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CHROMOPHORIC DETERMINATION OF PUTRESCINE, SPERMIDINE AND SPERMINE WITH DABSYL CHLORIDE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A fast and sensitive method for the determination of putrescine, spermidine, spermine and ammonia by high-performance liquid chromatography (HPLC) with dabsyl chloride is described. These compounds are converted to their chromophoric dabsyl derivatives and are separated by a normal-phase chromatographic column (μPorasil, 10 μm) with 2% acetone in chloroform as isocratic mobile phase. The sensitivity of the method is 20 pmoles. The present method was shown to be a straightforward procedure for estimating polyamines in various rat tissues.

The chromophoric derivatives of polyamines are also well separated by thin-layer chromatography (TLC) on silica gel, and the combination of the HPLC and TLC procedures provides a reliable method for qualitative and quantitative analysis of polyamines.

INTRODUCTION

Putrescine, spermidine and spermine are ubiquitous in living organisms. The amounts of these polyamines vary with cell type and the physiological state of the cells [1, 2]. The possible role of polyamines in cell metabolism at the replicational, transcriptional and translational levels has attracted much interest recently. Furthermore, it has been demonstrated that polyamine concentrations are usually increased in rapidly growing tissues [3—5] including cancerous tissues [6, 7]. Considerable effort has been made in the elucidation of the role of polyamines in neoplastic growth and proliferative diseases.

Widespread interest in the polyamines has led to the development of a number of methods for their rapid and sensitive assay. Radioimmunological methods [8] for polyamine determinations in clinical screening programs seem to be most promising, but the cross-reaction among their antibodies renders this method less clinically useful [9]. Ion-exchange column chromatography with

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automated instruments [10, 11] is a useful method for routine assay of polyamines in urine and body fluids. Reaction of polyamines with dansyl chloride has provided the basis for thin-layer chromatography (TLC) [12] and high-performance liquid chromatography (HPLC) [13, 14]. Tosyl chloride [15] and benzoyl chloride [16] were also used in the HPLC determination of polyamines. Gas chromatographic detection of the trifluoroacetyl and N-isobutyloxycarbonyl derivatives of polyamines have also been reported [17, 18]. The amino groups of polyamines are used for derivative formation and the specificity of the chemical methods is therefore exclusively based on the separation efficiency.

During recent years our laboratory has made several attempts to develop a routine procedure of polyamine determination based on dabsylation [19, 20]. Chromophoric derivatives of aliphatic amines and amino acids were formed by reaction with dabsyl chloride and subjected to HPLC; their separations were adequately rapid and reproducible in successive runs with authentic and tested samples [20, 21]. Good results were also obtained when the procedure was modified and employed in the estimation of polyamines in rat tissues such as liver, intestine, kidney, prostate, ovary, etc. This modified procedure and a routine HPLC system for polyamine determination are described in this paper.

MATERIALS AND METHODS

Apparatus

HPLC was accomplished with a μPorasil (fully porous particle size 10 μm) normal-phase column (30 cm × 3.9 mm I.D.,) a Waters ALC 201 liquid chromatograph equipped with a Model 6000A ~ ·nt delivery system, a Model U6K universal injector, a Model 660 solvent programmer, and a Model 450 variable-wavelength photometer set at 425 nm (all products of Waters Assoc., Milford, MA, U.S.A.). The recorder was an OmniScribe strip-chart recorder, Model B 5000 (Houston Instruments, Austin, TX, U.S.A.).

TLC was performed in a closed cylinder (10 cm high, 6 cm in diameter) with a ground-glass cover. The thin-layer plastic sheets precoated with silica gel 60 (0.25 mm thick) were purchased from E. Merck (Darmstadt, G.F.R.).

Chemicals

Dabsyl chloride (4-dimethylaminoazobenzene-4′-sulfonyl chloride; m.p. 186—188°C) was synthesized by reacting sodium 4-dimethylaminoazobenzene-4′-sulfonate with phosphorus pentachloride [19] or by chlorosulfonation of 4-dimethylaminoazobenzene [22]. Dabsyl chloride was also purchased from Polysciences (Warrington, PA, U.S.A.).

Putrescine 2HCl, spermidine 3HCl, spermine 4HCl and glycine were purchased from Sigma (St. Louis, MO, U.S.A.). Chemicals and solvents were of chemical pure grade (E. Merck).

