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Note

High-performance liquid chromatographic determination of putrescine, spermidine and spermine after derivatisation with 4-fluoro-3-nitrobenzotrifluoride

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The polyamines spermidine and spermine, and the diamine precursor putrescine are known to be widely distributed in nature[1]. The relationship between polyamines and cell proliferation has been a subject of intense interest in recent years; these amines are believed to play an important role in the control of cellular growth[2]. The assay of polyamines in various biological samples has been accomplished using several methods, many of which have been reviewed by Seiler[3].

Vessman and Strömberg[4] used 4-fluoro-3-nitrobenzotrifluoride (FNBT) as a reagent in the first stage of a two stage derivatisation of tranexamic acid. There appears to be no report of the use of this agent for the analysis of polyamines or application of any of its derivatives to high-performance liquid chromatography (HPLC).

This paper describes the development of a HPLC procedure for the determination of polyamines based upon the formation of nitrotrifluoromethylphenyl derivatives using FNBT.

EXPERIMENTAL

Apparatus

The system used for HPLC comprised a Constametric II G pump and Spectromonitor III variable-wavelength UV detector (Laboratory Data Control, Stone, Great Britain), fitted with a 25 cm × 4.5 mm I.D. stainless-steel column containing ODS (10 μm) Spherisorb.

Reagents

Acetonitrile was liquid chromatographic quality (Rathburn Chemicals, Walkerburn, Great Britain). Water was glass distilled shortly before use and the mobile phase was ultrasonicated immediately before chromatography. Diaminooctane and the hydrochlorides of putresince, spermidine and spermine were obtained from Sigma (London, Great Britain). FNBT was supplied by Kodak (Liverpool, Great Britain). All other reagents were of AnalaR grade.

HPLC

The isocratic mobile phase used was acetonitrile–water (80:20). The flow-rate was 3 ml/min at a constant temperature of 40°C.

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Derivatization procedure

The dry residue obtained from a sample solution containing putrescine, spermidine and spermine together with diaminooctane (internal standard), contained in a 100 × 18 mm Quickfit glass tube, was redissolved in 0.1 ml of 1 *M* sodium carbonate solution. This solution was treated with 0.3 ml of FNBT reagent (10 μl FNBT/ml dimethylsulphoxide). After mixing, the reaction was allowed to proceed at 60°C for 20 min. At the end of this time 40 μl of 1 *M* histidine in 1 *M* sodium carbonate was added to the reaction mixture and the incubation continued for a further 5 min.

After cooling the mixture, the N-2′-nitro-4′-trifluoromethylphenyl polyamine (NTP-polyamine) derivatives were extracted twice with 2 ml of 2-methylbutane. The organic phase was removed after centrifugation at 1500 *g* for 5 min and evaporated to dryness in a 115 × 17 cm conical, Quickfit glass tube. The residue was reconstituted with 50 μl of methanol; 10-μl volumes were applied to the liquid chromatograph.

RESULTS AND DISCUSSION

The optimum conditions for the reaction between polyamines and FNBT were found to be slightly different to those cited by Vessman and Stromberg[4] for the

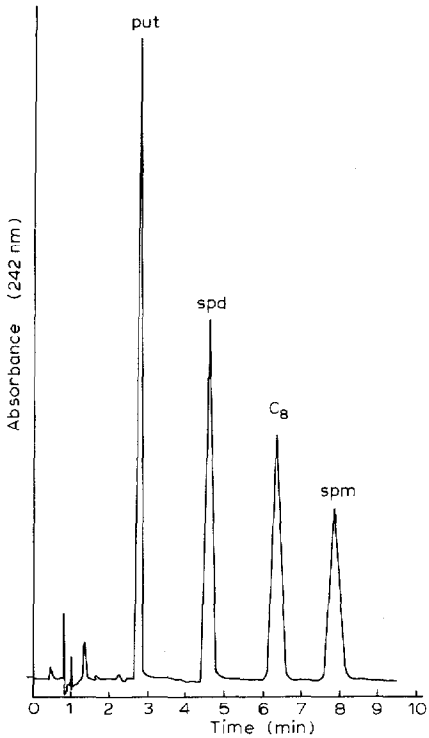

Fig. 1. A high-performance liquid chromatogram of an aqueous standard containing putrescine (put), spermidine (spd), diaminooctane (C_8) and spermine (spm) after derivatisation with FNBT is shown. Each peak represents 1.0 nmol of derivative. The sensitivity used was 0.2 absorbance units at full scale deflection. Experimental details are given in the text.

derivatisation of tranexamic acid with this reagent. An additional step was also required to remove excess reagent at the end of the reaction in order to avoid a large background peak interfering with the resolution of NTP-putrescine. Various compounds possessing amine groups were investigated as "scavenge" reagents for excess FNBT but histidine was found to be the most suitable. The NTP-polyamines could be extracted by a variety of organic solvents. 2-Methylbutane was selected because it extracted less of the reaction by-products and could easily be removed by evaporation. Under the conditions used, secondly amines did not appear to react with FNBT and compounds containing a polar group in addition to a primary amine, such as amino acids, were not extracted by the 2-methylbutane. Spectrophotometric analyses of the NTP-polyamines produced two peaks, one in the visible region of the spectrum (410 nm) and the other in the ultraviolet (242 nm). The NTP derivatives chromatographed well (Fig. 1) by reversed-phase liquid chromatography.

To investigate the linearity of the method, aqueous solutions of polyamines were dried and assayed using diaminooctane as the internal standard. The results produced linear (coefficient of determination 0.99) calibration graphs for the NTP derivatives of putrescine, spermidine and spermine from 0 to 250 nmol applied to the chromatographic column. It was possible to detect as little as 5 pmol of putrescine, 10 pmol of spermidine and 25 pmol of spermine. The precision of the method was determined from the mean peak height ratios obtained from 5 nmol samples of putrescine, spermidine and spermine with respect to a 5 nmol internal standard of diaminooctane. The coefficients of variation were found to be 1.1, 3.6, and 4% for putrescine, spermidine and spermine respectively, for a single batch of ten samples.

The HPLC determination of polyamines described in this paper has advantages in common with the method for the determination of polyamines described by Sugiura and co-workers[5,6] using a tosyl derivative. That is, the reaction proceeds in an aqueous medium, the derivatives are easily extracted from the reaction mixture and the polyamine derivatives may be detected with a UV monitor. Complete resolution of the NTP-polyamines was obtained on a 25 cm column in less than 10 min, which offered an improvement over the retention times reported for polyamine tosyl derivatives by Sugiura *et al.*[5] who obtained complete separation of their derivatives in 16 min using a 1 min column.

Further work is being carried out on the application of this method for polyamine analyses in biological samples. The possible use of FNBT for the determination of other amines by HPLC is also being investigated.

REFERENCES

1 S. S. Cohen, *Introduction to the Polyamines*, Prentice-Hall, NJ, 1971.
2 J. Janne, H. Poso and A. Raina, *Biochem. Biophys. Acta*, 473 (1978) 241.
3 N. Seiler, *Clin. Chem.*, 23 (1977) 1519.
4 J. Vessman and S. Strömberg, *Anal. Chem.*, 49 (1977) 369.
5 T. Sugiura, T. Hayashi, S. Kawai and T. Ohno, *J. Chromatogr.*, 110 (1975) 385.
6 T. Hayashi, T. Sugiura, S. Kawai and T. Ohno, *J. Chromatogr.*, 145 (1978) 141.