Journal of Chromatography, 110 (1975) 385–388

CHROM. 8281

Note

High-speed liquid chromatographic determination of putrescine, spermidine and spermine

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(First received November 21st, 1974; revised manuscript received February 25th, 1975)

The polyamines spermidine and spermine, and the precursor diamine putrescine, are considered to be generally distributed in the living world, and have been postulated to play an important role in the control of cellular growth. Russell and co-workers[1,2] reported that enhanced urinary excretions of these amines were observed in patients with different types of cancer.

The separation and/or determination of polyamines in various biological samples has been accomplished by several methods, such as fluorimetry[3–5], enzymology[6], thin-layer chromatography[7,8], gas chromatography[9,10] and ion-exchange chromatography[11–15].

This paper describes the development of a high-speed liquid chromatographic method for the determination of the polyamines based on the formation of tosylated derivatives. This derivatization enhanced the extraction, the chromatographic separation and the detection.

EXPERIMENTAL AND RESULTS

Apparatus

The apparatus was a Shimadzu–DuPont 840 liquid chromatograph equipped with an ultraviolet absorption detector. The separation was carried out with 1 m × 2.1 mm I.D. column of Zipax Permaphase ETH purchased from Shimadzu Seisakusho (Kyoto, Japan).

Reagents

Putrescine dihydrochloride and spermidine phosphate were obtained from Tokyo Organic Chemicals (Tokyo, Japan) and spermine phosphate, 1,10-diaminodecane and *p*-toluenesulphonyl chloride (TsCl) from Wako Chemicals (Osaka, Japan). Acetone, *n*-hexane and methanol were used after distillation. The other organic solvents and reagents used were of reagent grade.

Chromatographic system

The tosylation of the polyamines is considered to result in a large increase in their affinity for non-polar solvents, and various polar compounds occur in biological samples. Accordingly, the combination of this derivatization with reversed-

phase chromatography would seem to be appropriate for the determination of the polyamines in such samples and for this purpose, a 1-m column of Zipax Permaphase ETH was used.

The chromatographic behaviour of the tosylated polyamines has been studied on the ETH column in water–methanol and water–acetonitrile mixed solvents. The latter mixed solvent gave a better separation, and gave a more suitable flow-rate owing to its low viscosity.

Fig. 1 shows a typical chromatogram obtained from a standard mixture of these tosylated polyamines and tosylated 1,10-diaminodecane as internal standard. It is obvious from Fig. 1 that a good separation is achieved in only 20 min even without using a gradient elution technique.

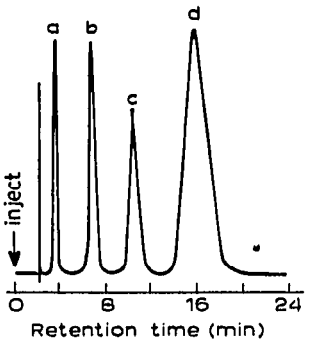

Fig. 1. Liquid chromatogram of a standard mixture of three tosylated polyamines and tosylated 1,10-diaminodecane (internal standard). Operating conditions: column, 1 m ETH; mobile phase, water–acetonitrile (6:4); Column temperature, 30°; flow-rate, 0.56 ml/min; detector, UV photometer. Peaks: a, Ts-putrescine; b, Ts-spermidine; c, Ts-1,10-diaminodecane; d, Ts-spermine.

Reaction system

An organic solvent was added to the reaction medium in order to aid the dissolution of TsCl, and the effect of organic solvents such as tetrahydrofuran, dioxan and acetone on the tosylation of the polyamines was investigated.

A water–acetone mixture (1:1) gave considerably higher yields in the tosylation reaction than the other mixtures, but this mixed solvent gave a substantial interfering background on the chromatogram, thus affecting the separation of tosylated putrescine. In the course of this work, it was fortunately found that the interfering background could be removed simply by warming the reaction mixture at about 70° for more than 30 min. Therefore, warming at 70° for 1 h was chosen so to ensure the complete removal of the interfering background. This condition was also sufficient for the tosylation to be completed.

