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Journal of Chromatography B, 666 (1995) 329–335

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

Rapid high-performance liquid chromatographic method for the quantitation of polyamines as their dansyl derivatives: application to plant and animal tissues

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First received 26 July 1994; revised manuscript received 19 December 1994; accepted 19 December 1994

Abstract

A rapid high-performance liquid chromatographic method for the separation of polyamines as their dansyl derivative has been developed. The chromatographic system used consisted of a reversed-phase column and a mobile phase of acetonitrile and water. The separation of 1,3-diaminopropane, putrescine, cadaverine, spermidine and spermine takes only 9 min. This method provides a good resolution between 1,3-diaminopropane and putrescine. It has been applied to quantify polyamines from seeds of wheat, petals of *Phalaenopsis* hybrids and various rat tissues.

1. Introduction

Polyamines, such as 1,3-diaminopropane (DAP), putrescine (PUT), cadaverine (CAD), spermidine (SPD) and spermine (SPM), are ubiquitous in all bacteria and plants. Animal cells mostly contain PUT, SPD and SPM. The interest in the physiological effects produced by polyamines in plants has increased over the last few years and it has been suggested that polyamine functions are involved in membrane stabilization, free radical scavenging, DNA, RNA and protein synthesis, RNase, protease and other enzyme activities, the interaction with ethylene biosynthesis and effects on second messengers [1]. Polyamines are required in large amounts by rapidly growing tissues and the importance of polyamines in tumour growth is widely recognised. Inhibiting polyamine biosynthesis in cancerous tissues is a major target for scientists involved in polyamine research.

Due to this interest many analytical methods for polyamine determination have been described. These include high-performance liquid chromatography (HPLC) [2–7], thin-layer chromatography (TLC) [8], thin-layer electrophoresis (TLE) [9] and paper electrophoresis (PE) [10].

Polyamines are aliphatic organic compounds containing two or more amino groups. Before these positively charged molecules can be separated by HPLC we must either incorporate an ion-pair reagent in the solvent system [11] or

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0378-4347/95/$09.50
SSDI 0378-4347(94)00586-9

derivatise the amino groups with a suitable reagent. The derivatising reagent will enhance the detection of polyamines by UV absorbance or fluorescence. A range of pre- and post-column derivatisation reagents have been described in the literature [2–6,10–16]. When we compared the relative merits of these methods with our need for a rapid screening of plant material and a rapid turnover on our HPLC system, we decided to investigate the pre-column dansylation technique.

Most HPLC techniques for the separation of dansyl polyamines described in the literature, require long analysis times (30–90 min) and an additional column equilibration cycle between runs. Only Walter and Geuns [3] reported a rapid analysis (5 min), but the separation of 1,3-diaminopropane (DAP) was not satisfactory.

We have been involved in the determination of polyamines from several monocot plant species including cereals and orchids. Monocots show high levels of DAP due to the oxidation of SPD and SPM by polyamine oxidase activity [1]. Therefore, complete resolution of DAP from other polyamines is essential for our studies.

In this report we describe a rapid and sensitive HPLC method with pre-column derivatisation with dansyl chloride for the determination of the six natural polyamines DAP, PUT, CAD, SPD and SPM in both plant and animal tissues.

2. Experimental

2.1. Apparatus

The HPLC system consisted of two pumps (Model 4004) programmed with a microprocessor controller (Model 1000S diode-array detector), Kratos Spectroflow 980, fluorescence detector (excitation at 252 nm, emission at 500 nm) all from Applied Biosystem (San José, CA, USA) and an autosampler Model Promis II from Spark Holland (Emmen, Netherlands).

The column was a Brownlee reversed-phase ODS Spheri-5 (C_{18}, 5 μm spherical, 80 Å pore size, 220 × 4.6 mm I.D.) supplied by Applied Biosystem protected by a guard column (20 × 4.5 mm I.D.) containing a silica-based C_{18} sorbent packing.

2.2. Reagents

Dansyl chloride, proline and polyamines in the form of hydrochlorides were obtained from Sigma (Madrid, Spain). Toluene, acetone, hydrochloric acid, perchloric acid and sodium carbonate were purchased from Panreac (Moncada i Rexac, Barcelona, Spain). Acetonitrile was provided by Scharlau (Barcelona, Spain).

