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High-performance liquid chromatographic method for the determination of dansyl-polyamines[a]

SUBHASH C. MINOCHA*, RAKESH MINOCHA[b] and CHERYL A. ROBIE[c]
Department of Plant Biology, University of New Hampshire, Durham, NH 03824-3597 (U.S.A.)
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ABSTRACT

This paper describes a fast reliable, and a sensitive technique for the separation and quantification of dansylated polyamines by high-performance liquid chromatography. Using a small 33 × 4.6 mm I.D., 3 μm particle size, C_{18} reversed-phase cartridge column and a linear gradient of acetonitrile–heptanesulfonate (10 *mM*, pH 3.4), at a flow-rate of 2.5 ml/min, the retention time for different polyamines was: N^8-acetyl-spermidine, 1.79 min; N^1-acetylspermidine, 1.82 min; putrescine, 2.26 min; cadaverine, 2.43 min; heptanediamine, 2.83 min; spermidine, 3.42 min; and spermine, 4.41 min. With an additional column regeneration time of 3–4 min, the complete cycle per sample took less than 8 min at room temperature. Using a fluorescence detector, the lower limit of detection was less than 1 pmol per 6 μl injection volume. The fluorescence response was linear up to 200 pmol per 6 μl for each polyamine. The method is suitable for separation of polyamines from animal, plant and fungal sources.

INTRODUCTION

The ubiquitous occurrence and psysiological importance of the polyamines in living systems has led to the development of a variety of analytical techniques for their separation and quantification. Thin-layer chromatography (TLC), thin-layer electrophoresis, gas chromatography and high-performance liquid chromatography (HPLC) have all been used[1]. The most widely used method of derivatization for quantifiation is dansylation[2–5]. Other methods of derivatization include benzoylation, tosylation, and dabsylation[6–10]. As compared to the separation of dansylated polyamines by TLC and quantification by fluorimetry[11,12], HPLC allows both a lower detection limit and a wider range of linearity[3,4,11,13–15]. Separation by HPLC

[a] Scientific contribution number 1669 from the New Hampshire Agricultural Experiment Station
[b] Present address: Louis C. Wyman Forestry Sciences Laboratory, Northeastern Forest Experiment Station, Durham, NH 03824, U.S.A.
[c] Present address: Biotechnica International Inc., 85 Bolton Street, Cambridge, MA 02140, U.S.A.

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generally involves a reversed-phase column and an isocratic or a gradient elution system using either water–methanol or water–acetonitrile as the solvent[14–18]. Further improvements in separation can be achieved by the use of an ion-pairing reagent such as octanesulfonic acid or heptanesulfonic acid.

Since a number of reliable protocols for separation of polyamines by HPLC are available, further improvements are directed mainly at decreasing the elution time and increasing the sensitivity and resolution of the technique. Protocols available at present generally require an elution time of 15–90 min depending upon the column and the solvent system. If a column regeneration time of 5–15 min per run is included, it takes at least 20–30 min for each sample. In a recent paper, Walter and Geuns[18] reported a relatively fast separation of dansylated polyamines by HPLC with a total elution time of about 5 min, using a 10-cm reversed-phase column and an isocratic solvent mixture at 50°C. At room temperature, the elution time was more than 10 min. Unfortunately, little information was provided on the parameters of separation and quantification such as the types of polyamines that can be separated, the limits of detection and the range of linearity, etc.

Using a smaller and a more efficient column, combined with a gradient elution, we have developed a rapid and a sensitive method of separation and quantification of dansylated polyamines from different sources. The method allows a complete elution of dansyl-polyamines in less than 5 min at room temperature. With a 3–4 min column regeneration time, the complete cycle per sample takes about 8 min. The elution time can be further reduced or the back pressure can be lowered at higher column temperatures. The limits of detection are less than 1 pmol per 6 μl injection volume, with a linearity of up to 200 pmol for each polyamine in the mixture. The method is suitable for the separation of polyamines from plant, animal and fungal sources[19,20].

EXPERIMENTAL

Materials

Dansyl chloride and all polyamine and acetylpolyamine standards were purchased from Sigma (St. Louis, MO, U.S.A.). Acetone, toluene (Photrex grade), HPLC-grade methanol and acetonitrile were supplied by J. T. Baker (Phillipsburg, NJ, U.S.A.).

