cell. Fluorescence activation at 360 nm; fluorescence measurement at 510 nm. Elution: three step water—methanol gradient (60 ml/h). (1) 57.5 to 67.5%, $\Delta_{methanol}$ = 0.5%/min (20 min). (2) 67.5% to 82.5%, $\Delta_{methanol}$ = 1.5%/min (10 min). (3) 82.5% to 100%, $\Delta_{methanol}$ = 3%/min (10 min). Equilibration before the next run for 10 min with 57.5% methanol. X = 5-Dimethylaminonaphthalene-1-sulfonic acid (Dns-OH); 1 = Dns-ammonia; 2 = N-Dns-ethanolamine; 3 = Dns-methylamine; 4 = Dns-2-oxopyrrolidine (reaction product of 4-aminobutyric acid); 5 = Dns-dimethylamine; 6 = bis-Dns-agmatine; 7 = Dns-2-phenylethylamine; 8 = bis-Dns-1,4-diaminobutane (putrescine); 9 = bis-Dns-1,5-diaminopentane (cadaverine); 10 = bis-Dns-1,6-diaminohexane; 11 = bis-Dns-histamine; 12 = O,N-bis-Dns-5-hydroxytryptamine (serotonin); 13 = O,N-bis-Dns-2-(4-hydroxyphenyl)ethylamine (*p*-tyramine); 14 = tris-Dns-spermidine; 15 = tetrakis-Dns-spermine. The derivatives were applied in equimolar amounts (1 nmol) dissolved in 20 μl of methanol—water (3/1, v/v).

viously [25, 33], fluorescence intensity of the Dns derivatives increases with the number of fluorophores attached to the molecule. However, this does not apply to the aryl ethylamine derivatives: even though the dansyl fluorophores are separated by the ethyl side-chain of histamine, serotonin, tyramine, etc., the fluorescence quantum yield of these amines is much lower than that of the corresponding N-mono-Dns derivatives, presumably owing to an intramolecular charge-transfer interaction of the fluorophores. Since the concentrations of

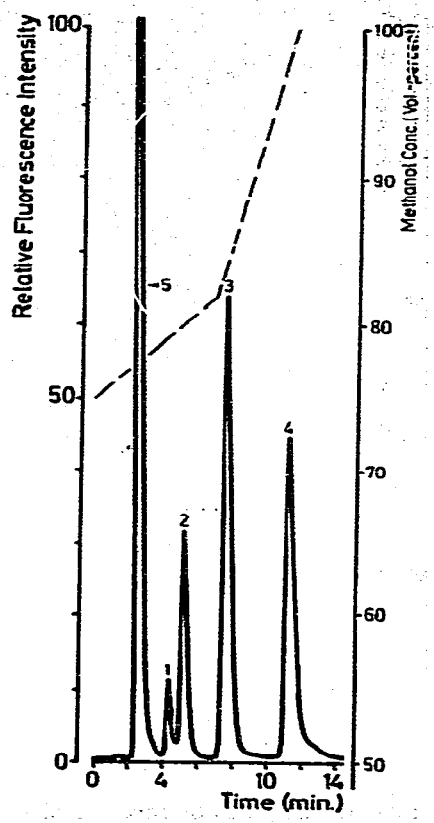

Fig. 2. Separation of the Dns-amine derivatives of a normal mouse liver using an elution program suited for the rapid determination of spermidine and spermine. The sample was identical with that shown in Fig. 4; however, about 0.3 nmoles of bis-Dns-1,4-diaminobutane and 1 nmole of bis-Dns-1,6-diaminohexane were added. The sensitivity of recording was identical with that shown in Fig. 4, dotted line. Elution program: two step water—methanol gradient (75 to 82%, $\Delta_{methanol}$ = 1%/min; 82 to 100%, $\Delta_{methanol}$ = 4%/min. 1 = Bis-Dns-1,4-diaminobutane (putrescine); 2 = bis-Dns-1,6-diaminohexane (internal standard); 3 = tris-Dns-spermidine; 4 = tetrakis-Dns-spermine; 5 = DNS-ammonia.

these amines are normally low in tissues, they do not interfere with the polyamine determinations.

