Plant Physiol. (1985) 78, 89-91
0032-0889/85/78/0089/91/$01.00/0

Separation and Quantitation of Polyamines in Plant Tissue by High Performance Liquid Chromatography of Their Dansyl Derivatives

Received for publication July 23, 1984 and in revised form January 15, 1985

MARY A. SMITH*[1] AND PETER J. DAVIES
Section of Plant Biology, Cornell University, Ithaca, New York 14853

ABSTRACT

High performance liquid chromatography in combination with fluorescence spectrophotometry can be used to separate and quantitate polyamines (putrescine, cadaverine, spermidine, spermine), prepared as their dansyl derivatives, from plant tissue. The procedure gives sensitive and consistent results for polyamine determinations in plant tissue. In a standard mixture, the minimal detection level was less than 1 picomole of polyamines.

Several methods have been employed to analyze polyamines in biological tissues. Chromatographic procedures including TLC, ion exchange, and HPLC have been used to separate colored or fluorescent derivatives of polyamines (2, 6). The most sensitive method for detecting polyamines is through their dansyl derivatives (8). Dansylated polyamines are highly fluorescent and detectable in small quantities. However, dansyl chloride (1-dimethylamino-naphthalene-5-sulfonyl chloride) is nonspecific since it reacts with amino groups of many compounds and with phenols and some alcohols (8). Generally, dansylated amines are separated by TLC. The advantage of this method is it allows the simultaneous separation of multiple samples, but they are difficult to quantify directly, so sensitivity is less. Several workers have reported successful separation of dansyl amines from animal tissues and fluids by HPLC (1, 5, 7). HPLC provides several advantages for the separation of dansyl amines including easy column regeneration for repeated separations of large numbers of samples over a short period of time, and the detection of low quantities of polyamines (previous reports have been in the order of 20 pmol). In this paper, a reverse phase HPLC method is described for the separation of dansylated polyamines prepared from plant extracts.

MATERIALS AND METHODS

Plant Material. G2 pea plants (*Pisum sativum*) were grown in clay pots containing a soilless mixture of vermiculite, peat moss, limestone, and fertilizer. After 3 weeks in the greenhouse, seedlings were transferred to growth chambers with full light (200 $\mu E/m^2 \cdot s$ at pot level) provided by a combination of fluorescent and incandescent lamps. Plants received an 18-h photoperiod and 19°C day and 17°C night temperature conditions.

Chemicals. Putrescine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride, and dansyl chloride were purchased from Sigma. Methanol and acetone (HPLC grade) were purchased from J. T. Baker (Phillisburg, NJ). Toluene used was distilled in glass from Burdick and Jackson (Muskegeon, MI).

Extraction of Tissues. Apical bud tissue was usually homogenized in chilled mortars with pestles in 0.2 N $HClO_4$ (100 mg tissue/ml acid). Smaller quantities of tissue were ground in 5-ml centrifuge tubes with ground glass conical bases using matching pestles (Kontes, Vineland, NJ). Hexanediamine at 1 μmol/g fresh weight of tissue was added to the extracts as an internal standard (7). The homogenates were centrifuged at 4°C in a clinical centrifuge. The supernatants were analyzed for polyamines. Samples were usually derivatized immediately or stored for no more than 2 weeks at −20°C.

Dansylation. The polyamines were derivatized according to the methods of Flores and Galston (3, and personal communication). Fifty- to 100-μl aliquots of the supernatant were added to 200 μl of saturated sodium carbonate and 400 μl of dansyl chloride in acetone (7.5 mg/ml) in a 5-ml tapered reaction vial. The mixture was incubated in a thermal reaction block at 60°C for 1 h in the dark. One hundred μl of proline was added to the mixture to remove the excess dansyl chloride. After 0.5 h, the polyamines were extracted with 500 μl of toluene with vigorous vortexing for 30 s. The mixture separated into two phases, aqueous and organic. The lower, aqueous phase was removed with a 1-ml syringe and discarded. The organic phase, containing the polyamines, was completely dried under nitrogen. The polyamine residue was dissolved in 1 ml of methanol, ultrafiltered through nylon membranes (0.2 μm pore; Rainin, Woburn, MA), and assayed immediately or stored (no more than 1 week) at −20°C. Aliquots of samples were diluted 5- to 20-fold before injection into the HPLC.

