ELSEVIER

Journal of Chromatography A, 824 (1998) 175–180

JOURNAL OF CHROMATOGRAPHY A

High-performance liquid chromatographic determination and standardization of agmatine

K. Bencsik[a], T. Kremmer[a,*], M. Boldizsár[a], J. Tamás[b], M. Mák[b], E. Páldi[c]

[a]National Institute of Oncology, Department of Biochemistry, P.O Box 21, H-1525 Budapest, Hungary
[b]Central Research Institute for Chemistry, Hungarian Academy of Sciences, P.O Box 17, H-1525 Budapest, Hungary
[c]Agricultural Research Institute of the Hungarian Academy of Sciences, P.O Box 19, H-2462 Martonvásár, Hungary

Received 14 July 1996; received in revised form 13 February 1997; accepted 14 February 1997

Abstract

In order to standardize the reversed-phase high-performance liquid chromatographic (RP-HPLC) determination of natural polyamines, particularly of agmatine, which is important in plant physiology, a simple dansylation and thin-layer chromatography (TLC) procedure was developed for the separation and purification of dansyl-agmatine. The monodansyl structure of the agmatine derivative was verified by mass spectrometry. Dansyl-agmatine was used as a reliable reference compound for the validation of an improved RP-HPLC method and to the quantitation of plant polyamines. In two varieties of wheat that had been stressed by cold treatment, significant changes were demonstrated in the agmatine content of plant seedlings. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Agmatine; Polyamines

1. Introduction

Natural polyamines, putrescine (Pu), spermidine (Spd), spermine (Spn), agmatine (Agm), cadaverine (Cad) and their acetyl derivatives, are generally considered as ubiquitous bioregulators of numerous cell functions, which are required particularly in cell growth, division and differentiation [1–6]. In spite of the theoretical and practical importance of common polyamines (Pu, Spd, Spn) in cancer, in the stress response of plants, etc., relatively few data are concerned with the intracellular concentration and role of Agm [7–10]. Analysis of Agm in plants was stressed by assuming that the alternative arginine–Agm metabolic pathway may play a significant role in the biosynthesis of Pu in wheat genotypes, especially under stress conditions.

To measure the polyamine levels in biological samples, a wide variety of pre- and post-column derivatization and reversed-phase high-performance liquid chromatographic (HPLC) techniques are commonly used [2,11–15]. Because of the reasonable stability and detection of derivatives and its simplicity, we applied dansylation to the HPLC analysis of polyamines [11]. On investigating the bioregulative role of Agm in plant tissues, we have found that no proper Agm standards were available, and commercial Agm products represented a mixture of various polyamines. In order to fulfil the requirements of quantitative standardization, we have prepared dansyl (Dns)-Agm using a simple thin-layer chromatographic (TLC) method and have used it as a

*Corresponding author.

0021-9673/98/$ – see front matter © 1998 Elsevier Science B.V. All rights reserved.
PII: S0021-9673(97)00193-3

reliable standard for the HPLC determination of Agm. The stability and structure of Dns-Agm was checked by mass spectrometry and it was found that mono- but no di-dansyl derivatives were present. According to these considerations, a modified RP-HPLC method, validated using Dns-Agm, provided the correct basis for the analysis of Agm in stressed plants.

2. Experimental

2.1. Apparatus

HPLC analyses were carried out using a Merck-Hitachi liquid chromatographic system consisting of an L6200 A Intelligent Pump with a dynamic mixing chamber, a Rheodyne 7161 injector (20 μl loop), an L4250 UV–Vis detector, and also equipped with an RF-350 fluorescence detector (Shimadzu). Programs and calculations were performed by a D2500 Merck Chromato-Integrator. TLC was performed in a Desaga chamber, saturated with the solvent system used.

2.2. Reagents and chemicals

All chemicals (perchloric and hydrochloric acids, sodium carbonate and phosphate, ammonium acetate) were of analytical grade. Hydrochlorides of Pu, Spd, Spn, Cad and dansyl chloride (5-dimethylamino-1-naphthalenesulfonyl chloride) were purchased from Sigma (St. Louis, MO, USA). Agm was obtained from several sources. The solvents used (acetone, acetonitrile, cyclohexane, ethyl acetate, methanol, toluene) were of HPLC quality (LiChrosolv, Merck, Darmstadt, Germany) and the water used was glass-distilled. TLC was performed on silica gel 60 pre-coated plates (Merck, HPTLC).