The effectivity of the standard separation procedure and the influence of the steepness of the water—methanol gradient on the separation and peak form is demonstrated in Fig. 3. The separation of the homologous bis-Dns diamines by the standard procedure was not complete (Fig. 3A). Bis-Dns-1,2-diaminoethane, bis-Dns-1,3-diaminopropane, and bis-Dns-1,4-diaminobutane (putrescine) overlapped. Bis-Dns-1,2-diaminoethane and bis-Dns-1,6-diaminohexane are non-natural compounds. They can be used as internal standards. The former compound was suggested for this purpose by Newton et al. [32]. Since bis-Dns-1,6-diaminohexane elutes between bis-Dns-cadaverine and tris-Dns-spermidine, we prefer this compound as standard. (Another reason is that

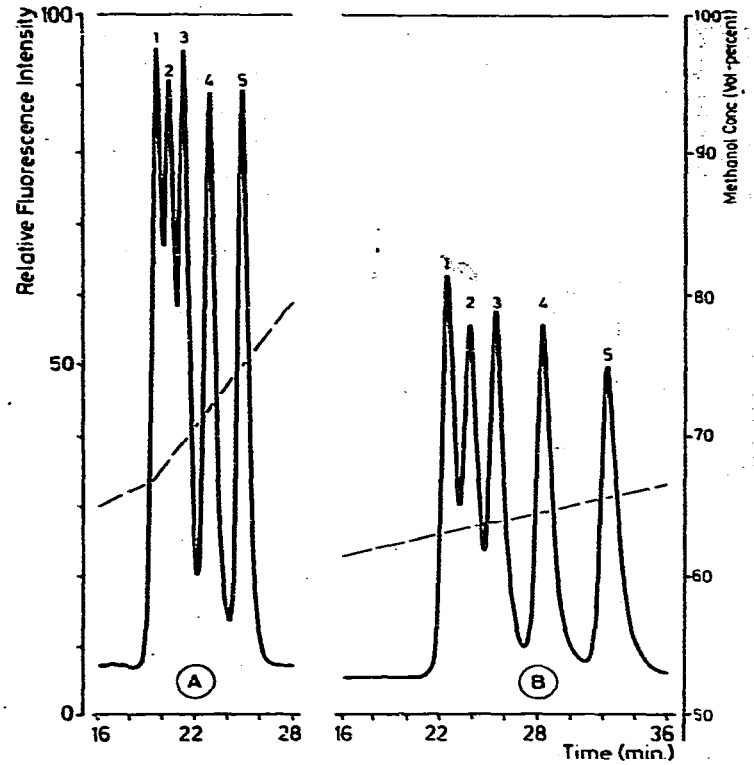

Fig. 3. Separation of the bis-Dns derivatives of the homologous diamines by reversed-phase HPLC. Column, chromatograph and detector as described in the legend to Fig. 1. Elution: water—methanol gradients; 60 ml/h. Two-step gradient (57.5 to 67.5 $\Delta_{methanol}$ = 0.5%/min (20 min); and 67.5% to 82.5%, $\Delta_{methanol}$ = 1.5%/min (10 min). (B) Linear gradient (57.5 to 67.5%, $\Delta_{methanol}$ = 0.25%/min (40 min). 1 = Bis-Dns-1,2-diaminoethane; 2 = bis-Dns-1,3-diaminopropane; 3 = bis-Dns-1,4-diaminobutane (putrescine); 4 = bis-Dns-1,5-diaminopentane (cadaverine); 5 = bis-Dns-1,6-diaminohexane. The amine derivatives were applied in equimolar amounts (1 nmol) dissolved in 20 µl of methanol—water (3:1, v/v). Detector sensitivity is identical in (A) and (B).

the same compound proved to be useful as standard for the mass spectrometric determination of putrescine and cadaverine [34].) Although the peaks of bis-Dns-putrescine and bis-Dns-1,3-diaminopropane overlap, their separation is nevertheless adequate for most purposes. By using a flat gradient, the separation of these overlapping compounds can be somewhat improved at the expense of sensitivity (Fig. 3B).

The method was applied for the determination of putrescine, spermidine and spermine in the livers of normal and 1,4-diamino-2-butyne-treated mice, and in chick embryo fibroblasts treated with the same putrescine analog. (A description of the results of this work will be given elsewhere.) The separation of tissue samples were recorded at two different sensitivities, in order to cover the enormous concentration difference between putrescine and the polyamines spermidine and spermine. As can be seen in Fig. 4, a peak is barely

visible beside the peaks of Dns-ammonia and the Dns derivatives of the polyamines, when recording is performed at reduced (1/20) sensitivity. This shows impressively the quantitative significance of the polyamines in tissues. At a detector sensitivity sufficient to measure less than 20 pmoles of the bis-Dns-diamines, there is still little background noise. It also appears from this figure that 1,3-diaminopropane and 1,5-diaminopentane (cadaverine) are present in this tissue, if at all, only in very low concentrations. Pre-treatment of the animals with 1,4-diamino-2-butyne (100 mg/kg, intraperitoneally) elevated putrescine concentration in liver considerably, probably owing to its inhibitory effect on diamine oxidase [35] (Fig. 5). Eight hours after its administration the compound was still detectable in the liver in amounts of 20 nmoles/g. Putrescine and polyamine concentrations of normal and transformed chick embryo fibroblasts were comparable (Fig. 6). Cadaverine was not detected in these cells.