HPLC Analysis. HPLC was performed on a system consisting of two solvent metering pumps (Altex, model 110A) programmed with a microprocessor controller (Altex, model 420). Samples were injected into a fixed 20-μl loop for loading onto a reverse phase C_{18} column. The columns used in these investigations included ODS[2] hypersil (5-μm particle diameter, 5 × 250 mm; Shandon, Cheshire, England) and ODS ultrasphere (5-μm particle diameter, 4.6 × 250 mm; Altex, Berkeley, CA). Samples were eluted from the column with a programmed water:methanol (v/v) solvent gradient, changing from 60% to 95% in 23 min at a flow rate of 1 ml/min. Elution was completed by 27 min. The column was washed with 100% methanol for 5 min and reequilibrated at 60% methanol for 5 min before the next sample was injected.

Detection and Quantification. Eluates from the column were

[1] Current address: MSU/DOE Plant Research Laboratory, Michigan State University, East Lansing, MI 48824.

[2] Abbreviation: ODS, octadecylsilane.

detected by an attached fluorescence spectrophotometer, equipped with an 8-μl flow through cell (model 650-10LC, Perkin Elmer). For dansyl polyamines, an excitation wavelength of 365 nm was used with an emission wavelength of 510 nm (7). Eluant peaks with their areas and retention times were recorded by an attached integrator (model 3390A, Hewlett Packard).

The scheme for extracting and assaying polyamines according to the method described above is shown in Figure 1.

RESULTS AND DISCUSSION

A typical chromatogram of dansyl polyamine standards separated by a C_{18} reverse phase HPLC column is illustrated in Figure 2A. Retention times in minutes shown at the tip of each peak are stable and consistently reproducible. Major side products of the dansyl reaction on the left side of the chromatogram are eluted by 12.5 min and are separated very clearly from derivatized polyamines. Two of these products may be dansyl ammonia and dansyl dimethylamine as the former occurs in high concentrations in tissues and the latter is the most abundant product of the dansylation reaction (7). Dansyl polyamines are completely eluted from the column by 27 min. The naturally occurring amines are putrescine, cadaverine, spermidine, and spermine. Hexanediamine was used as an internal standard (7). The amount of individual amine represented at each peak on the chromatogram is 2.5 pmol. As the fluorescence spectrophotometer sensitivity was set at 1.0 detection unit, 1/30 of the maximum sensitivity, lower amounts of polyamines could still be determined. The lowest levels tested and detected were 0.8 pmol for putrescine, cadaverine, spermidine, and spermine, injected as an aliquot from a dansylated mixture containing 200 pmol of each amine. Theoretically, the lower limit should be about 26 fmol. As tissues contain in the order of 1 μmol of amine/g fresh weight, higher concentrations of amine standards were dansylated and used to prepare standard curves.

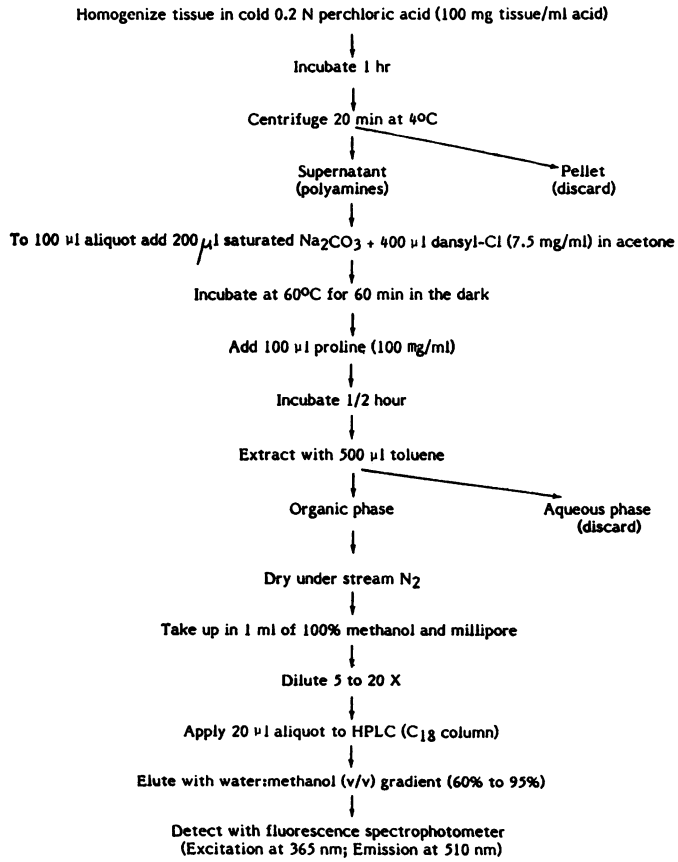

FIG. 1. Scheme for the extraction and assay of polyamines.

