Journal of Chromatography, 226 (1981) 208—212
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 978

Note

Rapid assay of spermidine synthase activity by high-performance liquid chromatography

R. PORTA*, C. ESPOSITO* and O.Z. SELLINGER*

Laboratory of Neurochemistry, Mental Health Research Institute, University of Michigan Medical Center, Ann Arbor, MI 48109 (U.S.A.)

(First received February 26th, 1981; revised manuscript received May 21st, 1981)

The final steps of spermidine and spermine biosynthesis involve the addition of an aminopropyl group from S-methyladenosylhomocysteamine (decarboxylated S-adenosyl-L-methionine) to putrescine and spermidine respectively [1—3]. The reactions, forming 5′-methylthioadenosine as a secondary product, are catalyzed by two distinct synthases [4—7]. Thus far, studies concerning polyamine metabolism have addressed primarily the decarboxylations of ornithine and S-adenosylmethionine (SAM), which are considered the rate-limiting steps in this metabolic process. Recent methodological improvements, including techniques for the preparation of decarboxylated SAM as well as for the separation of reactants from reaction products have provided important information regarding spermidine and spermine synthases [8—10]. Our interest in polyamines derives from our findings that brain levels of SAM are decreased prior to seizures elicited by a single administration of the chemical convulsant L-methionine-*d*,*l*-sulfoximine (MSO) [11]. This depletion of SAM is due to its increased utilization via transmethylation reactions [12]. Since the depletion of SAM levels by MSO could also limit the availability of decarboxylated SAM, the precursor of spermidine, we decided to investigate polyamine biosynthesis in the MSO epileptogenic mouse brain. In this paper we describe a rapid and economic high-performance liquid chromatographic (HPLC) method for the separation and quantitation of spermidine formed upon incubation of putrescine with a crude extract of mouse brain. This method thus permits the rapid measurement of spermidine synthase (EC 2.5.1.16) activity.

---

*Present address: Department of Biochemistry, 1st Medical School, via Costantinopoli 16, University of Naples, Naples, Italy.

0378-4347/81/0000—0000/$02.50 © 1981 Elsevier Scientific Publishing Company

EXPERIMENTAL

Equipment

A Waters Model ALC/GPC-204 liquid chromatograph equipped with a Model 6000A high-pressure pump, a U6K loop injector and Model 440 absorbance detector (254 nm) was used throughout the study. The chromatographic runs were recorded on an Omniscribe recorder (Texas Instruments, Austin, TX, U.S.A.). A pre-packed (30 × 0.4 cm I.D.) μBondapak C_{18} (10-μm particle size) column (Waters Assoc., Milford, MA, U.S.A.) was used to separate the benzoylated polyamines.

Materials

Putrescine dihydrochloride and spermidine trihydrochloride were from Sigma (St. Louis, MO, U.S.A.). [1,4-^{14}C]Putrescine dihydrochloride (122 mCi/mmol) and [^{14}C]spermidine (122 mCi/mmol) were from Amersham/Searle (Arlington Heights, IL, U.S.A.). Decarboxylated SAM was kindly supplied by Dr. G. Stramentinoli (Bio Research, Liscate, Italy). All other chemicals were of analytical grade quality.

Enzyme source

Brains from adult male Swiss—Webster mice (25—30 g) were homogenized in 5 volumes (w/v) of 25 m*M* potassium phosphate buffer (pH 7.2). The mixture was centrifuged at 100,000 *g* for 60 min and the supernatant used as the source of spermidine synthase. Protein content was measured by the method of Lowry et al. [13].

Spermidine synthase assay

Unless otherwise stated a standard reaction mixture (0.5 ml) contained 0.1 *M* sodium phosphate buffer (pH 7.4), 0.05 m*M* putrescine, 2.0 μM [1,4-^{14}C]putrescine, 0.06 m*M* decarboxylated SAM and 2 mg of enzyme protein. Blanks contained no decarboxylated SAM. After incubation at 37°C for 90 min the reaction was stopped by addition of 0.25 ml of 0.6 *M* perchloric acid. After centrifugation (10 min at 10,000 *g*), the supernatants were subjected to the benzoylation procedure and the extraction of the benzoylated polyamines as previously described [14,15]. The benzoylated polyamines were dissolved in 200 μl of methanol and aliquots (50 μl) were injected onto the μBondapak C_{18} column in order to separate putrescine from spermidine [15]. Elution was with 60% methanol (v/v) at a flow-rate of 2 ml/min. The quantitative determination of spermidine was carried out in the following two ways: (a) by radiometric determination of the [^{14}C]spermidine formed from [^{14}C]putrescine. In this case the HPLC eluates were collected (0.8 ml/fraction) into scintillation vials and counted after addition of ACS (a tissue solubilizer—scintillant mixture from Amersham/Searle), and (b) by measuring the increase in UV absorbance (254 nm) of the spermidine peak with respect to the blank (this correction is necessary when crude extracts, containing endogenous spermidine, are used as enzyme source). Calibration curves were generated by using [^{12}C]putrescine and spermidine in the presence of tracer amounts of [^{14}C]putrescine and spermidine to determine recoveries for each polyamine.

