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THIN-LAYER AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF P388/S TUMOR CELL AND HOST LIVER POLYAMINES

T. KREMMER*, L. HOLCZINGER and M. BOLDIZSÁR
Department of Biochemistry, Research Institute of Oncopathology, National Oncological Institute, H-1122 Budapest (Hungary)
L. SELMECI
Institute of Pathophysiology, Semmelweis University Medical School, H-1445 Budapest (Hungary)
and
S. BARDÓCZ
Department of Biochemistry, University Medical School, H-4012 Debrecen (Hungary)

SUMMARY

Thin-layer and high-performance liquid chromatographic (HPLC) methods using adsorption and reversed-phase techniques are compared for separation and quantitation of P388/S leukemia cell and host liver polyamines. An improved HPLC method on a silica gel column (MicroSil, LiChrosorb Si 100) using chloroform–methanol–water as mobile phase is described and some chromatographic parameters have been studied. A reliable and reproducible resolution and determination of Dns-polyamines has been achieved on a reversed-phase (LiChrosorb RP-8) columns by means of a programmed linear methanol–water gradient with detection at 254 nm. This method is suitable for the serial analysis of polyamines in P388/S leukemia cells, in host liver and body fluids.

INTRODUCTION

The biological importance of polyamines in cell proliferation and differentiation with respect to malignancy has been demonstrated both in experimental cancer studies and in human pathology[1–4]. The polyamine patterns of different cells and body fluids have been determined by several methods including thin-layer (TLC) and high-performance liquid chromatography (HPLC)[5–14] techniques which were recently reviewed by Seiler[15].

In the present work some conventional methods based on TLC coupled with spectrofluorimetry and HPLC using adsorption or reversed-phase techniques are compared and adapted for the separation and quantitation of Dns-polyamines, in order to determine the concentrations of putrescine, spermidine and spermine in P388/S leukemia cells and in the liver of tumor-bearing animals.

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EXPERIMENTAL

Chemicals

Laboratory chemicals were of analytical grade and purchased from commercial sources: 5-dimethylamino-1-naphthalenesulphonyl chloride (Dns chloride) (Calbiochem, Pierce); putrescine (free base), spermidine trihydrochloride, spermine tetrahydrochloride, 1,6-diaminohexane (DAH) (Calbiochem); 1,3-diaminopropane (DAP) (Koch-Light); Dowex 50-X8, 200–400 mesh (Serva). All solvents including water were purified and redistilled twice according to the HPLC standards. Chloroform was dried over molecular sieve 5A and methanol was distilled from active charcoal (Norit A, Serva). The water content of the eluents was determined by the Karl-Fischer method.

Thin-layer chromatography

For the TLC separation of Dns-polyamines, 20 × 20 cm silica gel 60 plates (pre-coated plastic or aluminium sheets, Merck) were used throughout and developed by ascending chromatography in an ethyl acetate–cyclohexane (2:3, v/v) solvent system. Dns derivatives were detected under UV light and identified by use of the reference compounds.

High-performance liquid chromatography

HPLC was accomplished using a Hewlett-Packard 1084B liquid chromatograph equipped with a Model 79875A variable-wavelength detector and a 79850 LC Terminal. The flow-rate was 1 ml/min throughout, except for experiments on the relationship of linear flow velocity to plate height.

Dansylated polyamines were separated by adsorption on Microsil (SY-LAB, Micromeritics; 30 × 0.5 cm, 7.5 μm) or LiChrosorb Si 100 (Merck, 20 × 0.46 cm; 5 μm) pre-packed columns using isocratic chloroform–methanol–water ternary mobile phase with varying methanol (0.3–2%, v/v) and water (300–1500 ppm) contents. Reversed-phase separations were performed on a prepacked LiChrosorb RP-8 column (Merck; 20 × 0.46 cm, 7 μm) with a linear 70% methanol–water to methanol gradient programmed at a rate of 3% methanol per min.

In the experiments on the relationship of the linear flow velocity to the plate height, isocratic conditions were used with 85% (v/v) methanol in water as mobile phase. In all HPLC determinations, detection was carried out at 254 nm. Calibration was performed in arbitrary area units with the reference polyamines as well as with 1,3-diaminopropane (for SiO_2) and 1,6-diaminohexane (for RP-8) as internal standards.