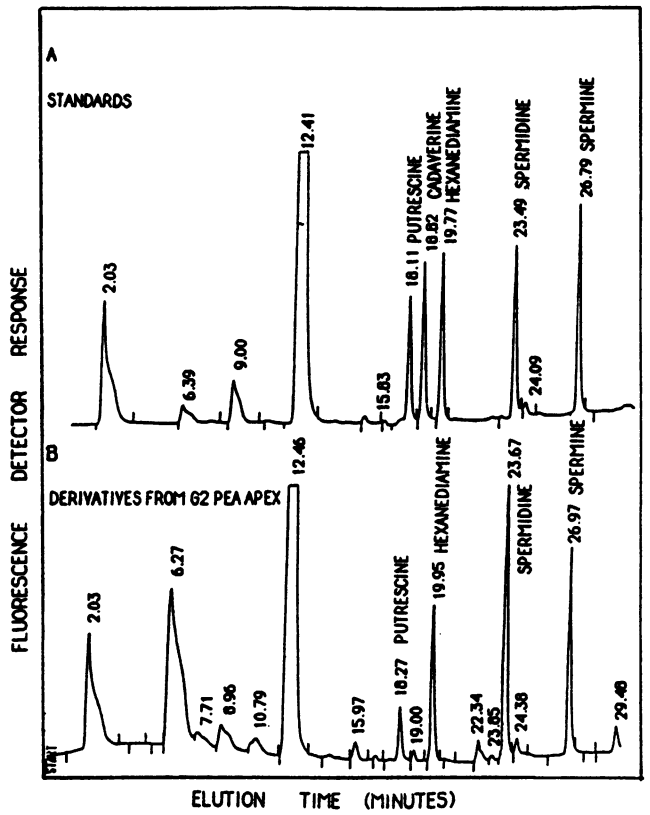

FIG. 2. Separation of dansyl amine derivatives by HPLC. Column: reverse phase (C_{18} ODS Hypersil). Gradient: water:methanol (v/v) 60 to 95% over 23 min at a flow rate of 1 ml/min. V_o = 2.03 min. Detector: Perkin-Elmer fluorescence spectrophotometer (excitation 365 nm, emission 510 nm). Numbers above peaks designate elution times in minutes from injection, starting from the left. A, Peak areas of standards represent 2.5 pmol. B, Polyamine derivatives from apical buds of mature G2 pea plants (7 weeks from sowing) grown under an 18-h photoperiod.

The chromatogram in Figure 2B shows the separation of naturally occurring polyamines from apical tissue of a mature G2 pea plant including the hexanediamine internal standard. The identities of the peaks were confirmed by adding polyamine standards to the extract of the plant tissue.

The procedure used in this study to separate dansylated polyamines appears to be faster and more sensitive, at least for plant tissues, than the HPLC method reported by Seiler and Knodgen (7) for mammalian tissues. Using a Lichrosorb RP-8 reverse phase column with a different methanol-water gradient, these investigators separated dansyl derivatives in less than 20 pmol amounts in 40 min. Detection of putrescine from mammalian tissues in the order of 10 to 20 μmol/g fresh weight required purification of the dansyl derivative on silica gel columns before HPLC. In addition, separations were recorded at two different sensitivities to detect putrescine, and the other two amines (spermidine and spermine) at the wide range of concentrations found in animal tissues. Other workers, using a Micropak CN-10 column (5) separated dansyl derivatives prepared from animal tissues in 22 min with sensitive detection of putrescine at 40 pmol amounts and spermidine or spermine at levels on the order of 20 pmol. Differences between the procedure used in this study and other HPLC methods may be related to the type of tissue being analyzed and the sensitivity of the detector.

Quantitation Procedure. Polyamines in tissues were quantitated by using a relative calibration procedure according to