2.3. Sample preparation

Stock solutions of reference polyamines (10 nmol/ml) were prepared in distilled water and used throughout. Fresh plant tissues (corn, wheat) were pulverized in liquid nitrogen and homogenized in a phosphate buffered saline solution (PBS; 100 mg/ml). Tissue homogenates were extracted consecutively three times with ice-cold 0.7 *M* perchloric acid and the residue was separated by centrifugation (1000 *g*, 20 min, O°C). Combined supernatants were pre-separated by solid-phase extraction on a micropreparative Dowex 50-X8 column (3×0.5 cm), as described previously [11]. Eluates with 6*M* HCl containing polyamines were evaporated, the dry residue was dissolved in 1% sodium hydrogencarbonate and dansylated immediately.

2.4. Dansylation and TLC separation of Dns-Agm

Saturated sodium carbonate (800 μl) and dansyl chloride (1600 μl, 10 mg/ml in acetone) were mixed in sealed tubes with solutions (800 μl) of the reference polyamines or biological samples in 1% sodium hydrogencarbonate, and kept for 1 h at 56°C in the dark. After evaporation of the acetone, dansyl polyamines were extracted into toluene (1500 μl) and were used for TLC. For quantitative HPLC, an aliquot (1000 μl) of the toluene extract was dried under vacuum and dissolved in methanol (100–200 μl). Dns-Agm was purified on a preparative scale by ascending TLC, using a cyclohexane–ethyl acetate (1:1, v/v) solvent system. Dansyl derivatives were detected under UV light and Dns-Agm, positioned near the start (R_F=0.1), was collected and extracted from the silica gel with methanol–toluene (2:1, v/v). Dns-Agm was dried under vacuum, measured by weight, protected from light and kept at −20°C until required.

2.5. HPLC of dansyl polyamines

Dansyl polyamines were separated and measured by RP-HPLC on a Nucleosil C_{18} column (20×0.46 mm I.D., 5 μm) using gradient elution with the following solvent systems:

(A) 10 m*M* ammonium phosphate buffer (pH 4.4)–acetonitrile (45:55, v/v);

(B) acetonitrile–methanol (37:3, v/v).

The gradient program developed was 0–15% of B for 20 min, then 100% B for 10 min, and equilibration again with A for 10 min. The flow-rate was 1.0 ml/min and 20 μl volumes of the samples were injected. Fluorescence detection was at an excitation wavelength of 340 nm and an emission wavelength of 515 nm. For the quantitative measurement of Pu,

Spd and Spn, external calibration curves were plotted using individual polyamine standards.

2.6. Mass spectrometry

Dns-Agm, isolated by HPLC from the dansylation reaction mixture, was investigated by mass spectrometry using the fast atom bombardment (FAB) technique. The measurements were made using a VG ZAB-2 SEQ type tandem mass spectrometer (Fisons) of a B/E/Q/Q geometry with a Cs^{+} gun of 20 keV ion energy. Glycerol was used as the matrix solvent. The protonated molecule (m/z 364) was also studied by high energy collision using neutral Ar gas in the second-field-free region. The fragment ions produced were investigated by the collision-induced dissociation–mass analyzed ion kinetic energy spectroscopy (CID–MIKE) technique.

3. Results and discussion

3.1. Chromatographic procedures

The aim of this study was to provide a reliable standard for the HPLC determination of Agm in various biological samples, especially in plant tissues. In practice, we observed that Agm was relatively labile and commercial products were heavily contaminated by several impurities (Fig. 2B) that