Chromatographic characteristics, capacity factor, selectivity, plate number and height of columns, peak resolution, were calculated in the conventional way[16].

Materials

P388/S leukemia cells were grown either *in vivo* in the peritoneal cavity of BDF_1 inbred male mice, or *in vitro* in Fischer's medium at 37°C in a 7% CO_2 atmosphere, and were harvested in the logarithmic phase of tumor cell proliferation. Tumor cells, usually $30 \cdot 10^6$–$50 \cdot 10^6$ cells per ml of ascites fluid or $1.5 \cdot 10^5$–$3 \cdot 10^5$ cells per ml of culture medium were counted, centrifuged and homogenized by son-

ication on a MSE Ultrasonic Disintegrator P6100, at maximum output (for 1 min) in phosphate-buffered salt solution (PBS), pH 7.4, at 4°C prior to the extraction of polyamines.

Livers of the tumor-bearing animals were prepared and homogenized by sonication as above, using 1 g of wet tissue in 10 ml of PBS. The protein content of homogenates was determined according to Hartree[17].

Methods

Perchloric acid extraction and pre-separation of polyamines on a Dowex 50-X8 column was performed by the methods of Inoue and Mizutani[5] and Seiler and Knödgen[6,11].

Dansylation of the polyamine solutions in 1% NaHCO was carried out according to the procedure previously described[7,8,10,11] using saturated Na_2CO_3 and Dns chloride (15 mg/ml) in acetone, except that instead of the overnight reaction an incubation at 54°C for 60 min was used[12]. To eliminate its high absorbancy at 254 nm, acetone was evaporated from the reaction mixtures, and Dns derivatives were extracted with ethyl acetate or toluene and dissolved in methanol for HPLC.

RESULTS AND DISCUSSION

Very few data are available[18,19] on the polyamine pattern of leukemia cells (L1210, P388/S) used frequently in drug screening and experimental chemotherapy. In our studies concerning the metabolism of polyamines it was necessary to determine the concentrations of spermidine, spermine and putrescine in tumor cells as well as in the different compartments, liver, ascites plasma, serum, etc., of tumor-bearing animals. In order to compare some conventional methods, polyamines of P388/S tumor cell and host liver homogenates were extracted with perchloric acid, pre-separated on a Dowex 50-X8 column and dansylated in parallel with the reference compounds, *e.g.*, putrescine, spermidine, spermine, 1,3-diaminopropane and 1,6-diaminohexane.

For the separation and quantitation of Dns-polyamines a TLC technique was applied first, in which the fluorescent spots were removed by cutting the plate into zones, eluted and measured by spectrofluorimetry[5,6]. The results obtained by this method are summarized in Table I. Apart from the characteristic distribution of the prominent polyamines, spermidine, spermine and putrescine, in the different tissues, cells and body fluids[1,3], our figures are comparable with the data reported by others[18–21] for liver and various tumor cells.

Considering the advantages of HPLC, *i.e.*, high resolution and versatility, several attempts were made to separate Dns-polyamines on silica gel particles, *e.g.*, Micropak Si-10, Corasil II and μPorasil, using simple, isocratic mobile phases such as chloroform–triethylamine (50:1)[9], chloroform–dioxane–triethylamine (100:10:1)[10] or chloroform containing 2% acetone[14]. As shown in Fig. 1, an equally good resolution was obtained for all of the Dns-polyamine pairs on a LiChrosorb Si 100 column (20 × 0.46 cm, 5 μm) by means of a ternary solvent system of chloroform–methanol (99.3:0.7, v/v) containing 1000 ppm water. It seems that the exceptionally good resolutions can be accounted for in terms of the successive increases in the capacity factors, k', of Dns derivatives.

TABLE I

COMPARISON OF TLC AND HPLC METHODS

Mean ± S.D. given in nmol per 10^6 P388/S leukemia cells, and in nmol per mg of liver protein.