2.3. Plant material and sample preparation

Phalaenopsis hybrids (Orchidaceae) were grown under a controlled environment in a greenhouse. Plants received a natural photoperiod and 25 ± 2°C day and 19 ± 2°C night temperature conditions. Humidity was 60 ± 5%. Seeds of wheat (*Triticum aestivum*), provided by Svalöf (Sweden), were also analysed.

Petals of *Phalaenopsis* hybrids and seeds of wheat were extracted in 5% cold perchloric acid (PCA) (300 mg fresh weight/ml) in an ice bath. After extraction the samples were centrifuged at 27 000 *g* for 20 min. The supernatant, containing the free polyamines (S-fraction), was stored at −20°C until dansylation. The pellet was resuspended in 1 *M* NaOH (300 mg fresh weight/ml), and stored at −20°C.

A 300-μl aliquot of the supernatant or pellet fractions and 300 μl of 12 *M* HCl were mixed into an injectable vial, which was sealed with a flame and heated at 100°C for 20 h. The resulting mixture was filtered and dried in vacuum. In this way, the SH- and PH-fractions containing polyamines liberated from PCA-soluble and PCA-insoluble conjugated polyamines, respectively, were obtained. The dried material was redissolved in 300 μl of 5% perchloric acid and stored at −20°C until dansylation.

2.4. Animals and tissue preparation

Thirty day-old Hooded-Lister spf (specific pathogen-free) rats from the Rowett colony,

weighing 82.2 ± 2.3 g were selected, kept separately in cages and fed (6 g rat $^{-1}$ day $^{-1}$) on semi-synthetic, good quality diets containing lactalbumin (100 g kg^{-1} diet) as the sole protein source for three days. Rats were killed with overdose of ether and their small intestine, colon, caecum, pancreas, liver, stomach and hind leg gastrocnemius muscles were immediately removed, blotted, weighed, then freeze-dried. The freeze-dried tissues were extracted in 1,7-diaminoheptane (HTD) with 10% (w/v) perchloric acid (15 mg dry tissue per milliliter) for 30 s at 0°C and then centrifuged for 30 min at 4°C at 10 000 *g*. Following appropriate dilution the samples were ready for dansylation.

2.5. Dansylation

The polyamines were derivatised according to the method of Seiler and Wiechmann [17] with some modifications, as follows: 200 μl of the perchloric acid extracts were mixed with 40 μl of 0.05 m*M* diamino heptane (HTD) as internal standard. Then, 200 μl of a saturated solution of sodium carbonate and 400 μl of dansyl chloride (5 mg ml^{-1} acetone) were added.

The resulting mixture was either incubated in the dark overnight at room temperature or for 10 min at 70°C [18]; then 100 μl of proline solution (100 mg ml^{-1} water) was added and the mixture was incubated in the dark for 30 min. Dansylated polyamines were extracted in 500 μl of toluene, and mixed for 30 s. After the mixture had separated into two phases, 400 μl of the organic phase was removed, dried and re-dissolved in 800 μl of acetonitrile. Finally, the sample was passed through a 0.45-μm pore size syringe filter and 20 μl was injected onto the HPLC system.

2.6. HPLC procedure

Dansylated polyamines injected onto the column were eluted with acetonitrile and water both of which had been previously passed through a 0.45-μm filter. The gradient used in the HPLC method developed in this study is shown in Fig. 1. The initial conditions were 70% acetonitrile and 30% water pumping at a flow-rate of 1.5 ml min^{-1}. This mixture was pumped for 4 min, then the concentration of acetonitrile was raised to 100%. These conditions were optimised for the elution of SPD and SPM, which are both strongly retained on the column. This concentration of acetonitrile was kept constant for 10 min, then returned to the initial conditions.

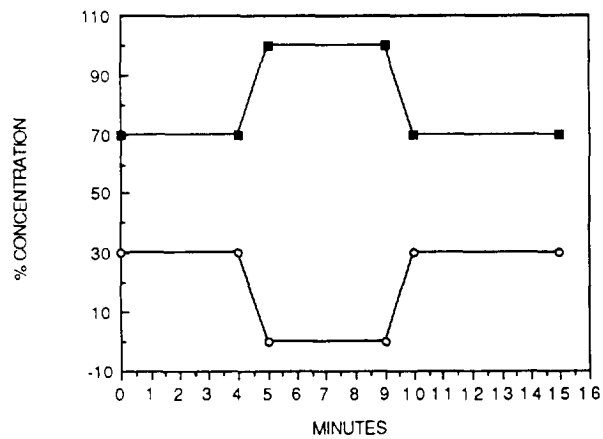

Fig. 1. Diagram of the gradient used in the HPLC method: percent concentration acetonitrile and water in the mobile phase vs. time in minutes. (■) Acetonitrile, (○) water. For details of operating conditions see text.