Peaks were evaluated by measurement of peak height and peak width at

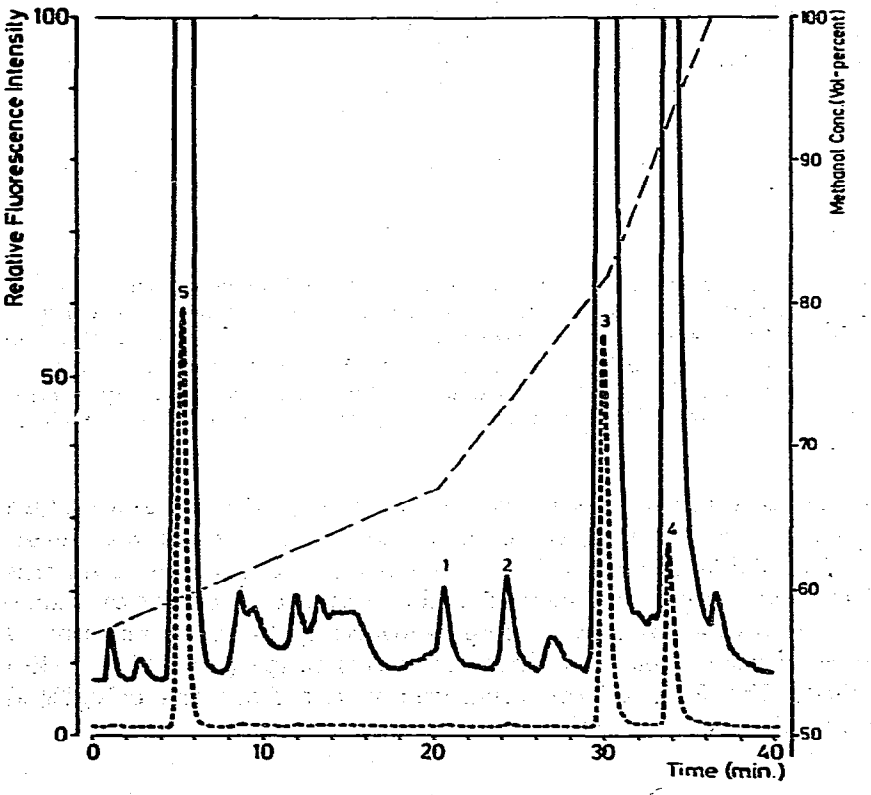

Fig. 4. Separation of the Dns-amine derivatives of a normal mouse liver in the system described in the legend to Fig. 1. 1 = Bis-Dns-1,4-diaminobutane (putrescine); 2 = bis-Dns-1,6-diaminohexane (internal standard, 16.6 pmoles); 3 = tris-Dns-spermidine; 4 = tetrakis-Dns-spermine; 5 = Dns-ammonia. The separated sample corresponded with 2.7 mg of liver. Dotted line: recording at reduced (1/20) sensitivity.