Extraction and dansylation of polyamines

Polyamines were extracted from various tissues and dansylated following modifications of the procedure of Smith and Davies[4]. Stock solutions of the various compounds were made in 5% perchloric acid and diluted to obtain the necessary final concentrations. Aliquots (50 μl) of each solution or the centrifuged tissue extract were placed in 1.0-ml Reactivials (Pierce, Rockford, IL, U.S.A.) containing 100 μl of a saturated sodium carbonate solution. A 100-μl volume of dansyl chloride solution in acetone (10 mg/ml) was added to each vial. The vials were capped tightly and incubated in the dark in a water bath at 60°C for 1 h. A 50-μl volume of proline (100 mg/ml) was added to the reaction mixture to remove excess dansyl chloride. After an additional 30 min incubation, acetone was evaporated from each vial by spinning under vacuum for 2 min in SpeedVac Evaporator (Savant, Farmingdale, NY, U.S.A.). A 400-μl volume of toluene was then added to the solution and each vial was

vortex-mixed for 30 s. The vials were centrifuged at 500 *g* for 2 min. After the aqueous and organic phases had separated, 200 μl of the toluene layer were transferred to an Eppendorf tube. Toluene was completely evaporated in the SpeedVac and the residue dissolved in 1 ml of methanol or acetonitrile.

HPLC apparatus

The liquid chromatographic system consisted of a Perkin-Elmer series 400 pump, a Rheodyne injector valve fitted with a 6-μl loop, a Perkin-Elmer Pecosphere-3 × 3 CR C_{18}, 33 × 4.6 mm I.D. cartridge column (3 μm particle size), and a fluorescence detector (LS-1, Perkin-Elmer). The excitation and emission wavelengths were set at 340 and 510 nm, respectively. Peak areas were calculated using a LCI-100 integrator (Perkin-Elmer).

A helpful hint: When heptanesulfonate solution was left in the bottle for several days, the growth of microorganisms (not identified) in the connecting tubes caused problems with chromatography. The inclusion of 10% (v/v) acetonitrile in the heptanesulfonate solution eliminated this problem. Appropriate adjustments should be made in the gradient profile to achieve the desired concentrations at each step.

RESULTS AND DISCUSSION

Isocratic vs. gradient elution

The first important objective of the study was to obtain a clean separation of the three major polyamines (putrescine, spermidine and spermine) within as short a time as possible. Based on information in the literature, a reversed-phase C_{18} column was used with (1) an isocratic or (2) a linear gradient elution system. In order to achieve a faster elution time, while also maintaining peak resolution, a 3-μm, 33-mm column was selected for separation. The isocratic solvent system of water–acetonitrile (28:72) used by Walter and Geuns[18] produced an unsatisfactory separation of the three polyamines with this column. Whereas putrescine and spermidine eluted within 2 min, with distinct but overlapping peaks, spermine did not elute until 7.2 min (Fig. 1A). Increasing the column temperature, as suggested by the authors, did reduce the total elution time, while at the same time making the peaks of putrescine and spermidine even less distinct. A significant improvement in separation as well as elution time was seen when water was replaced by a 10 m*M* solution of heptanesulfonate, pH 3.4. The elution times for putrescine, spermidine and spermine were 1.34, 4.05 and 5.83 min, respectively (Fig. 1B). Furthermore, all three peaks were well-separated. Increasing the concentration of acetonitrile to 90% in heptanesulfonate caused a faster separation of the compounds but resulted in poor resolution of the peaks.

