CHROM. 5565

A MICRO METHOD FOR THE QUANTITATIVE ESTIMATION OF PUTRESCINE IN TISSUES

N. SEILER AND A. ASKAR

Max-Planck-Institut für Hirnforschung, Arbeitsgr. Neurochemie, Frankfurt-am-Main, (G.F.R.)

(Received June 20th, 1971)

SUMMARY

Putrescine can be quantitatively estimated in tissue after its extraction by pre-separation of the amine mixture by ion-exchange chromatography on small columns, reaction of the putrescine fraction with 1-dimethylaminonaphthalene-5-sulphonyl chloride, two-dimensional thin-layer chromatographic separation of the dansylated amines, and fluorimetry of the bis-DANS-putrescine extracted from the carrier. Recovery of putrescine from tissue is 93–94%, independent of the amount of putrescine applied to the columns. Losses occur during ion-exchange chromatography. The sensitivity of the method is higher by a factor of 50–100 compared with existing methods. Reproducibility is ±1–3% for reference samples. Concentrations of putrescine were determined in mouse brain and liver.

INTRODUCTION

Putrescine (1,4-diaminobutane) is supposed to be as generally distributed in the living world as are spermidine and spermine, since it is the physiological precursor of these polyamines. Although quite a number of methods have been described for the determination of putrescine in tissue, quantitative data are relatively scarce and in many cases not very reliable. One reason for this may be that general interest in the naturally occuring polyamines has grown only recently[1], despite the fact that remarkable features of these amines have been known for some time[2]. Another reason for the lack of data on putrescine may be sought in the relative insensitivity of the available methods. Most of them depend on the colour reaction with ninhydrin[2–7]; some authors suggested the use of 2,4-dinitrophenyl derivatives for photometric determination[2,8–11], and the recently described gas–liquid chromatographic method[12] can measure only microgram quantities. The unreliability of the data derives from the fact that the spots on electropherograms and the fractions eluted from columns were normally investigated for uniformity only by the use of further chromatographic procedures, and not by independent methods, so that in some cases even the identity of the substances determined with putrescine may be questionable.

During the last few years we have devised in our laboratory solvent systems for the thin-layer chromatographic (TLC) separation of the highly fluorescent 1-dimethylaminonaphthalene-5-sulphonyl (DANS) derivatives of more than 100 biogenic amines and of amines of pharmacological interest, including a series of polyamines[13,14].

The principles of the quantitative determination of the DANS derivatives on thin-layer plates have been described[15]. One-dimensional systems for the separation of the DANS derivatives of spermidine and spermine, among others, from all other fluorescent spots of comparable intensity in dansylated tissue extracts were used as the basis for the quantitative determination of spermidine and spermine in different tissues[16–19].

In the present work, a sensitive fluorimetric method based on the same principles is described for the determination of putrescine in tissue. A similar procedure to that described here has been applied recently to the separation of putrescine and some of its metabolites on a large scale[20].

MATERIAL AND METHODS

Experimental animals

Male albino mice (NMRI) (Süddeutsche Versuchstierfarm, H. Voss K.G., Tuttlingen, G.F.R.) of weight 30–35 g were used.

Preparation of the tissue extracts

The animals were killed by decapitation. The brain and liver were removed as quickly as possible and immediately homogenized with ice-cold 0.2 *N* $HClO_4$ (1:25 w/v). Samples of the homogenates (2 ml, corresponding to *ca.* 77 mg of tissue) were centrifuged for 15 min at 800 *g* and the sediments re-extracted with 2 ml of 0.2 *N* $HClO_4$. The combined supernatants were mixed with 10 ml of ethanol and stored at 3° for 16 h to complete protein precipitation.

Ion-exchange chromatography

The cleared tissue extracts were applied to small columns (6 × 60 mm) with 2.0 ml of Dowex 50-W X8, 100–200 mesh (H^+ form), at a flow rate of 1.5–2 ml/h. The columns were washed with 20 ml of 1 *N* HCl at a flow rate of 3 ml/h. These eluates were discarded. Putrescine and some other amines were eluted from the resin with 10 ml of 3.5 *N* HCl at a flow rate of *ca.* 1.5 ml/h. The putrescine fractions were evaporated almost to dryness by a stream of air at 60–70°.