Procedure

To 1.0 ml of 2% perchloric acid solution containing putrescine, spermidine and spermine (10—250 nmol), 30 mg of sodium carbonate and 1.6 mg of dabsyl chloride in 1.0 ml of acetone were added sequentially. The mixture was

thoroughly mixed and allowed to stand at ambient temperature (25—26°C) for 1 h. Then additional dabsyl chloride (0.8 mg in 0.5 ml of acetone) was added and kept at the same temperature for 30 min to ensure the completeness of dabsylation. The excess dabsyl chloride was consumed by reaction with 5 mg of glycine in 0.5 ml of water for 30 min. The resulting mixture was extracted with 5 ml of benzene and the organic layer was washed with 5 ml of water twice and dehydrated with anhydrous sodium sulfate (0.3—0.5 g). A 5—20-μl aliquot of the clear benzene extract was taken for HPLC and TLC analyses.

Analysis of tissue polyamines

Male or female rats of Sprague—Dawley strain weighing 200—300 g were used. The experimental animals were sacrificed and their organs were removed, weighed and chilled on an ice-bath. A 10% tissue homogenate was made from each organ by homogenizing it with a sufficient quantity of 2% perchloric acid in a Polytron homogenizer. After centrifuging at 800 *g* for 15 min, 2 ml of the supernatant were taken and mixed with 2 ml of acetone. The resulting mixture was centrifuged again at 800 *g* for 15 min; 1-ml aliquots of the clear supernatant were used for the determination of polyamines as described above.

RESULTS

HPLC separation of dabsyl polyamines

Due to their high molar absorptivity in the yellow color region, spectrophotometric monitoring at 425 nm after separation by HPLC was considered a favorable approach to quantitation of dabsyl polyamines.

After equilibration of the μPorasil column at a rate of 1 ml/min with 2% acetone in chloroform, 5—20 μl of the dabsylated polyamine solutions were injected. The HPLC elution was performed isocratically with the same solvent mixture. Fig. 1 represents the separation pattern of dabsyl polyamines. Spermidine and ammonia were inadequately separated in this system. Fortunately, the

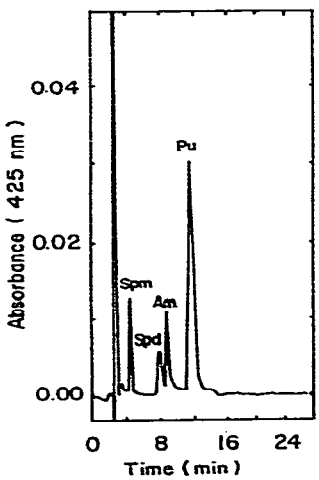

Fig. 1. Separation of dabsyl spermine (Spm), dabsyl spermidine (Spd), dabsyl ammonia (Am) and dabsyl putrescine (Pu). Chromatographic conditions: mobile phase, 2% acetone in chloroform; flow-rate, 1 ml/min; detector, tungsten lamp, 425 nm; a.u.f.s., 0.1.

ammonia content in most tissues did not seriously interfere with the appearance of spermidine.

Effects of mobile phases

In addition to 2% acetone in chloroform, several other solvent systems such as various combinations of ethanol—chloroform or hexane—chloroform were also evaluated for the separation of dabsyl polyamines. Fig. 2 shows the effect of the addition of ethanol to the mobile phase on the retention of dabsyl polyamines and dabsyl ammonia. From the shapes of the curves, regions can be selected where small incremental changes in ethanol concentration have large effects on the capacity factor, k', and therefore on the chromatographic separation. It appeared that the regions at 0.7—0.8% and 1.4—1.6% of ethanol in the mobile phase would provide a clear-cut separation of these amines. The resolution of dabsyl polyamines could be increased gradually when the concentration of *n*-hexane was increased stepwise in the ethanol—chloroform system (Fig. 3).