For quantitative purposes, HTD was used as internal standard. This is an artificial polyamine, which resolves well from derivatives of endogenous polyamines and elutes between the polyamines of interest.

Under these conditions, SPM was the last peak eluted from the column (approximately 8.30 min), and the column was re-equilibrated in the remaining 6.30 min.

3. Results and discussion

3.1. Chromatographic parameters

Retention times of the different dansylated polyamines were as follows: 4.3 min (DAP), 4.6 min (PUT), 5.2 min (CAD), 6.4 min (HTD), 7.1 min (SPD) and 8.2 min (SPM). These values were reproducible, their standard deviations

varying from 0.43% (SPD) to 1.47% (CAD), and within acceptable limits for day-to-day variation. Resolution (R_S) values were all above the acceptable limits ($R_S = 1$).

Fig. 2 shows a chromatogram of a polyamine standard mixture. The amount injected for all standards is 30 pmol (1.50 nmol ml^{-1}).

3.2. Detection limits

The range of sensitivity depends on the detector used. With our apparatus for amounts injected above 175 pmol (8.75 nmol ml^{-1}) the signal was beyond the range for DAP, PUT and CAD. For SPD, SPM and HTD the maximum quantifiable concentration is 35 pmol (1.17 nmol ml^{-1}). The limit of detection was considered as the lowest concentration injected onto the system that gave a coefficient of variation (C.V.) less than 2 for several determinations.

According to this criterion, the minimum quantifiable concentration is 0.5 pmol (0.025 nmol ml^{-1}) for SPD, SPM and HTD, and a little higher, 1 pmol (0,05 nmol ml^{-1}) for CAD, PUT and DAP.

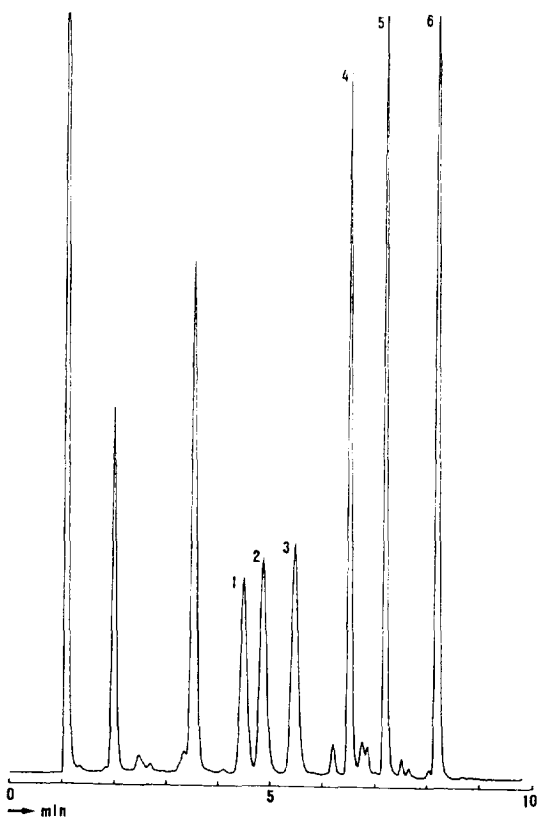

Fig. 2. Chromatogram of dansylated standard polyamines. Peaks: 1 = DAP; 2 = PUT; 3 = CAD; 4 = HTD; 5 = SPD; 6 = SPM. The amount injected for all standards is 30 pmol (1.50 nmol ml^{-1}). For details of the separation conditions see text.

3.3. Linearity

To establish the linearity of the relationship between the polyamine level and the area measured, five different solutions of standard polyamines were prepared in concentrations ranging from 3 nmol ml^{-1} to 12 nmol ml^{-1}. Each of these standard solutions was run five times on two consecutive days.

In the regression equation x is the ratio between the areas of polyamine and HTD and y is the ratio between the injected amounts of polyamine and HTD. The equations for these linear curves were as follow: for DAP, $y = 3.5654 \cdot 10^{-2} + 0.51949x$ ($r = 0.999$); for PUT, $y = 2.7031 \cdot 10^{-2} + 0.75967x$ ($r = 1.000$); for CAD, $y = 2.1721 \cdot 10^{-2} + 0.68921x$ ($r = 1.000$); for SPD, $y = 5.4143 \cdot 10^{-2} + 1.08410x$ ($r = 0.999$); and for SPM, $y = 4.3472 \cdot 10^{-2} + 1.27550x$ ($r = 1.000$). The correlation coefficients (r) calculated were optimum since they were close to or equal to 1.