Dansylation

The residues of the putrescine fractions were dissolved in 1 ml of distilled water. To these solutions, 10 mg of DANS-Cl dissolved in 2.5 ml of acetone were added, and the reaction mixtures saturated with $Na_2CO_3 \cdot 10H_2O$. After completion of the dansylation (normally by reaction overnight at room temperature), 10 mg of proline dissolved in 0.2 ml of water were added in order to react with the excessive DANS-Cl. After 2h, the dansylated amines were extracted from the reaction mixtures with 2 × 10 ml of benzene or toluene. The organic layers were taken to dryness in a stream of air at 40° and the residues were dissolved in 0.1 ml of toluene–ethyl acetate (7:3).

Thin-layer chromatography

Each sample with the dansylated amine mixture was quantitatively applied on to a separate 20 × 20 cm thin-layer plate (200 μm layers of Silica Gel G, E. Merck, Darmstadt, G.F.R.). The plates were developed in two directions in the manner

described in detail earlier[13]. The solvents for the first direction were: (a) trichloroethylene–methanol (19:1) and (b) benzene–methanol (19:1). The solvent for the second direction was chloroform–triethylamine (10:1), which should be prepared immediately before use.

Reference samples of pure bis-DANS-putrescine were run on the plate edges to allow the localization of the putrescine spot on the two-dimensional chromatogram.

Quantitation

The thin-layer plates were dried for 10 min at room temperature. After the putrescine spot was marked under a UV lamp, the corresponding plate area was scraped out quantitatively with the zone extractor described earlier[21], and the silica gel was collected in 10-ml centrifuge tubes. DANS-putrescine was extracted from the carrier by shaking with 5 ml of benzene–acetic acid (90:1)[14,15,21]. After centrifugation at 800 *g* for 15 min, the fluorescence intensity of 3-ml portions of the extracts was measured at 500 nm; fluorescence was excited at a wavelength of 365 nm. (A Zeiss spectrophotometer PMQII was used for the fluorescence measurements in combination with the fluorescence accessory ZFM4).

Standardisation

Each determination of putrescine in tissue was made on at least two parallel samples; to two further homogenate samples, known amounts of putrescine·2HCl (Th. Schuchardt, München, G.F.R.) were added as internal standards. Calculations of the amounts of endogenous putrescine were based on these internal standards.

Mass spectrometry

Mass spectra were prepared with a Varian MAT CH5 mass spectrometer under the usual conditions, with an electron beam energy of 70 eV. For details of the mass spectrometry of DANS-amides, see ref. 22.

RESULTS

Fig. 1 shows the chromatogram obtained from a dansylated brain extract prepared as described above and subsequently separated by two-dimensional TLC. The chromatograms obtained from liver tissue were of comparable complexity. The fluorescing spot corresponding in its chromatographic behaviour to bis-DANS-putrescine exhibited a mass spectrum identical with the spectra of authentic samples of bis-DANS-putrescine, M^+ at $m/e = 554$ (ref. 22). It was ensured in this way that only putrescine was determined by our quantitative procedure.

It is well established that simple primary or secondary amines react quantitatively with DANS-Cl under favourable reaction conditions[14,15,21,23]. Further it has been demonstrated that a linear relationship is obtained between fluorescence intensity and the amount of DANS-derivative. This is demonstrated for bis-DANS-putrescine in Fig. 2 not only for reference samples, which were directly dansylated and measured after their TLC separation (curve A), but also for putrescine that was added to tissue homogenates in known amounts and then recovered from the tissue together with the endogenous putrescine in the manner described (curve B). From the slope of this curve and from Table I it can be calculated that the recovery of