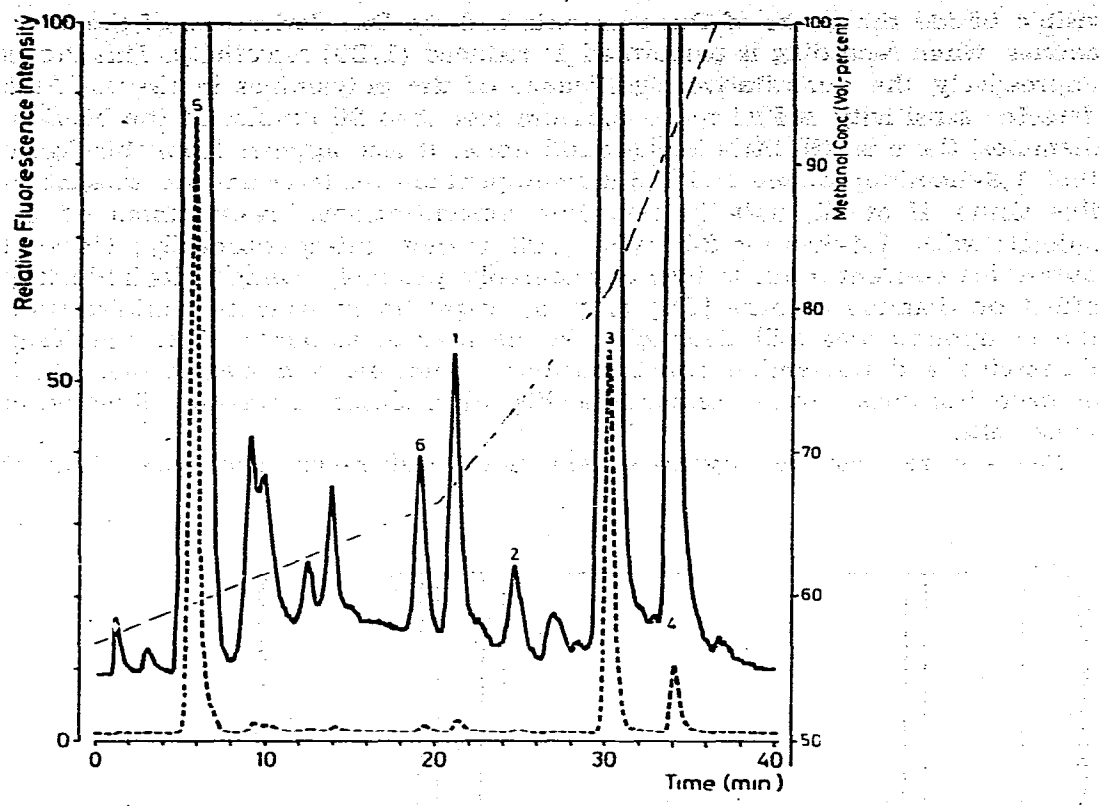

Fig. 5. Separation of the Dns-amine derivatives of mouse liver, 8 h after treatment of the animal with 1,4-diamino-2-butyne at 100 mg/kg. Separation conditions are identical with those in Figs. 1 and 3. Peaks: 1 = bis-Dns-1,4-diaminobutane (putrescine); 2 = bis-Dns-1,6-diaminohexane (internal standard, 16.6 pmoles); 3 = tris-Dns-spermidine; 4 = tetrakis-Dns-spermine; 5 = Dns-ammonia; 6 = bis-Dns-1,4-diamino-2-butyne. The separated sample corresponded with 2.7 mg of liver. Dotted line: recording at reduced (1/20) sensitivity.

half height. Although this method is of limited accuracy, the mean standard deviation (S.D.) of measurements repeated on three different days was nevertheless less than ±7%. Table I summarizes these results. It appears from this table that the S.D. was about the same for the whole concentration range from 20 pmoles to 2 nmoles of amine. The relationship between amount of substance and recorded peak areas was linear over this range (linear regression coefficient > 0.988). The S.D. of the measured values from the calculated regression curve was within ±2% for all amines.

DISCUSSION

Estimation of polyamines as their Dns deviations in tissues and body fluids by thin-layer chromatography has proved its usefulness as a sensitive and reliable method [26, 28, 29]. Using automated application of sample and fluorescence scanning in situ, especially of HPTLC plates [30], makes the

