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Note

High-pressure liquid chromatographic determination of putrescine, cadaverine, spermidine and spermine

JOHN WILLIAM REDMOND and ALBERT TSENG

School of Chemistry and Biomolecular Science Unit, Macquarie University, North Ryde, N.S.W. 2113 (Australia)

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In connection with structural studies of components of the outer membrane of Gram negative bacteria[1], it became necessary for us to estimate the levels of diamines and polyamines in various membrane fractions.

Recently published procedures for analysis of these amines by high-pressure liquid chromatography (HPLC) have relied on separation of tosyl[2,3] or 5-dimethylaminonaphthalene-1-sulphonyl (Dns) derivatives[4–7]. The tosyl derivatives are prepared by a simple, but lengthy, procedure and allow good separation of putrescine, spermidine and spermine using either an isocratic or gradient system. Cadaverine was not included in these studies. The Dns derivatives provide excellent sensitivity and allow fairly good estimation of both putrescine and cadaverine, provided rather complex gradient profiles are used. It is, however, desirable to carry out derivative purification prior to chromatography[4,7].

Since preliminary experiments had indicated the presence of significant amounts of cadaverine in our materials[8], it was essential that we had available a routine HPLC system which provided good separation of putrescine and cadaverine.

A recent publication reports elevated blood levels of cadaverine, and more particularly acylcadaverines, in schizophrenic patients[9]. These amines were estimated to be the DNS derivatives by a combination of thin-layer chromatography and mass spectrometry. We report here a simple method for estimation of biological amines, including cadaverine, which lends itself readily to routine analysis.

EXPERIMENTAL

Equipment

Two LDC Constametric pumps (Models I and IIG) with a dynamic gradient mixer were operated in conjunction with an LDC Gradient Master. A Rheodyne Model 7120 syringe-loading injector was used and detection was by means of an LDC UV III monitor (Model 1203) operating at 254 nm. A 25-cm Brownlee RP-8 column (10 μm) was used.

Materials

Amines were purchased from Sigma (St. Louis, Mo., U.S.A.). Analytical-grade

methanol was distilled from 2,4-dinitrophenylhydrazine through a 100-cm helix-packed column. The absorbance at 254 nm was *ca.* 0.01.

Procedure

To a sample (0.1–1 ml) containing up to 0.5 μmole amines and no more than 1 mequiv. mineral acid, was added a solution of 1,6-diaminohexane (as internal standard) and 2 *N* sodium hydroxide (1.0 ml) followed by benzoyl chloride (5 μl). The mixture was shaken briefly using a vortex mixer and allowed to stand for 20 min. Saturated sodium chloride solution (2 ml) was added and the solution extracted with diethyl ether (2 ml). After centrifugation to separate the layers, the upper organic phase was removed and evaporated in a stream of nitrogen. The residue was dissolved in methanol (100 μl) and aliquots examined by HPLC (see Fig. 1).

RESULTS AND DISCUSSION

The benzoyl derivatives of the amines were chosen for study because of their chemical simplicity. It was hoped then that separation of benzoylated putrescine and cadaverine might be less difficult than with the more complex Dns derivatives. Furthermore, the classical Schotten–Baumann benzoylation procedure is simple, rapid and economical. The excess benzoyl chloride is readily destroyed in the alkaline

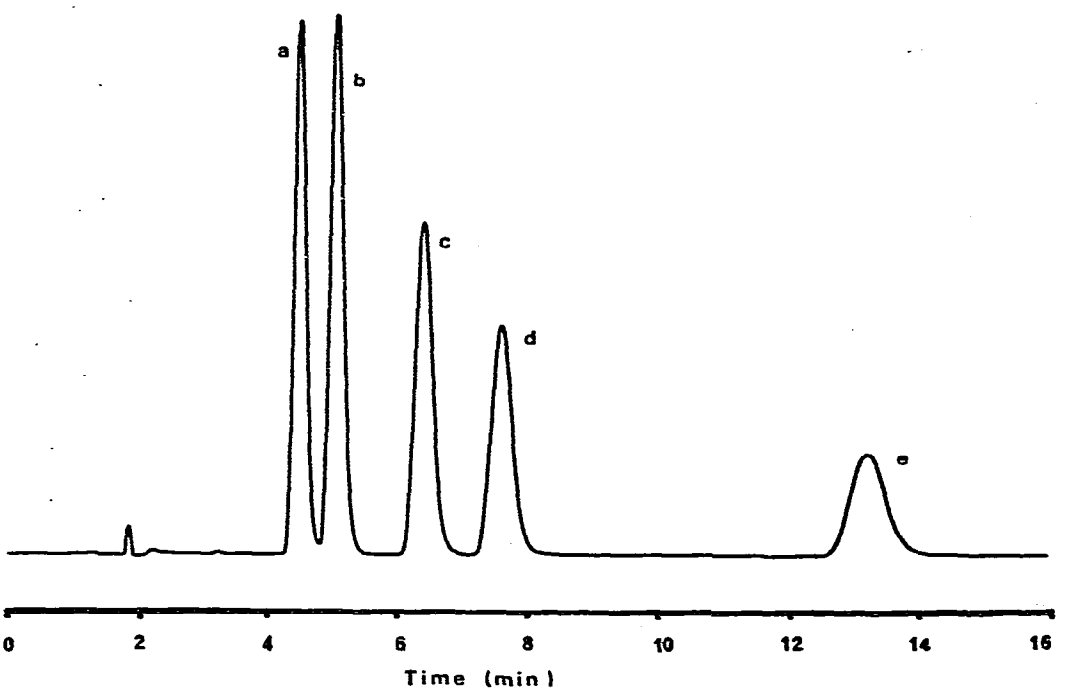

Fig. 1. Separation of benzoylated polyamines by HPLC. Operating conditions: column, 25 cm RP-8 (10 μm); mobile phase, methanol–water (58:42); column temperature, ambient; flow-rate, 1.5 ml/min. Peaks: a = putrescine; b = cadaverine; c = 1,6-diaminohexane (internal standard); d = spermidine; e = spermine.

medium and the side product, benzoic acid, is retained in the aqueous layer during ether extraction. The benzoyl derivatives are stable and no special precautions are required for storage.

By comparison with the purified reference benzamides, it was shown that a single derivatization, using 5 μl of benzoyl chloride, takes place with 94% efficiency. Treatment with an additional amount of benzoyl chloride achieves complete acylation. The degree of incompleteness of derivatization is the same for each of the amines studied. Therefore, apart from a marginal improvement in sensitivity, there is no advantage in using larger amounts of benzoyl chloride.

The RP-8 reversed-phase column proved particularly suitable for these benzamides and complete separation of the putrescine and cadaverine derivatives was achieved using an isocratic system. Moreover, the precise methanol–water proportions are not critical and a range of mixtures gives good separations. Furthermore, the dependence of the height equivalent to a theoretical plate (HETP) on flow-rate is not great: flow-rates of up to 3 ml/min still allow good separation of the putrescine and cadaverine derivatives.

The use of an isocratic system provides optimal baseline stability so that sensitive detector settings can be used. This advantage, in addition to excellent peak shape, confers good sensitivity on the method. Using the maximum detector sensitivity (0.002 a.u.f.s.), a full scale peak is obtained for 16 ng (approx. 200 pmoles) of putrescine.

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