Samples	*Method*	*Putrescine*	*Spermidine*	*Spermine*
P388/S Leukemia cells *in vivo* (n = 5)	TLC	0.217 ± 0.113	3.864 ± 1.088	2.036 ± 1.101
	HPLC	0.293 ± 0.107	5.225 ± 1.876	2.755 ± 0.542
P388/S Leukemia cells from tissue culture (n = 7)	TLC	0.119 ± 0.046	1.175 ± 0.121	0.486 ± 0.068
	HPLC	0.116 ± 0.067	1.892 ± 0.291	1.047 ± 0.142
Liver of P388/S tumor-bearing mice (n = 5)	TLC	0.409 ± 0.161	3.619 ± 0.813	2.048 ± 0.727
	HPLC	0.411 ± 0.199	4.588 ± 0.187	2.797 ± 0.358

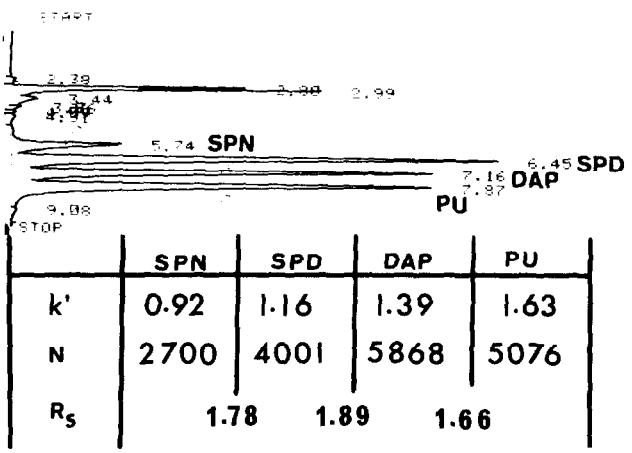

Fig. 1. HPLC separation of Dns derivatives of spermine (SPN), spermidine (SPD), 1,3-diaminopropane (DAP) and putrescine (PU) on a LiChrosorb Si 100 column (20 × 0.46 cm, 5 μm). Mobile phase: chloroform–methanol (99.3:0.7, v/v) containing 1000 ppm of water. Flow-rate: 1 ml/min. Detection: 254 nm.

In a further study with Dns 1,3-diaminopropane as a model compound it was found that the k' values gradually decrease with increasing methanol or water concentrations, and are more sensitive to water at low concentrations of methanol (Fig. 2). This effect is less pronounced at relatively high methanol concentrations (over 0.6%), but by increasing the amount of methanol in chloroform over 1% it limits the resolution because of the shortening of retention times. Obviously, there is an optimum range of the methanol concentration, 0.6–0.9% (v/v), for the best resolution, and the water content of chloroform (and of methanol!) proved to be a critical parameter.

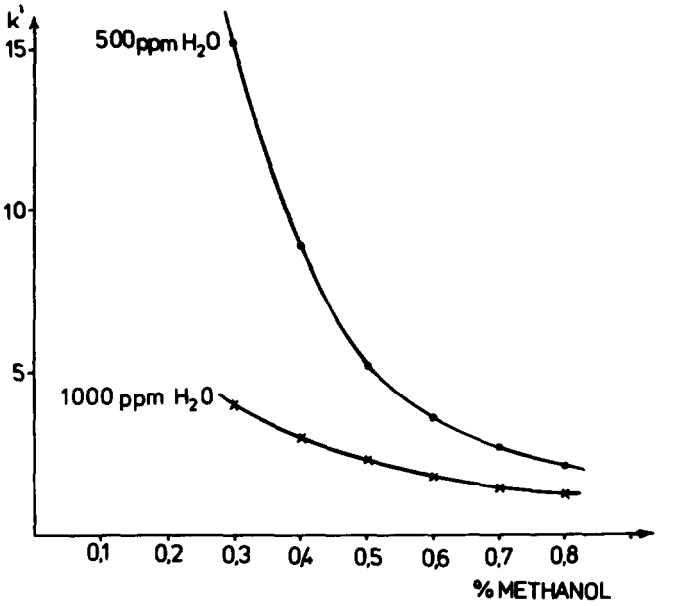

Fig. 2. Effect of methanol and water content of chloroform on the capacity factor, k', of Dns-1,3-diaminopropane on a LiChrosorb Si 100 column. Chromatographic conditions as in Fig. 1.