A much faster and cleaner separation of the three polyamines was obtained by using a linear gradient of acetonitrile and heptanesulfonate with the parameters shown in Table I. Retention times of 1.84, 2.61 and 3.28 min, were seen for putrescine, spermidine and spermine, respectively (Fig. 1C). A flow-rate of 2.5 ml/min was found to be optimal for separation. At this flow-rate the back pressure ranged from 45 bar at 100% acetonitrile to 110 bar at 50% acetonitrile. When a mixture of histamine and seven polyamines (including two of the acetyl derivatives and heptanediamine, a commonly used internal standard) was injected, the separation was unsatisfactory using the above gradient. A modification of the gradient profile as shown in Table II

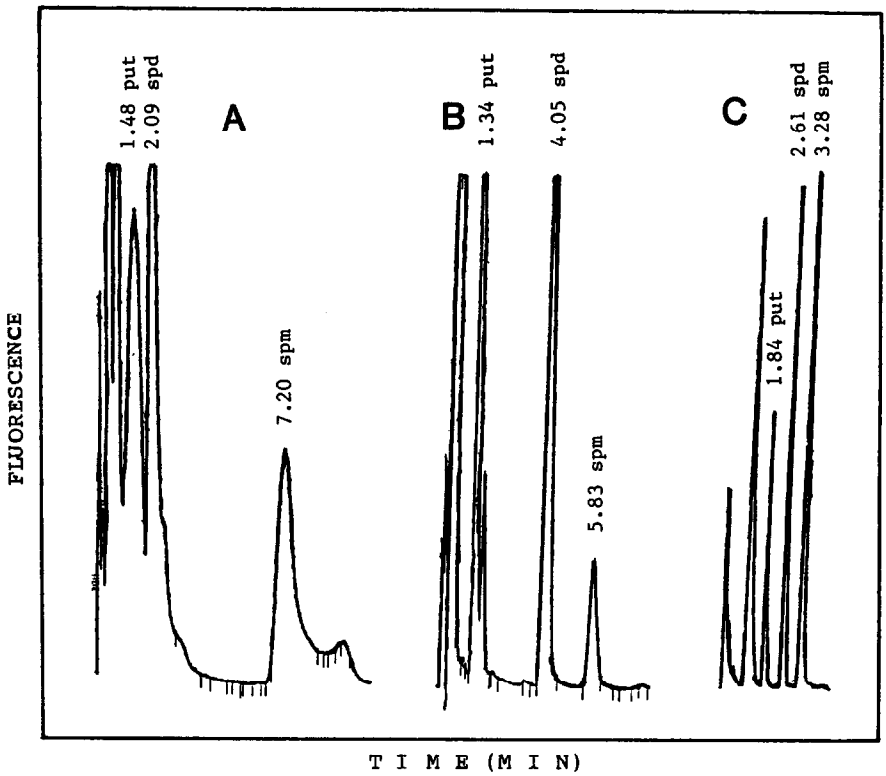

Fig. 1. Separation of standard dansyl polyamines on a 3-μm, 3.3-cm Pecosphere C_{18} reversed-phase column using an isocratic or a gradient elution. Each injection (6-μl loop) contained the equivalent of 50 pmol each of putrescine (put), spermidine (spd) and spermine (spm). Flow-rate was 2.5 ml/min. The fluorescence detector was set at 340 nm and 510 nm for absorption and emission, respectively. The solvent systems were: (A) isocratic with acetonitrile–water (72:28); (B) isocratic with acetonitrile–heptanesulfonate, 10 m*M*, pH 3.4 (72:28); (C) linear gradient of acetonitrile and heptanesulfonate. Parameters of the gradient are given in Table I.

TABLE I

GRADIENT PROFILE OF THE SOLVENT FOR THE SEPARATION OF DANSYLATED PUTRESCINE, SPERMIDINE AND SPERMINE

Step	*Time*[a]	*Acetonitrile (%)*	*Heptanesulfonate (%)*[b]
1	0.1	50	50
2	1.5	80	20
3	2.0	100	0
4	1.0	100	0
5	0.1	50	50
6	2.0	50	50

[a] Time is not cumulative from the start of the run.
[b] Heptanesulfonate concentration: 10 m*M*, pH 3.4.

TABLE II

GRADIENT PROFILE OF SOLVENT FOR THE SEPARATION OF DANSYL-POLYAMINES AND THEIR ACETYL DERIVATES

Solvent A = 100% acetonitrile; solvent B = heptanesulfonate (10 m*M*, pH 3.4)-acetonitrile (90:10).