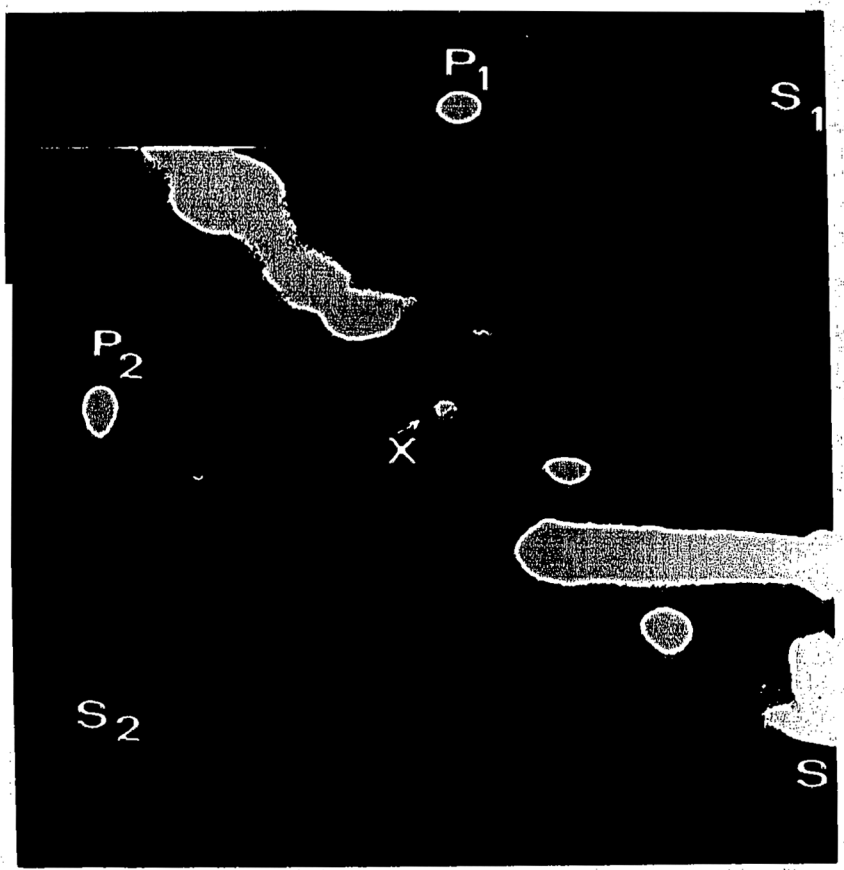

Fig. 1. Two-dimensional chromatogram of DANS-amides from the putrescine fraction of mouse-brain extract. Solvents: 1st dimension, (a) trichloroethylene–methanol (10:1), (b) benzene–methanol (10:1); 2nd dimension, chloroform–triethylamine (10:1). S, S_1, S_2 = starting points; X = spot corresponding to endogenous putrescine; P_1, P_2 = reference samples of bis-DANS-putrescine.

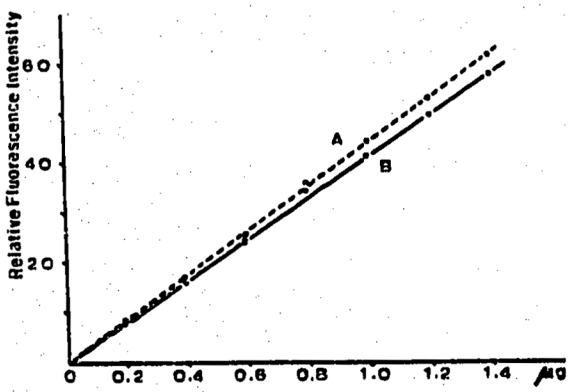

Fig. 2. Relationship between relative fluorescence intensity and amount of bis-DANS-putrescine. Curve A: directly dansylated reference samples; curve B: recovered putrescine from tissue samples. Ordinate: relative fluorescence intensity (arbitrary units); abscissa: amount of putrescine·2HCl (µg).

TABLE I

RECOVERY OF PUTRESCINE FROM THE ION-EXCHANGE COLUMNS

Samples were applied to the ion-exchange columns with known amounts of putrescine·2HCl; tissue extracts corresponding to 77 mg of tissue (see MATERIAL AND METHODS section), and tissue extracts with known amounts of added putrescine·2HCl. Calculations of recovery are based on the relative fluorescence intensities of the reference samples that were directly dansylated without passing the columns.