It has been observed earlier[9,10,14,22] and is also demonstrated in this work that the sorption properties of different silicas are highly sensitive to the polar components of the mobile phase. The influence of moderators, *e.g.*, water and alcohols, on the adsorption parameters of silicas with a relatively non-polar eluent like chloroform has been reviewed by Engelhardt[23] and demonstrated quite recently by Szepesy *et al.*[16]. It is now considered that it is difficult to optimize the water content of the stationary and mobile phases in a reproducible way, and that a narrow range of critical values is usually found.

In conclusion, adsorption chromatography of Dns-polyamines on silica gel proved not to be suitable for the serial determinations. Apart from the time-consuming equilibration of silica gel, a poor reproducibility of the resolution caused by the uncontrollable water consumption of solvents was found. The instability of the mobile phase is also a problem, bearing in mind that halogenated hydrocarbons are not welcome in HPLC equipment and that as a result of their degradation, highly polar, corrosive compounds are formed. In addition, irreversible changes (inactivation) in the sorption properties of silica gel were observed which limited considerably the lifetime of expensive columns.

HPLC techniques have been reported for the separation of apolar dansyl polyamines on reversed-phase columns by means of different gradient systems and fluorimetric detection[7,10,12,15]. By use of the method of Seiler *et al.*[7], we have obtained a sufficiently good and reproducible separation of the dansylated reference compounds putrescine, spermidine, spermine and 1,6-diaminohexane using a programmed, relatively simple, linear methanol-in-water gradient and UV detection at 254 nm. The relationship between the linear flow velocity and the plate height of the

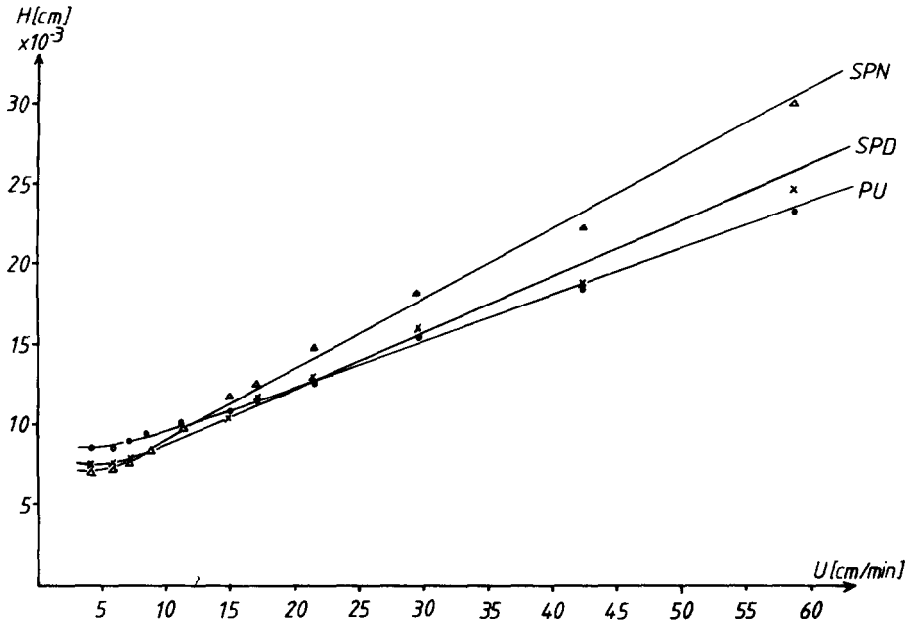

Fig. 3. Relationship of the linear flow velocity, U, to the plate height, H, of a LiChrosorb RP-8 column (20 × 0.46 cm, 7 μm) using 85% (v/v) methanol in water as mobile phase. Abbreviations as in Fig. 1.