Step	*Time*[a]	*Solvent A (%)*	*Solvent B (%)*
0	0.1	40	60
1	2.0	70	30
2	3.3	100	00
3	2.0	100	00
4	0.1	40	60
5	1.0	40	60

[a] Time is not cumulative from the start of the run.

resulted in a clean separation of all the compounds. The retention times for various compounds were as follows: N^8-acetylspermidine, 1.79 min; N^1-acetylspermidine, 1.82 min; putrescine, 2.26 min; cadaverine, 2.43 min; heptanediamine, 2.83 min; spermidine, 3.42 min; and spermine, 4.41 min (Fig. 2). Histamine eluted in two peaks (0.86 and 1.36 min) and did not interfere with the peaks of other polyamines. Including a column cleaning and regeneration time of 3–4 min, the total time per sample was about 8 min. The identity of each peak was confirmed by running each compound separately as well as by spiking the mixture individually with each compound. Using the above method, it was not possible to clearly separate N^1- and N^8-acetylspermidines. However, further modification of the gradient resulted in an excellent separation of these two derivatives, but it took a total of about 15 min per sample[21].

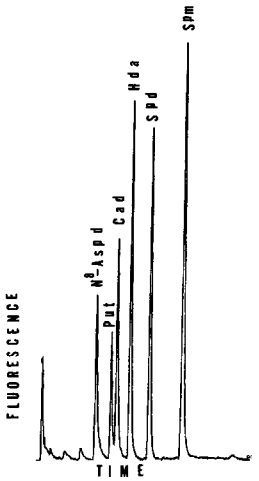

Fig. 2. Separation of a mixture of seven dansyl-polyamines with the gradient profile of the solvent presented in Table II. Each sample contained the equivalent of 20 pmol of each compound per 6 μl injection. N^8-Aspd = N^8-Acetylspermidine; Put = putrescine; Cad = cadaverine; Hda = heptanediamine; Spd = spermidine; Spm = spermine.

The retention time for each peak was highly repeatable for samples run on the same day (average standard error less than 2%), although it varied slightly from one day to another. Increased flow-rate resulted in somewhat faster elution but increased back pressure (*e.g.* at 3.0 ml/min, the pressure was about 130 bar); decrease flow-rate had the opposite effects. Raising the column temperature to 50°C, while maintaining the flow-rate at 2.5 ml/min, lowered the back pressure by 25–35% and decreased the total elution time to 2.65 min, but produced poor resolution.

Quantification of dansylated polyamines

Both the peak height and the peak area have been used for quantification of polyamines and other compounds separated by HPLC. The selection of the method depends largely on the mode of integration and the range of concentrations. Peak areas were used to determine the quantities of polyamines in the present study. The range of linearity for accurate quantification was from 1 to 200 pmol per injection (6-μl loop). The detection limit for putrescine (the least sensitive of the three major polyamines with respect to fluorescence) was at least an order of magnitude lower than 1 pmol, however, the increased noise-to-signal ratio affected the accuracy of quantification at lower concentrations. Concentrations higher than 200 pmol were not tested. A similar correlation between concentration and peak area was also observed for heptanediamine, making it suitable for use as an internal standard[19,20].

Analysis of biological samples

Extracts from a variety of sources including animal, plant, and fungal tissues were tested for separation of dansylated polyamines using the above chromatographic conditions. All parameters of pump (gradient profile), detector, and integrator were kept constant for comparison. In each case excellent separation and quantification of the three polyamines was obtained using heptanediamine as an internal standard[19,20].

CONCLUSION

In conclusion, we have developed a fast, sensitive, and highly quantitative technique for the analysis of dansylated polyamines. The technique is suitable for quantification over a wide range of concentrations of polyamines from a variety of sources. Separation of the three common polyamines is achieved in less than 3.5 min, a complex mixture containing acetylpolyamines and an internal standard (*e.g.* heptanediamine) can all be separated within about 5 min. This is a considerable improvement over previously published separation times of 15–90 min[3,4,9,10,14,15,17,18]. The cartridge column used here is very efficient and inexpensive as compared to larger columns. Fast separation also results in considerable saving of solvents and allows a large number of samples to be run within a normal working day. The method is suitable for the analysis of polyamines as well as their acetyl derivatives from a variety of biological samples in conjunction with the use of an internal standard[19,20].

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