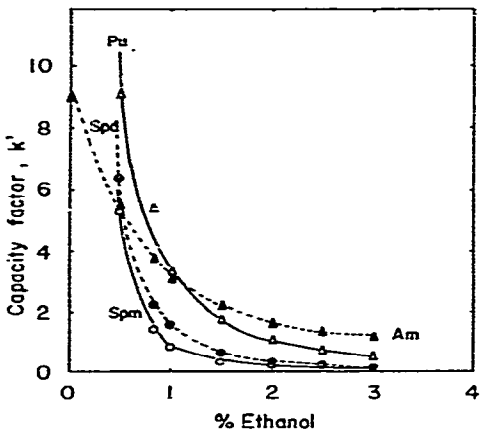

Fig. 2. Capacity factor (k') for dabsyl polyamines vs. ethanol concentration in mobile phase containing chloroform. Am, dabsyl ammonia; Pu, dabsyl putrescine; Spd, dabsyl spermidine; and Spm, dabsyl spermine.

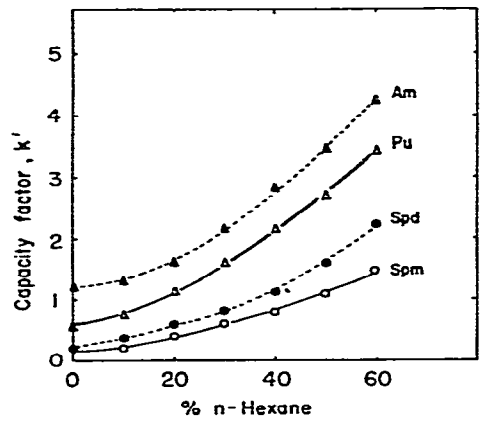

Fig. 3. Capacity factor (k') for dabsyl polyamines vs. hexane concentration in mobile phase containing 3% ethanol in chloroform. Am, dabsyl ammonia; Pu, dabsyl putrescine; Spd, dabsyl spermidine; and Spm, dabsyl spermine.

Linearity and sensitivity

Linear calibration curves passing through the origin were obtained with known amounts of polyamines ranging from 40 to 1000 pmol, corresponding to the range for tissue samples obtained from experimental animals. The equations for these linear curves were found to be: dabsyl putrescine, $y = 0.1946x + 0.078$ ($r = 0.9999$); dabsyl spermidine, $y = 0.3894x + 2.1064$ ($r = 0.9716$); and dabsyl spermine, $y = 0.6607x - 0.565$ ($r = 0.9995$). The minimum detectable concentration of polyamines in tissue extracts was about 2.5 nmol/ml. This produced 2% deflection of the recorder pen when the recorder was set at 0.1 a.u.f.s. The volume of sample injected was 20 μl. The sensitivity of this analysis approaches 1 pmol per sample if the recorder is set at 0.01 a.u.f.s.

Precision

We assessed within-run precision of the assay by processing aliquots of dabsylated polyamine solutions through the procedure during a single day. The coefficients of variation (n = 7) were 4.6% for ammonia at 20 nmol/ml, 4.9% for putrescine at 56 nmol/ml, 3.3% for spermidine at 21 nmol/ml, and 5.2% for spermine at 20 nmol/ml. The results indicate that the precision obtained is adequate. Between-run precision of the assay was evaluated over a two-month period by assaying the pooled dabsylated polyamine solutions. The coefficients of variation (n = 16) were 3.2% for ammonia at 25 nmol/ml, 5.4% for putrescine at 58 nmol/ml, 6.1% for spermidine at 23 nmol/ml, and 6.9% for spermine at 22 nmol/ml. The data obtained by two different personnel performing the assay during this period indicate acceptable precision.

Analysis of tissue samples

The contents of polyamines in the tissues of rats were estimated by the assay procedure described. Fig. 4 depicts typical profiles of polyamines in liver and intestinal tissues.

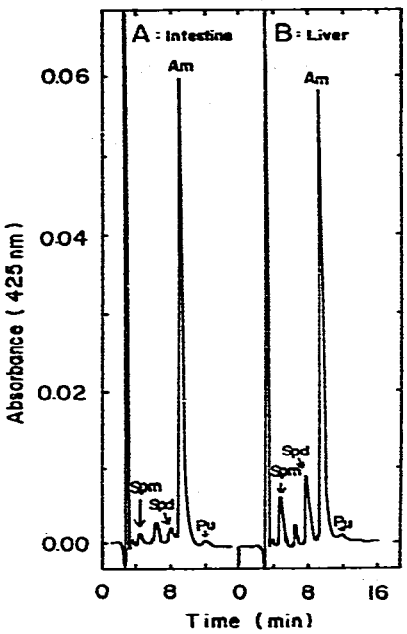

Fig. 4. HPLC analyses of dabsyl polyamines extracted from the intestine (A) and liver (B) of rats. Abbreviations and chromatographic conditions as in Fig. 1.