LiChrosorb RP-8 column (Fig. 3) suggested an optimum flow-rate of *ca.* 1 ml/min and this was used throughout the further work. In order to minimize the analysis time we systematically modified the gradient profile as shown in Fig. 4. It could be concluded that the resolution of the critical pair Dns-putrescine/1,6-diaminohexane was increased with decreasing steepness of the methanol gradient and that an incre-

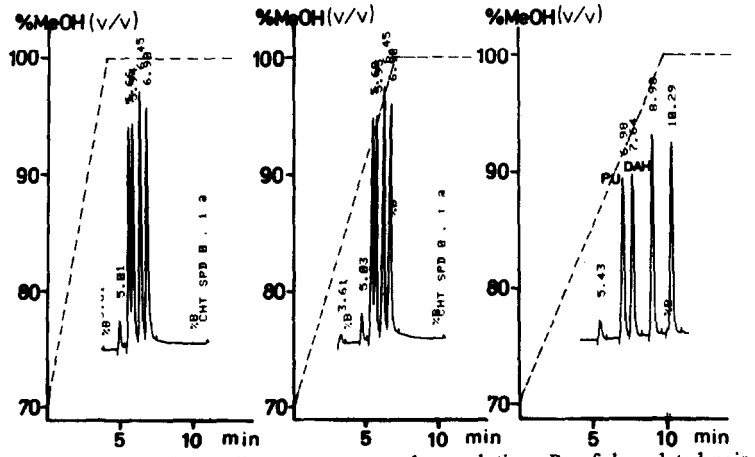

Fig. 4. Effect of the gradient steepness on the resolution, R_s, of dansylated pair 1,6-diaminohexane/putrescine (DAH/PU) on a LiChrosorb RP-8 column (20 × 0.46 cm, 7 μm). Flow-rate: 1 ml/min. Detection: 254 nm. Methanol gradients, left to right: 7.5% (v/v) per min, R_s = 0.707; 4.3% (v/v) per min, R_s = 0.685; 3.0% (v/v) per min, R_s = 1.284.

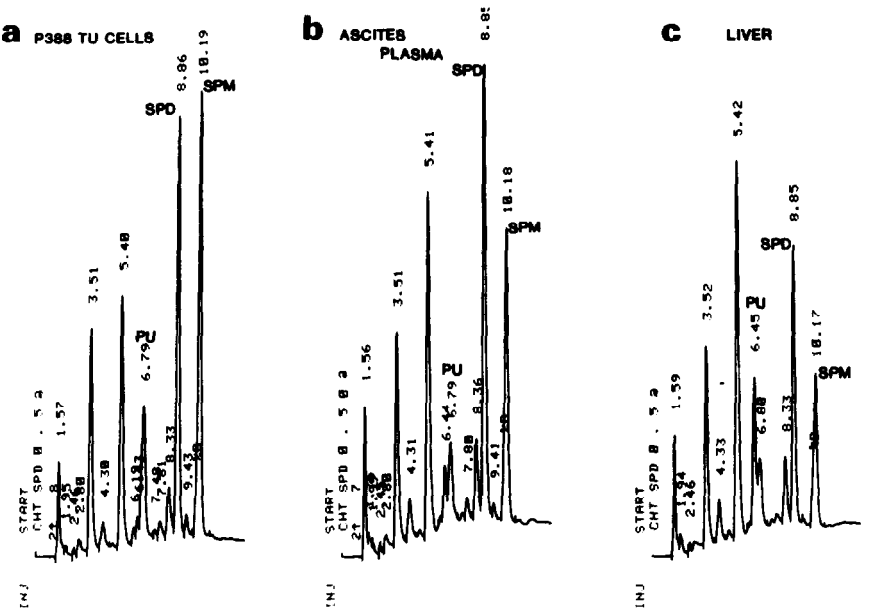

Fig. 5. HPLC separation of the Dns derivatives of putrescine (PU), spermidine (SPD) and spermine (SPN) contained in P388/S leukemia cells grown *in vivo* (a), ascites plasma (b) and liver of tumor-bearing mice (c). Chromatographic conditions as in Fig. 6.

ment of 3% methanol per min (from 70% to 100% in 10 min) was suitable. Good separations were obtained for the Dns-polyamines originating from more complex biological materials such as tumor cells, ascites fluid and liver as shown in Fig. 5.