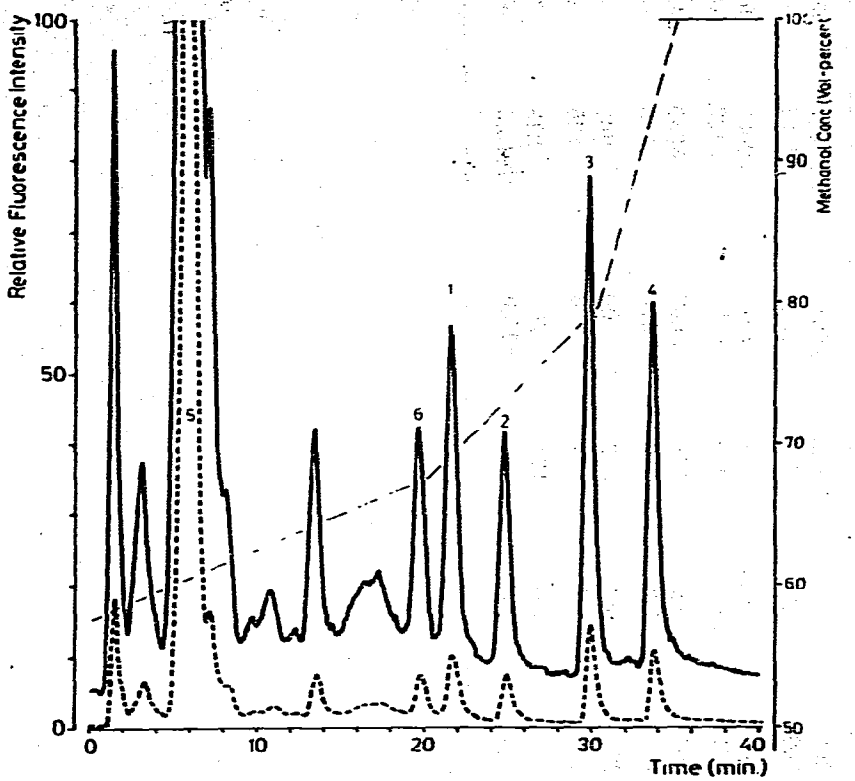

Fig. 6. Separation of the Dns-amine derivatives of chick embryo fibroblasts transformed with Rous sarcoma virus (Schmidt—Ruppin) and treated with 0.1 m*M* 1,4-diamino-2-butyne for 24 h. The separated sample corresponded with 0.044 mg of cell protein. Separation conditions were identical with those described in the legend to Fig. 1. Peaks: 1 = bis-Dns-1,4-diaminobutane (putrescine); 2 = bis-Dns-1,6-diaminohexane (internal standard, 125 pmoles); 3 = tris-Dns-spermidine; 4 = tetrakis-Dns-spermine; 5 = Dns-ammonia; 6 = bis-Dns-1,4-diamino-2-butyne. Dotted line: recording at reduced (1/5) sensitivity.

method very rapid. Many samples can be handled in parallel. From this point of view, sequential separation methods, such as HPLC, are of comparable usefulness only if their superior separatory quality is decisive or if they are automated. For various reasons the method described in this paper is a favorable alternative to TLC. (1) Adequate separations of the homologous diamines and polyamines are achieved within a short time. TLC of these amines is not adequate in a one-dimensional separation. For complete separation a two-dimensional separation is required [25, 36]. (2) The reversed-phase column allows rapid regeneration. Repeated separations are possible within a short time. This is an advantage over the otherwise highly effective ion-exchange column separations with advanced technology [15—17]. (3) The Dns derivatives can be collected from the column outflow and characterized by mass spectrometry and by other suitable methods. (4) Since radioactivity measurements can be achieved on collected fractions, HPLC separations of Dns-amines

TABLE I

REPRODUCIBILITY OF HPLC DETERMINATIONS OF Dns-AMINE DERIVATIVES

Mean values ± S.D. of three determinations; relative fluorescence unit = peak height × peak width at half height of the recorded curves.

Amount (nmoles)	Ammonia	Ethanol-amine	Methyl-amine	Dimethyl-amine	Agmatine	β-Phenyl-ethylamine	Putrescine	Cadaverine	Hexamethylene-diamine	Spermidine	Spermine
2.0	4025 ±385	4617 ±161	5125 ±156	5300 ±229	13125 ±675	6800 ±606	16617 ±176	15625 ±715	15818 ±588	20625 ±1231	20017 ±825
1.0	2321 ±203	2522 ±376	2725 ±220	2500 ±173	6138 ±376	3100 ±241	8104 ±188	7659 ±420	7675 ±435	10358 ±429	9367 ±555
0.5	1099 ±69	1223 ±48	1465 ±131	1297 ±91	3430 ±93	1653 ±134	4227 ±162	4002 ±238	3667 ±510	5360 ±688	4880 ±450
0.075	188 ±15	204 ±32	236 ±10	200 ±11	490 ±14	269 ±10	605 ±16	638 ±24	600 ±48	868 ±25	731 ±61
0.02	50 ±4.6	60 ±6.5	71 ±9.6	57 ±3.0	134 ±6.1	75 ±4.9	182 ±14.9	171 ±5.1	201 ±13.0	228 ±13.3	211 ±4.6

may prove useful in metabolic studies and for quantitation using double-isotope methods [6]. Although the equipment used in the present work was not automated, the separation method is suitable for full automation. The ease with which the separations can be adapted to special requirements may be a further aspect in favor of reversed-phase HPLC as a routine method.

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