The percentage analytical recovery of polyamines (50 μg each) added to different tissue extracts (1 ml) were determined and are given in Table I.

The application of the present procedure in assaying the contents of polyamines in rat tissues, including brain, trachea, heart, lung, liver, stomach, small intestine, spleen, kidney, adrenal, large intestine, bladder, prostate, uterus, testis, ovary and leg muscle, was found to be straightforward and satisfactory. With this method, we have run several types of liver samples including male,

TABLE I

RECOVERY OF POLYAMINE SPIKED IN DIFFERENT TISSUE EXTRACTS

Recovery analysis: 50 μg of polyamine were added to 1 ml of tissue extract in 2% perchloric acid and was dabsylated as described in the text. The percentage recovery was calculated as [(amount found)/(amount added)] × 100, and expressed as mean ± S.D. (n = 6).

Tissue extract	Recovery (%)		
	Putrescine	Spermidine	Spermine
Prostate	88.2 ± 5.2	93.1 ± 4.4	86.3 ± 5.9
Liver	91.5 ± 9.2	87.2 ± 5.7	84.2 ± 6.7
Kidney	93.2 ± 4.6	91.4 ± 6.9	88.4 ± 7.3
Small intestine	89.1 ± 5.9	86.3 ± 9.5	84.1 ± 7.6
Ovary	92.6 ± 7.8	90.1 ± 7.3	91.2 ± 9.2

female, castrated male and ovariectomized female rats; the ratios of spermidine or spermine to putrescine vary depending on the sex and hormonal status of the experimental animals (Table II). We have also studied the effects of estrogen and androgen on the formation of polyamines in several tissues of rats and the results will be published elsewhere. Polyamine concentrations are usually increased in rapidly growing tissues [3, 4, 6, 7]; it is suggested that the applicability of this method in the determination of polyamines in various malignant tissues of cancer patients should be explored.

TABLE II

POLYAMINES IN RAT LIVER

The liver extract (1 ml) in 2% perchloric acid was dabsylated and analyzed by HPLC as described in the text.

Experimental rat (Sprague—Dawley strain)	Liver polyamine (nmol/g of tissue; mean ± S.D., n = 5)		
	Putrescine	Spermidine	Spermine
Male	124 ± 24	488 ± 26	174 ± 12
Female	161 ± 27	1293 ± 475	325 ± 58
Castrated male*	194 ± 31	786 ± 56	593 ± 51
Ovariectomized female*	32 ± 12	969 ± 175	172 ± 89

*The male and female rats were subjected to orchiectomy and ovariectomy, respectively, and sacrificed 10 days later. Their livers were removed for polyamine estimation as soon as possible.

TLC separation of dabsyl polyamines

The chromophoric properties of dabsyl polyamines render their detection on the TLC plate an easy operation. This prompted us to investigate their separation on silica-gel plates. The results of TLC on silica gel with five solvent systems are listed in Table III. The mobile phases were selected after studying numerous results reported in the literature or obtained in our laboratory. Data for dabsyl chloride, dabsyl ammonia, dabsyl ornithine and dabsyl hydroxide

are also listed. With these solvents, the chromatographic mobilities of these dabsyl derivatives on thin layers of silica gel are inversely correlated with their polarity.

The polarity of the solvent mixture exerted a profound effect on the mobilities of dabsyl polyamines, as demonstrated in Fig. 5. As observed for the HPLC system, the migration rate of dabsyl polyamines on the silica-gel plate increased as the concentration of ethanol in chloroform was increased.