Apart from the higher sensitivity of fluorimetric detection[6,8,10,11,15], UV absorption at 254 nm has also been reported[9] for determining Dns-polyamine patterns. We obtained linear correlations of area *versus* nmol of Dns-spermidine, -spermine and -putrescine at 254 nm (Fig. 6) in the range 0.1–15 nmol of amine in 10 μl of sample. The reproducibility was on average $\pm 8.4\%$ ($n = 5$). The lower molar ratio of (bis-)Dns groups in the putrescine derivative compared with spermidine and spermine was indicated by its less steep calibration curve.

The results obtained by TLC and HPLC (reversed-phase) determination of the polyamine composition of P388/S leukemia cells and of livers of tumor-bearing animals are compared in Table I. It could be concluded that TLC combined with spectrofluorimetry of dansyl polyamines provided lower figures than HPLC, mainly for spermidine and spermine, presumably due to some sophistication of the TLC technique applied, *e.g.*, removal of spots by cutting the plate into zones, elution, etc. There is no doubt that *in situ* fluorodensitometric scanning of dansyl polyamines separated by TLC[6,8,11] may result in data more comparable with those obtainable by HPLC, but this technique needs special care and equipment. It is notable that the P388/S leukemia cells grown *in vivo* show much (*ca.* two-fold) higher polyamine concentrations than the same tumor cell line proliferating in tissue culture.

In conclusion, by comparison of TLC and HPLC methods for the separation and quantitation of Dns-polyamines it was confirmed that with the appropriate

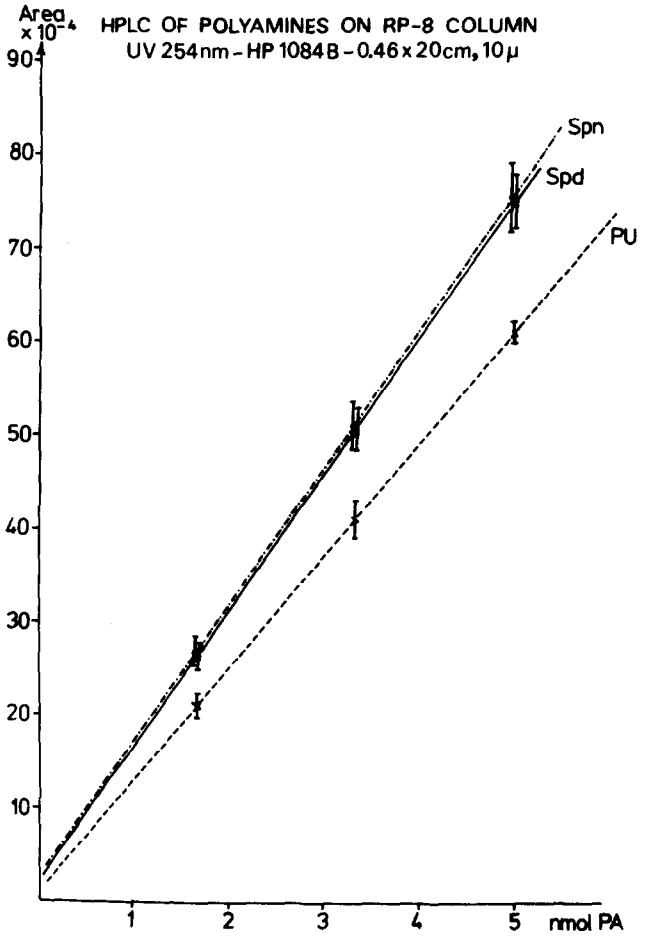

Fig. 6. Calibration curves for the quantitation of Dns-polyamines, putrescine (PU) spermidine (SPD) and spermine (SPN) separated by HPLC on a LiChrosorb RP-8 column with a linear gradient of 70% methanol–water to methanol (3% methanol per min). Flow-rate: 1 ml/min. Detection: 254 nm.

equipment and the advantages of automation and the gradient-programmed mobile phase, an improved and relatively simple version of the HPLC technique of Seiler *et al.*[7] on a reversed phase (RP-8) is the most reliable for determination of the prominent polyamines in tumor cells, liver and other biological samples.

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