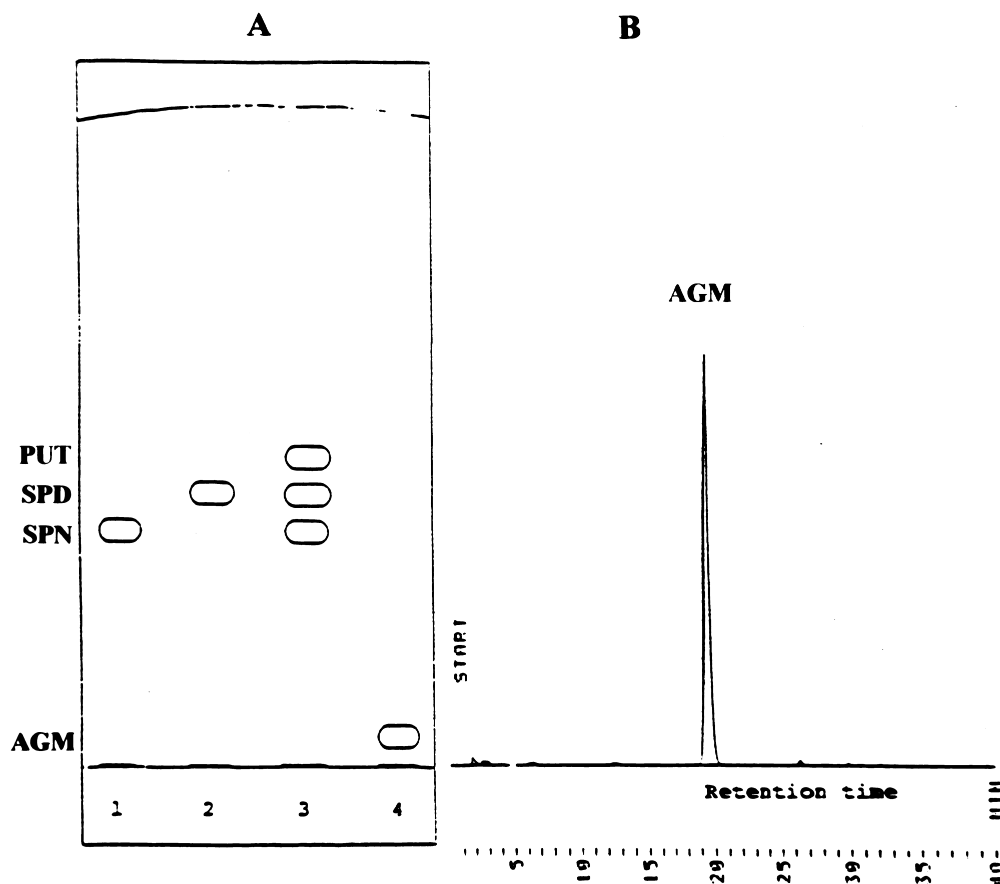

Fig. 1. TLC and HPLC separation of dansyl (Dns) polyamines. (A) TLC separation of Dns-polyamines on a silica gel 60 plate in cyclohexane–ethyl acetate (1:1, v/v). Detection, UV fluorescence. PUT, SPD, SPN and AGM indicate the dansyl derivatives of putrescine, spermidine, spermine and agmatine, respectively. (B) Purity control (HPLC) of Dns-Agm obtained from TLC (Lane 4). For the HPLC conditions, see Fig. 2.

made it impossible to prepare stock solutions with the correct Agm content. Fig. 1A demonstrates that the simple procedure of dansylation and preparative TLC on high-resolution silica gel plates using cyclohexane–ethyl acetate (1:1, v/v) as the mobile phase [2] was suitable for the isolation and purification of Dns-Agm. RP-HPLC with a modified gradient elution program using ammonium phosphate buffer (10 m*M*, pH: 4.4)–acetonitrile (45:55, v/v) and acetonitrile–methanol (37:3, v/v) mobile phases verified the purity and chromatographic position of Dns-Agm obtained from TLC (Figs. 1B and 2A). In contrast to other derivatives [7], Dns-Agm proved to be stable for weeks when stored in methanol, in a cool place and in the dark, without detectable deterioration. In five separate experiments, an average recovery of 87% (R.S.D.=5.6%) was obtained for dansyl derivatives following toluene extraction. Accordingly, we thoroughly calibrated the HPLC method with a stock solution (10^{-2} M) of Dns-Agm and a linear correlation (r=0.995) was obtained in a range of 2.5–250 nmol Agm vs. area. HPLC determination of Dns-Agm was reproducible, with a precision of R.S.D. values of 4.2 and 6.1% at 50 and 100 nmol of Dns-Agm, respectively (seven experiments each). Under the recommended chromatographic conditions, the retention time of Dns-Agm was t_R= 19.9±0.14 min (R.S.D.=0.7%) and the minimum detectable concentration was 100 pmol Agm/20 μl of sample.