TABLE III

TLC ANALYSIS OF DABSYL POLYAMINES ON SILICA-GEL PLATES

Dabsyl derivative	R_F in solvent system*				
	A	B	C	D	E
Dabsyl chloride	0.92	0.94	0.97	0.99	0.87
Dabsyl spermine	0.87	0.94	0.82	0.78	0.78
Dabsyl spermidine	0.70	0.87	0.71	0.58	0.51
Dabsyl putrescine	0.66	0.72	0.61	0.27	0.48
Dabsyl ammonia	0.62	0.60	0.55	0.35	0.46
Dabsyl ornithine	0.37	0.39	0.33	0.04	0.25
Dabsyl hydroxide	0.02	0.01	0.02	0.03	0.00

*Solvent systems: A, chloroform—dichloromethane—acetone—95% ethanol (5:2:0.5:1); B, chloroform—dichloromethane—acetone—absolute ethanol (5:2:0.5:1); C, chloroform—dichloromethane—absolute ethanol (5:2:1); D, chloroform—trimethylamine (5:1); and E, chloroform—dichloromethane—acetone—absolute ethanol—*n*-hexane (5:2:1:0.5:3).

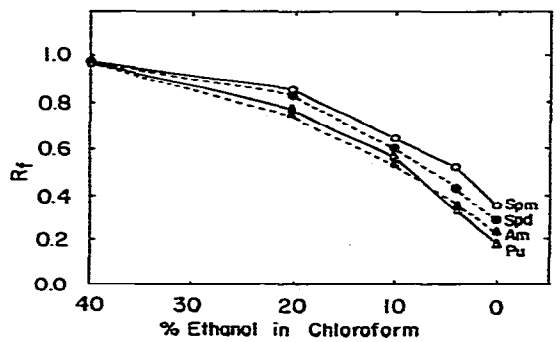

Fig. 5. Effect of the concentration of ethanol on the mobility of dabsyl polyamines on the silica-gel plate. Spm, dabsyl spermine; Spd, dabsyl spermidine; Pu, dabsyl putrescine; and Am, dabsyl ammonia.

DISCUSSION

Several chromatographic procedures for the determination of polyamines have been published since 1973. Table IV summarizes the characteristics of these procedures. The important parameters such as type of chromatography, form of derivative, sampling time, running time, sensitivity and recovery are listed and compared. Among the published procedures, the HPLC of dansyl

TABLE IV

COMPARISON OF PUBLISHED AND PRESENT METHODS FOR THE DETERMINATION OF POLYAMINES

Author (year)	Type of chromatography	Derivative	Sampling time (h)	Running time (min)	Sensitivity	Recovery (%)	Reference
Gehrke et al. (1973)	Gas chromatography	Trifluoroacetyl	20	20	20 ng	55–90	17
Morton et al. (1973)	Amino acid analysis	Ninhydrin	20	95	1 nmol	100	10
Sugiura et al. (1975)	HPLC	Tosyl	1.5	20	6–30 μg	–	15
Fleisher and Russell (1975)	TLC	Dansyl	Overnight	210	5 nmol	–	12
Saeki et al. (1978)	HPLC	Dansyl	16	20	30 pmol	91–95	14
Seiler et al. (1978)	HPLC	Dansyl	Overnight	40	16.6 pmol	–	13
Makita et al. (1978)	Gas chromatography	N-Isobutyloxycarbonyl	19	35	6 nmol	94–99.6	18
Redmond and Tseng (1979)	HPLC	Benzoyl	2	16	200 pmol	–	16
Kneip et al. (1979)	Cation-exchange chromatography	o-Phthaldehyde	24	120	20 nmol	90–98	11
Lin and Lai (1981)	HPLC	Dabsyl	2	14	20 pmol	84–93	Present work

polyamines as described by Seiler et al. [13] and Saeki et al. [14] has proved to be a sensitive and reliable method. For various reasons the method described in this paper may on some occasions be a favorable alternative to the dansyl procedure: (A) Dansyl derivatives are highly fluorescent under light illumination and the intensity of the fluorescence is markedly modified by environmental factors such as temperature, pH, solvent polarity and exposure to light; dabsyl derivatives are visible and photostable [19], and this property provides the basis for the good reproducibility of chromatographic separation and quantitation. (B) The chromophoric properties of dabsyl derivatives allow spectrophotometric estimation in the visible region (425 nm); this monitoring will avoid the troublesome interference caused by UV-absorbing substances in the biological samples. (C) The colored dabsyl derivatives can be collected from the column outflow and characterized directly by TLC on silica-gel plates and by other suitable methods. (D) The isocratic elution used in the present method is more time-saving and easier to operate compared to the stepwise elution [13] or gradient elution [14] used in the dansyl procedure.

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