RESULTS AND DISCUSSION

Fig. 1 illustrates a typical HPLC pattern of benzoylated polyamines as derived from the enzymatic assay sample and its corresponding blank. The separation of putrescine and spermidine was highly satisfactory and the HPLC run was completed in 6 min. The increase in absorbance of the spermidine peak as well as the appearance of a radioactive peak corresponding to spermidine are well evident in the experimental sample. It should be noted that since the sensitivity of the detection of the benzoylated spermidine by UV absorbance is sufficiently high (about 0.1 nmol) it is possible to avoid, if desired, the use of radio-labelled putrescine in the enzymatic assay. Fig. 2 shows the time course of spermidine formation. The reaction was linear for up to 90 min using the enzymatic assay conditions described in the text, whether spermidine formation is determined radiometrically or by UV absorbance. Also, the reaction was linear with up to 2 mg of protein in the reaction mixture (Fig. 3). Fig. 4 shows the apparent K_m of the enzyme for putrescine. The apparent K_m values (2.2 and 3.3 · 10^{-5} *M*), calculated using either procedure of product quantitation, are in good agreement with those previously reported for mammalian spermidine synthase [16,17].

The recent widespread interest in the aminopropyltransferase reactions has led to various attempts to develop new and more rapid procedures for their in vitro assays. Among the various assay procedures recently reviewed [16], those using HPLC separation of polyamines require a flow cell for radioactivity measurements of the formed spermidine or spermine and a total elution time of at least 15 min for a single analysis [18,19]. Benzoylation appeared to be a much simpler and economical tool for the derivatization, separation and quantitation of polyamines produced in vitro upon incubation of the appro-

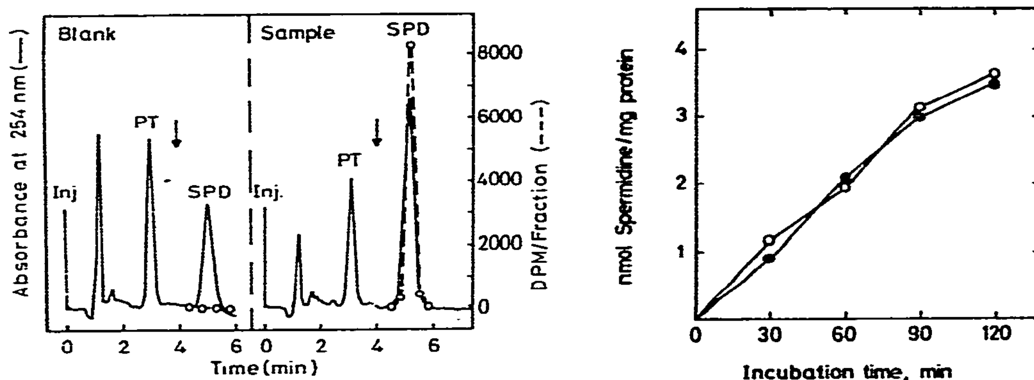
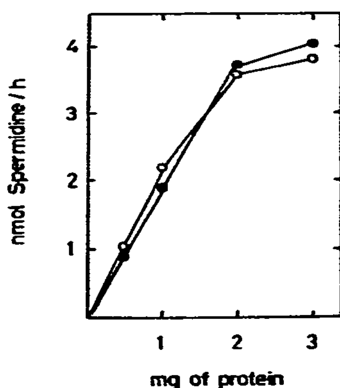


Fig. 1. HPLC separation of the benzoyl derivatives of putrescine (PT) and spermidine (SPD) contained in a standard reaction sample and in the corresponding blank after 60 min of incubation at 37°C. Details of the enzymatic assay and of the chromatographic run are described in the text and also in ref. 15. Fractions (0.8 ml) were collected for determination of radioactivity. The arrows indicate a change in detector sensitivity from 0.2 to 0.05 a.u.f.s.

Fig. 2. Mouse brain spermidine synthase activity as a function of time. The assay conditions are as described in the text. Both the absorbance (o——o) and the radiometric (•——•) determination of product formation represent the mean value of duplicate assays. The reproducibility was within 10%.