3.4. Reproducibility and recovery

Reproducibility of the method was estimated using three different concentrations of standard solution and five plant samples representing different cell polyamine fractions extracted from *Phalaenopsis* petals and wheat seeds. Each sample was run five times over two consecutive days. The coefficients of variations were all satisfactory (below 3%).

Table 1 shows the polyamine content of *Phalaenopsis* petals and wheat seeds. Optimal values for coefficients of variation were generally obtained, except for SPD in the soluble fraction of Orchid petals, which was higher than ex-

Table 1
Reproducibility with Orquid petals and wheat seeds

	DAP		PUT		CAD		SPD		SPM	
	nmol/g	C.V.	nmol/g	C.V.	nmol/g	C.V.	nmol/g	C.V.	nmol/g	C.V.
Orquid petals										
S[a]	77.98 ± 2.33[b]	3.00	40.74 ± 1.12	2.75	6.07 ± 0.34	5.62	5.38 ± 0.40	7.56	0.75 ± 0.05	6.78
SH[c]	ND[d]	–	234.75 ± 6.84	2.92	ND	–	13.60 ± 0.15	1.13	ND	–
Wheat seed										
S[a]	ND[d]	–	37.96 ± 0.22	0.56	ND	–	105.70 ± 3.29	3.11	29.00 ± 0.52	1.82
SH[c]	17.49 ± 1.03	5.89	173.64 ± 5.44	3.13	ND	–	95.59 ± 2.65	2.78	20.88 ± 0.29	1.38
PH[e]	4.92 ± 0.07	1.43	49.23 ± 0.60	2.99	ND	–	19.52 ± 0.19	1.78	3.46 ± 0.05	4.30

[a] S = Non hydrolyzed PCA supernatant containing the free, soluble PAs; see text for details of analysis method.
[b] Mean value of 3 replicates ± standard deviation.
[c] SH = Hydrolyzed PCA supernatant containing PAs liberated from soluble conjugates; see text for details.
[d] ND = Not detected.
[e] PH = Hydrolyzed PCA pellet containing PAs liberated from insoluble conjugates; see text for details.
For operating conditions, see text.

Table 2
Recoveries of polyamines

	Added (nmol)	Recovered (nmol)	Recovery (%)	Mean recovery ± S.D. (%)
DAP	0.646	0.591	91.50	93.25 ± 6.47
	2.588	2.545	98.35	
	5.176	4.391	84.83	
	7.765	7.634	98.31	
PUT	0.625	0.570	91.28	94.98 ± 2.71
	2.502	2.442	97.60	
	5.004	4.746	94.84	
	7.504	7.218	96.19	
CAD	0.672	0.638	95.00	97.09 ± 1.55
	2.690	2.648	98.43	
	5.382	5.211	96.83	
	8.070	7.916	98.09	
SPD	0.674	0.617	91.58	92.68 ± 4.89
	2.697	2.693	99.85	
	5.395	4.866	90.19	
	8.091	7.208	89.08	
SPM	0.720	0.637	88.57	89.47 ± 2.25
	2.880	2.663	92.47	
	5.758	5.019	87.16	
	8.638	7.748	89.70	

Table 3
Contens of polyamines in plant and animal tissues

	Polyamine (nmol g^{-1} fresh wt)				
	DAP	PUT	CAD	SPD	SPM
PLANT					
Orchid flower					
Sepal	128.5 ± 63.2[a]	163.4 ± 3.6	–	61.3 ± 38.6	3.7 ± 3.8
Petal	123.6 ± 28.3	144.1 ± 45.2	–	55.1 ± 19.6	3.9 ± 0.7
Lip	137.9 ± 39.4	172.3 ± 12.5	–	14.3 ± 1.9	2.8 ± 0.5
Column	210.7 ± 5.7	165.7 ± 25.0	–	20.0 ± 15.9	2.5 ± 0.4
Ovary	132.0 ± 30.7	157.5 ± 3.9	–	25.6 ± 19.1	1.7 ± 0.6
Wheat seed					
Winter wheat	3.5 ± 1.3	52.4 ± 20.9	4.6 ± 3.9	162.4 ± 9.1	24.4 ± 1.8
Spring wheat	4.4 ± 2.9	37.4 ± 9.0	7.6 ± 2.6	162.1 ± 36.9	28.4 ± 5.8
ANIMAL					
Jejunum	–	136 ± 17	–	400 ± 62	338 ± 41
Heart	–	2 ± 0.5	–	31 ± 6	51 ± 6
Pancreas	–	24 ± 6	–	1692 ± 25	647 ± 54
Liver	–	57 ± 16	–	2055 ± 195	1210 ± 165
Colon	–	17 ± 1	–	320 ± 37	276 ± 26
Kidney	–	9 ± 1	–	334 ± 10	331 ± 7
Ceacum	–	18 ± 5	–	353 ± 16	290 ± 25