Tissue	*Number of experiments (tissues from different animals)*	*Amount of putrescine·2HCl (μg)*	*Relative fluorescence intensity (arbitrary units)*			*Recovery of putrescine from the columns (%)*
			Reference samples	*Tissue extract without added putrescine·2HCl*	*Chromatographed samples with added putrescine·2HCl*	
—	9	0.4	17.2 ± 0.27	-	16.1 ± 0.15	93.4 ± 0.87
—	3	0.6	25.2 ± 0.50	-	23.9 ± 0.45	95.2 ± 1.43
Liver	1	0.2	8.8	4.0	12.7	92.3
	10	0.4	17.3 ± 0.20	13.0 ± 0.04	30.6 ± 1.43	93.2 ± 5.4
	5	0.6	25.9 ± 1.0	19.3 ± 2.56	41.0 ± 1.08	95.7 ± 3.5
	1	0.8	36.0	11.0	46.2	97.7
	1	1.0	44.3	11.0	52.5	93.8
	1	1.2	53.3	11.0	60.8	93.8
	1	1.4	62.1	10.0	67.7	92.0
Brain	11	0.4	40.2 ± 1.5	14.0 ± 2.0	51.0 ± 3.38	93.4 ± 1.81

putrescine from the columns was 93–94 %, independent of the amount of putrescine applied to the columns and also independent of whether putrescine solutions were separated by ion-exchange chromatography or whether the putrescine was added to tissue. The reproducibility of the determinations was also independent of these parameters for reference samples: $s = \pm 1$–3 % (for 0.4 μg of putrescine·2HCl). This value is in agreement with previous results for quantitative determinations of different amines by the extraction procedure[14, 15, 21, 24].

Since the DANS derivative of putrescine bears two independently fluorescing dansyl groups per molecule of amine, its molar fluorescence efficiency is twice that of a monoamine[15]. The detection sensitivity for putrescine is therefore high. The detection limit of the described method is, however, dependent on several parameters, so that no absolute values can be given. With the fluorimeter used in our experiments, 0.1 nmole of bis-DANS-putrescine could be determined without difficulty. Concentration of the bis-DANS-putrescine solution to 0.5 ml and the use of microcuvettes can lower the detection limit by approximately one order of magnitude.

In mouse brain and liver, the following values for putrescine content were determined from a total of 15 animals: for brain, 10.0 ± 1.2 nmoles/g of fresh tissue; and for liver, 25.2 ± 3.0 nmoles/g of fresh tissue.

DISCUSSION

The suggested pre-separation of the tissue extracts by ion-exchange chromatography can be performed with almost unlimited numbers of samples without any difficulty. The two-dimensional separation of each sample on a separate plate, how-

ever, is somewhat laborious and expensive, since for a duplicate determination four plates are needed: two plates for the samples without added putrescine and two for the internal standards. TLC is therefore the rate-limiting step in our procedure. However, the specificity of analytical methods that use non-specific physical or chemical detection methods relies only on the efficiency of the separation techniques used. The careful separation of bis-DANS-putrescine from other fluorescent spots of comparable intensity by two-dimensional TLC therefore seems necessary to us for the quantitative determination of putrescine. One-dimensional chromatography of the DANS derivatives, as suggested by DION AND HERBST[19] for the determination of putrescine as well as spermidine and spermine, should give satisfying results only in special cases, since the amine mixtures present in tissues are normally very complex. On the same grounds, we think it is further necessary in the case of chromatographic procedures to ensure the identity and uniformity of the substances to be determined by independent methods. For the identification of the small amounts of DANS derivatives normally available from tissue, mass spectrometry was the most suitable method, since it gives additional information about the presence and the type of other compounds in the chromatographically separated spots.

Putrescine has been identified in brain extracts by several authors[25–28]. Quantitative data have only been published recently by KREMZNER *et al.*[11] for sheep brain. More data are available on liver putrescine[3,29,30], especially in rat liver[5,31]. Our data on the putrescine content of mouse brain and liver cannot be compared with existing data for the mouse. Compared with the published figures for sheep brain[11] and rat liver[31], the concentrations of putrescine found by us in the mouse organs were lower by a factor of 0.25–0.3.

A comparison of the presently available methods for the determination of putrescine reveals that the sensitivity of detection of the dansyl method is higher compared with those of the other procedures by a factor of *ca.* 50–100.