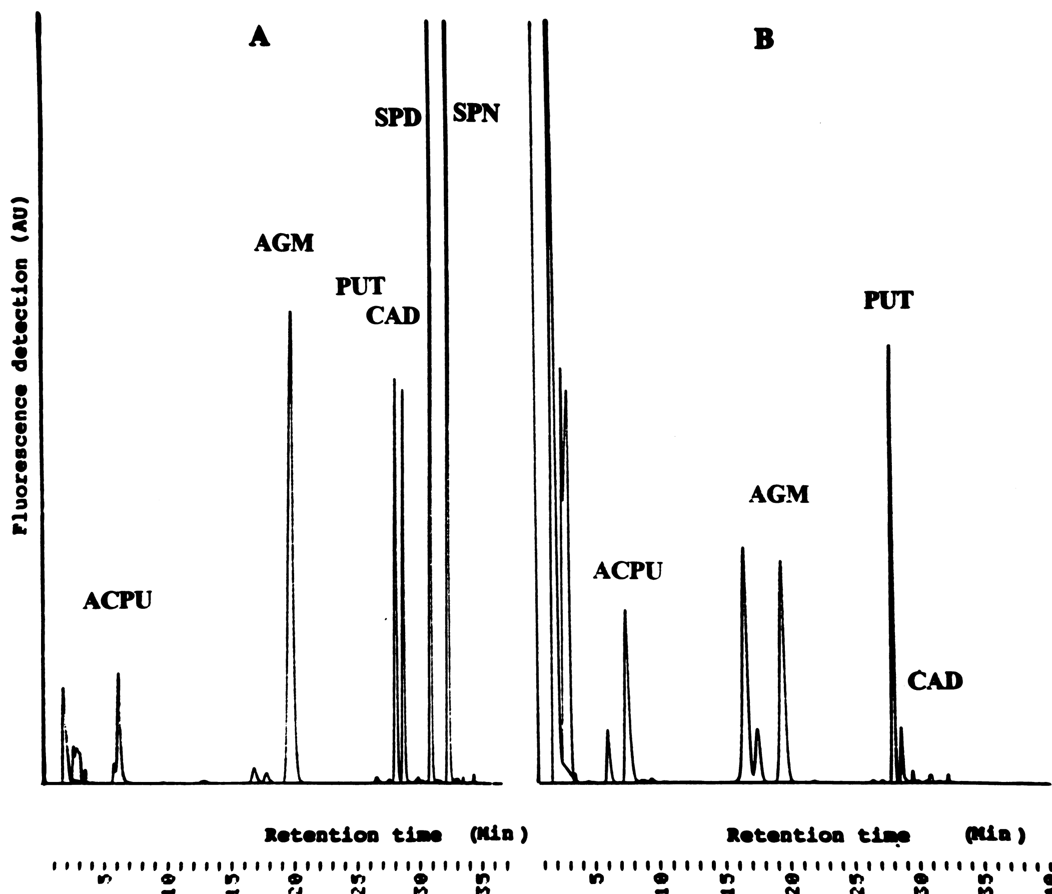

Fig. 2. HPLC separation of Dns-polyamines on a Nucleosil C_{18} column (20×0.46 mm I.D., 5 μm) with gradient elution (see Section 2). (A) Dansyl derivatives of acetyl putrescine (ACPU), agmatine (AGM), putrescine (PU), cadaverine (CAD), spermidine (SPD) and spermine (SpN), respectively. (B) Polyamine pattern of a commercial Agm.

3.2. Structure elucidation

The mass (FAB) spectra of Dns-Agm samples obtained either from TLC or isolated by HPLC showed a very abundant peak at m/z 364, corresponding to the protonated molecule of monodansyl-Agm. At the same time, no signal exceeding the background noise (approx. 1%) was detectable at m/z 597 in this spectrum, which should be characteristic of didansyl-Agm. Consequently, the dansylation procedure recommended here resulted in monodansyl-Agm exclusively. Further structural information for the monodansyl-Agm was achieved from the CID–MIKE spectrum of the m/z 364 peak. This spectrum exhibited abundant fragments at m/z 170 and 347. The former corresponds to the formation of a dimethylaminonaphthyl cation, which is significant for dansylated polyamines, while the latter can be attributed to the loss of an NH_3 molecule from the parent m/z 364. The elimination of ammonia proved to be characteristic for polyamines containing guanidine moieties [16]. Both of these reactions are in accordance with the monodansyl-Agm structure.