[a] Mean values from 3 replicates ± S.D.
For sample preparation conditions, see text.

pected. The greater variability in the samples compared to the standard solution is an illustration of the difficulty to obtain representative samples from the seeds and petals. For recovery determinations two standard solutions were prepared, one dilute (3.5 nmol ml^{-1}) and one more concentrated (12.5 nmol ml^{-1}). Four different amounts of the concentrated solution were added to the dilute solution. In this way four different mixtures of increasing concentration were tested. Each mixture was run three times. Table 2 shows that the recoveries of the polyamines were excellent (89.47–97.09%) with a standard deviation of 1.55–6.47%.

3.5. Application

To demonstrate the validity of the method, the polyamine content of petals of *Phalaenopsis* hybrids, seeds of wheat and rat tissues was determined (see Fig. 3 and Table 3). The peaks were identified by adding a mixture of reference compounds to the sample solution before injection. Table 3 gives the results expressed as nmol g^{-1} (fresh weight). It is known that DAP does not occur in animal tissue but it is interesting to note the large variation in the DAP levels between wheat seed and Orchid flower. Cadaverine was quantifiable only in wheat seeds and did not appear in any of the animal tissues. The levels of the different polyamines appear to be reasonably consistent throughout the various parts of the Orchid flower but there is a large variation in the distribution of polyamine levels in the various rat tissues.

4. Conclusions

The method developed in this study is capable of separating the polyamines DAP, PUT, CAD, SPD and SPM in less than 9 min. It is also possible to process a large number of samples in a short time, since a sample can be injected

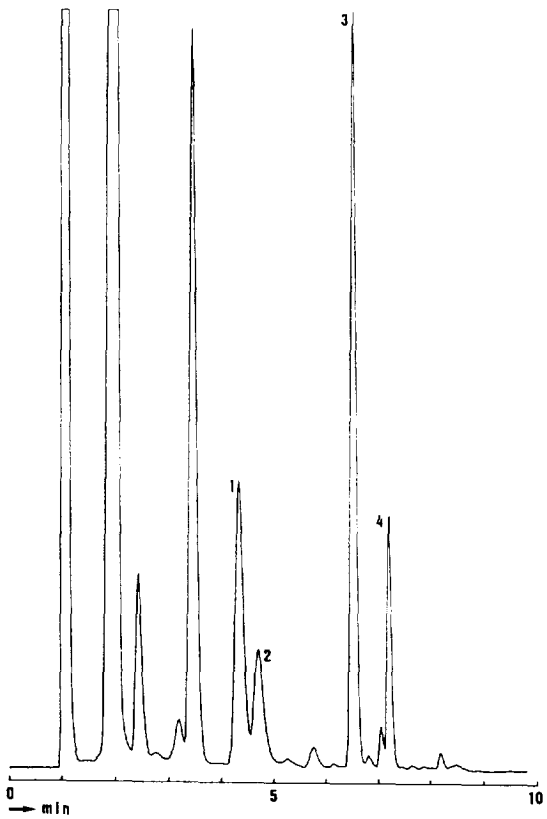

Fig. 3. Separation of dansylated polyamines in perchloric acid extract of petals of *Phalaenopsis*. Peaks: 1 = DAP; 2 = PUT; 3 = HTD; 4 = SPD. For details of operating conditions see text.

every 15 min. This method has been tested by quantifying polyamines from plant samples and animal tissues. We have shown that this method provides a fast alternative to other published HPLC methods for the quantitation of polyamines from various biological sources.

Acknowledgements

The work has been financed by the Spanish CICYT-PTR-91-70 (AFT) and by EC Concerted Action Programme (AIR-1-CT-92-0569) (AFT and DSB).

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