REFERENCES

1 E. J. HERBST AND U. BACHRACH (Editors), *Metabolism and Biological Functions of Polyamines*, *Ann. N.Y. Acad. Sci.*, 171 (1970) 691–1069.
2 H. TABOR AND C. W. TABOR, *Pharmacol. Rev.*, 16 (1964) 245.
3 F. G. FISCHER AND H. BOHN, *Hoppe-Seyler's Z. Physiol. Chem.*, 308 (1957) 108.
4 A. RAINA AND S. S. COHEN, *Proc. Nat. Acad. Sci. U.S.A.*, 55 (1966) 1587.
5 D. H. RUSSELL, V. J. MEDINA AND S. H. SNYDER, *J. Biol. Chem.*, 245 (1970) 6732.
6 J. E. HAMMOND AND E. J. HERBST, *Anal. Biochem.*, 22 (1968) 474.
7 D. R. MORRIS, K. C. KOFFRON AND C. J. ORSTEIN, *Anal. Biochem.*, 30 (1969) 449.
8 C. W. TABOR AND S. M. ROSENTHAL, *Methods Enzymol.*, 6 (1963) 615.
9 H. SHIMIZU, Y. KAKIMOTO AND I. SANO, *J. Pharmacol. Exp. Ther.*, 143 (1964) 199.
10 S. HOLDER AND H. J. BREMER, *J. Chromatogr.*, 25 (1966) 48.
11 L. T. KREMZNER, R. E. BARRETT AND M. J. TERRANO, *Ann. N.Y. Acad. Sci.*, 171 (1970) 735.
12 T. A. SMITH, *Anal. Biochem.*, 33 (1970) 10.
13 N. SEILER AND M. WIECHMANN, *J. Chromatogr.*, 28 (1967) 351.
14 N. SEILER AND M. WIECHMANN, in A. NIEDERWIESER AND G. PATAKI (Editors), *Progress in Thin-Layer Chromatography and Related Methods*, Vol. 1, 1970, Ann Arbor-Humphrey Science Publishers, Ann Arbor, London, p. 95.
15 N. SEILER AND M. WIECHMANN, *Z. Anal. Chem.*, 220 (1966) 109.
16 N. SEILER AND M. WIECHMANN, *Hoppe-Seyler's Z. Physiol. Chem.*, 348 (1967) 1285.
17 N. SEILER, G. WERNER, H. A. FISCHER, B. KNÖTGEN AND H. HINZ, *Hoppe-Seyler's Z. Physiol. Chem.*, 350 (1969) 676.
18 N. SEILER AND J. M. SCHRÖDER, *Brain Res.*, 22 (1970) 81.
19 A. S. DION AND E. J. HERBST, *Ann. N.Y. Acad. Sci.*, 171 (1970) 723.
20 N. SEILER, M. WIECHMANN, H. A. FISCHER AND G. WERNER, *Brain Res.*, 28 (1971) 317.

21 N. SEILER, *Methods Biochem. Anal.*, 18 (1970) 250.
22 N. SEILER, H. SCHNEIDER AND K.-D. SONNENBERG, *Z. Anal. Chem.*, 252 (1970) 127.
23 A. A. BOULTON, *Methods Biochem. Anal.*, 16 (1968) 327.
24 M. PESEZ AND J. BARTOS, *Talanta*, 10 (1960) 331.
25 H. KEWITZ, *Naturwissenschaften*, 40 (1950) 495.
26 T. L. PERRY, S. HANSEN, J. G. FOULKS AND G. M. LING, *J. Neurochem.*, 12 (1965) 397.
27 T. L. PERRY, S. HANSEN AND L. MACDOUGALL, *J. Neurochem.*, 14 (1967) 775.
28 C. M. CALDARERA, B. BARBIROLI AND G. MORUZZI, *Biochem. J.*, 97 (1965) 84.
29 R. H. WEAVER AND E. J. HERBST, *J. Biol. Chem.*, 231 (1958) 937.
30 D. H. RUSSELL, S. H. SNYDER AND V. J. MEDINA, *Life Sci.*, 8 (1969) 1247.
31 J. JÄNNE, *Acta Physiol. Scand. Suppl.*, 300 (1967) 7.