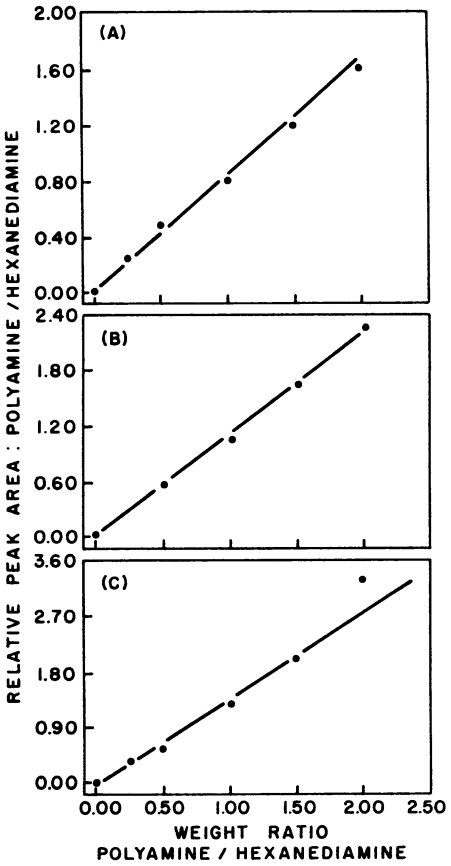

FIG. 3. Correlation graph for the determination of (A) putrescine, (B) spermidine, and (C) spermine. Each line was generated from the dansylation of 10 nmol of hexanediamine of which 1/2000 (or 5 pmol in 20 μl) was injected into the HPLC. The linear relationship between the area and weight ratios is (A) $y = 0.014 + 0.792x$, $r^2 = 0.998$; (B) $y = -0.0049 + 1.13x$, $r^2 = 0.999$; (C) $y = -0.110 + 1.55x$, $r^2 = 0.977$.

McNair and Bonelli (4). Known weights (nmol) of individual polyamines and the internal standard, hexanediamine, were dansylated and chromatographed. Weight ratios of individual polyamines to the internal standard were plotted against area ratios as determined from the area units given by the integrator attached to the fluorescence detector. Three curves were generated by this procedure, one each for putrescine, spermidine, and spermine (Fig. 3).

Quantifying the unknown amounts of amines in the samples required adding accurate amounts of the internal standard to the tissue at the time of extraction. The mixture was chromatographed and area ratios were determined. Weight ratios were determined from area ratios and the regression equations for the standard curves. The nmol ratios were then multiplied by the nmol of internal standard added to the extract. The internal standard was generally added in the order of 0.20 to 1 μmol/g fresh weight. This method of quantification eliminates the need for exact measurements of the injection quantities and avoids problems encountered with changes in detector response, since area ratios are constant (4). Hexanediamine appeared to be an appropriate internal standard, as it resolved well from other substances in the mixture, and eluted close to the naturally occurring amines.

The HPLC method in combination with fluorescence spectrophotometry allowed the separation and quantitation of polyamine levels in plant tissues similar to those detected by UV by Flores and Galston (3), although increased sensitivity over UV detection is clearly possible. The analysis is fast and allows for repeated assays in a short period of time. Amounts as low as 0.8 pmol of standard polyamine were detected, although higher amounts are found in plant tissues. The method was used routinely for measuring hundreds of samples from pea tissues.

Acknowledgments—The authors are grateful to Dr. Hector Flores for providing the dansylation procedure and useful suggestions with respect to the HPLC technique.

LITERATURE CITED

1. ABDEL-MONEM MM, K OHNO 1975 Separation of DNS derivatives of polyamines and related compounds by thin-layer and high-pressure liquid chromatograph. J Chromatogr 107: 416–419
2. BACHRACH U 1978 Analytical methods of polyamines. *In* RA Campbell, DR Morris, D Bartos, G Daves, F Bartos, eds, Advances in Polyamine Research, Vol 2. Raven Press, New York, pp 5–12
3. FLORES HE, A GALSTON 1982 Analysis of polyamines in higher plants by high performance liquid chromatography. Plant Physiol 69: 701–706
4. MCNAIR HM, EJ BONELLI 1969 Basic gas chromatograph. Consolidated Printer, Berkeley, CA, pp 150–151
5. NEWTON NE, K OHNO, M ABEL-MONEM 1976 Determination of diamines and polyamines in tissues by high-pressure liquid chromatography. J Chromatogr 124: 277–285
6. SEILER N 1971 Identification and quantitation of amines by thin-layer chromatography. J Chromatogr 63: 97–112
7. SEILER N, B KNODGEN 1978 Determination of di- and polyamines by high performance liquid chromatographic separation of their dimethylaminonaphthalene-1-sulfonyl derivatives. J Chromatogr 145: 29–39
8. SEILER N, M WIECHMANN 1970 TLC analysis of amines as their dans-derivatives. *In* A Niederwieser, G Pataki, eds, Progress in Thin-Layer Chromatography and Related Methods, Vol 1. Ann Arbor Humphrey Science, Ann Arbor, MI, pp 95–144