3.3. Applications

In this work, the determination of plant polyamines, particularly of Agm, was attempted in order to discover the changes that are induced in the polyamine composition of wheat during a long period of low temperature treatment. The data in Table 1 show that in winter wheat (Mironovskaya 808), the quantity of Agm increased substantially as the treatment progressed, while in spring wheat (Super X), such an increase could not be observed. On the other hand, reversed changes in the Pu and Spd levels previously reported in various wheat genotypes [17], in maize [18] and Zucchini squash [19] were observed in both varieties of cold-stressed wheat. These results seem to indicate that in wheat genotypes that behave differently in response to low temperature, the significance and role of the alternative biosynthetic route for Pu via Agm may differ considerably.

Summarizing our results, it can be concluded that the HPLC determination of dansyl polyamines using Dns-Agm for standardization can be applied successfully to the investigation of the bioregulative role of polyamines in plant physiology.

Acknowledgements

The authors thank Mrs. Zsuzsa Németh and Andrea Napholcz for valuable technical assistance. This work was supported by the Hungarian Research Foundation (OTKA T-4375) and the Ministry of Welfare (ETT T-412/90).

Table 1
Effect of cold treatment on the polyamine content in seedlings of two wheat varieties

Wheat varieties	Polyamines[a]	Time-schedule of cold treatment (weeks)[c]				
		Control[b]	3	5	6	7
Miranovskaya 808	Agm	123±18	198±20	220±22	634±57	77±69
	Pu	388±27	421±39	479±36	506±49	511±52
	Spd	240±25	237±28	234±33	195±25	116±20
	Spn	86±11	91±13	87±13	90±14	75±9
Super X	Agm	40±8	86±10	87±11	89±9	25±5
	Pu	301±35	440±42	484±34	492±31	505±46
	Spd	198±23	122±17	107±14	85±11	63±9
	Spn	26±6	32±7	74±9	33±6	20±5

Concentrations are expressed as nmol polyamines per g of wet plant tissue.
Figures are averages (±SE) of three separate determinations.
[a] Agm=Agmatine, Pu=putrescine, Spd=spermidine, Spn=spermine.
[b] No treatment.
[c] 72 hour-old etiolated seedlings were treated at 0°C in the dark.

References

[1] A. Pegg, Cancer Res. 48 (1988) 759.
[2] N. Seiler, B. Knödgen, J. Chromatogr. 164 (1979) 155.
[3] N. Seiler, J. Chromatogr. 379 (1986) 157.
[4] A.W. Galston, in U. Babrach and Y.M. Heimer (Editors), The Physiology of Polyamines, Vol. II, CRC Press, Boca Raton, FL, 1988, p. 99.
[5] H.E. Flores, C.M. Protacio, M.W. Signs, Recent Adv. Phytochem. 23 (1989) 329.
[6] A.W. Galston, R.K. Sawhney, Plant Physiol. 94 (1990) 406.
[7] R.D. Slocum, H.E. Flores, A.W. Galston, L.H. Weinstein, Plant Physiol. 89 (1989) 512.
[8] H.E. Flores, A.W. Galstone, Plant Physiol. 69 (1982) 701.
[9] S. Regunathan, D.L. Feinstein, W. Raasch, D.J. Reis, NeuroReport 6 (1995) 1897.
[10] M.J. Lortie, W.F. Novotny, O.W. Peterson, V. Vallon, K. Malvey, M. Mendonca, J. Satriano, P. Insel, S.C. Thompson, R.C. Blantz, J. Clin. Invest. 97 (1996) 413.
[11] T. Kremmer, M. Boldizsár, E. Paulik, Chromatographia 26 (1988) 423.
[12] P. Koski, I.M. Helander, M. Sarvas, M. Vaara, Anal. Biochem. 164 (1987) 261.
[13] C.F. Verkoelen, J.C. Romijn, F.H. Schroeder, W.P. Van Schalkwijk, T.A.W. Splinter, J. Chromatogr. 426 (1988) 41.
[14] C. Löser, U. Wunderlich, U.R. Fölsch, J. Chromatogr. 436 (1988) 249.
[15] T. Bartók, G. Börcsök, F. Sági, J. Liq. Chromatogr. 15 (1992) 777.
[16] G.J. Feistner, J. Mass Spectrom. 30 (1995) 1546.
[17] I. Rácz, M. Kovács, D. Lásztity, O. Veisz, G. Szalai, E. Páldi, J. Plant Physiol. 148 (1996) 368.
[18] G. Szalai, T. Janda, T. Bartok and E. Páldi, Plant Physiol. Biochem., special issue (1996) 257.
[19] G.F. Kramer, C.Y. Wang, Physiol. Plant 76 (1989) 479.