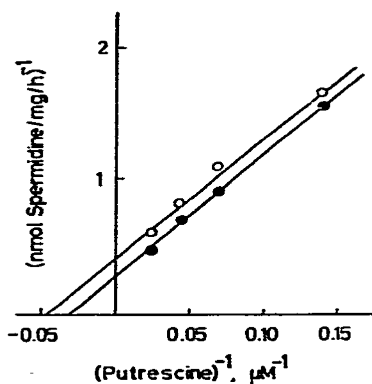


Fig. 3. Spermidine production as a function of protein concentration. The details of the assay conditions are as described in the text. Both the absorbance (o——o) and the radiometric (•——•) determination of product formation represent the mean value of duplicate assays. The reproducibility was within 10%.

Fig. 4. Graphic estimation of the apparent K_m for putrescine (Lineweaver—Burke plot). The assay conditions were as described in the text. Both the absorbance (o——o) and the radiometric (•——•) determination of product formation represent the mean value of duplicate assays. The reproducibility was within 10%.

priate aminopropyltransferase assay mixture. This paper specifically describes an assay procedure for spermidine synthase although, as shown elsewhere [15], it is theoretically possible to use the same methodology for the determination of spermine synthase activity. The purity of the samples after derivatization, the brevity of the chromatographic run and the isocratic elution schedule are the favorable characteristics of the assay technique described herein. The most significant improvement over existing methods, however, is that the high sensitivity of the assay obviates the determination of the radioactivity in the spermidine peak for an accurate quantitation of spermidine synthase activity. Indeed, as shown by our findings, there was virtual identity between spermidine synthase activity values obtained with the non-isotopic and the isotopic procedure of product quantitation.

Results of applications of the HPLC method in studies of polyamine biosynthesis in the brain of animals subjected to convulsant stimuli [15,20] have been presented elsewhere.

ACKNOWLEDGEMENT

This work was supported by the United States Public Health Service, Grant NS-06294.

REFERENCES

1 H. Tabor and C.W. Tabor, Advan. Enzymol. Relat. Areas Mol. Biol., 36 (1972) 203.
2 C.W. Tabor and H. Tabor, Ann. Rev. Biochem., 45 (1976) 285.
3 A. Raina and J. Janne, Med. Biol., 53 (1975) 121.
4 A.E. Pegg and H.G. Williams-Ashman, J. Biol. Chem., 244 (1969) 682.

5 J. Janne, A. Schenone and H.G. Williams-Ashman, Biochem. Biophys. Res. Commun., 42 (1971) 758.
6 A. Raina and P. Hannonen, FEBS Lett., 16 (1971) 1.
7 P. Hannonen, J. Janne and A. Raina, Biochem. Biophys. Res. Commun., 46 (1972) 341.
8 R.L. Pajula, A. Raina and T. Eloranta, Eur. J. Biochem., 101 (1979) 619.
9 H. Hibasami, R.T. Borchardt, S.Y. Chen, J.K. Coward and A.E. Pegg, Biochem. J., 187 (1980) 419.
10 V. Zappia, G. Cacciapuoti, G. Pontoni and A. Oliva, J. Biol. Chem., 255 (1980) 7276.
11 R.A. Schatz and O.Z. Sellinger, J. Neurochem., 24 (1975) 63.
12 R.A. Schatz, K. Frye and O.Z. Sellinger, J. Pharmacol. Exp. Ther., 207 (1978) 794.
13 O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 265.
14 J.W. Redmond and A. Tseng, J. Chromatogr., 170 (1979) 479.
15 R. Porta, R.L. Doyle, S.B. Tatter, T.E. Wilens, R.A. Schatz and O.Z. Sellinger, J. Neurochem., in press.
16 H. Hibasami, M. Tanaka, J. Nagai and T. Ikeda, FEBS Lett., 116 (1980) 99.
17 K. Samejima and Y. Nakazawa, Arch. Biochem. Biophys., 201 (1980) 241.
18 V. Zappia, G. Cacciapuoti, G. Pontoni, F. Della Ragione and M. Carteni-Farina, in C.M. Caldarera, V. Zappia and U. Bachrach (Editors), Advances in Polyamine Research (Vol. 3), Raven Press, New York, 1981, p. 39.
19 J.K. Coward, N.C. Motola and J.D. Moyer, J. Med. Chem., 20 (1977) 500.
20 R. Porta, R.A. Schatz and O.Z. Sellinger, Trans. Amer. Soc. Neurochem., 12 (1981) 150.