Extraction system

As the excess of TsCl used as a reagent gave a large peak that overlapped with those of tosylated putrecine and spermidine, thus interfering in their separation ,its prior removal had to be effected. Preliminary experiments demonstrated that TsCl could be extracted with *n*-hexane from an aqueous alkaline medium, while the tosyl-

ated polyamines were not extracted, so that the excess of TsCl could thus be removed. It was also found that the tosylated polyamines could be easily extracted with some organic solvents, such as dichloromethane, chloroform, carbon tetrachloride and ethyl acetate. Of these solvents, chloroform was chosen as one of the most suitable for extraction, as it clearly separated from the aqueous phase as a lower layer, which was convenient for the separation procedure. Condidering further the effects of TsCl and sodium hydrogen carbonate concentrations on the tosylation, the following optimal conditions were adopted in the recommended procedure for the high-speed liquid chromatographic determination of putrescine, spermidine and spermine.

Procedure

To 1.00 ml of an aqueous sample solution containing putrescine, spermidine and spermine, 1 ml of 0.5 *M* sodium hydrogen carbonate and 20 mg of TsCl dissolved in 2 ml of acetone are added. The mixture is then warmed in a water-bath at about 70° for 1 h. After cooling, 100 μl of internal standard solution (1 mg of tosylated 1,10-diaminodecane in 1 ml of methanol) and 10 ml of 1 *N* sodium hydroxide solution are added. The mixture is washed with four 5-ml volumes of *n*-hexane and, after adding 15 ml of 1 *N* hydrochloric acid, the tosylated polyamines are extracted with 10 ml of chloroform. The organic phase is dried over sodium sulphate and chloroform is evaporated on a rotary evaporator. The residue is re-dissolved in a few drops of methanol and 10 μl of the resulting solution are subjected to the high-speed liquid chromatography. The operating conditions are given in the legend to Fig. 1.

Fig. 2 illustrates the calibration graphs obtained by the over-all procedure. Relative peak height ratios of tosylated putrescine, spermidine and spermine to an internal standard were plotted against the amount of each polyamine in the solution. For each polyamine, a good linear relationship was obtained, at least in the concentration range shown in Fig. 2. Nine replicate determinations on an identical mixture containing putrescine (6 μg), spermidine (9 μg) and spermine (30 μg) gave standard deviations of 2.75, 0.90 and 2.57%, respectively.

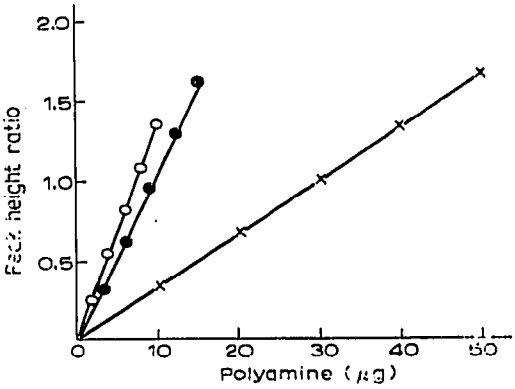

Fig. 2. Calibration graphs for the determination of polyamines. ○, Putrescine; ●, spermidine; ×, spermine.

CONCLUSION

The high-speed liquid chromatographic determination of putrescine, spermidine and spermine using tosylation has been developed, the use of this derivatization having the following advantages:

(1) it proceeds in an aqueous medium;

(2) it enables the polyamines to be easily extracted from the reaction mixture;

(3) it gives derivatives convenient for the chromatographic separation, as the tosylated polyamines can be completely separated in only 20 min without a gradient elution technique; and

(4) the derivatives of the polyamines can be detected forms with a UV (254 nm) monitor.

We are carrying out further studies on the application of this method to various biological samples.

REFERENCES

1 D. H. Russell, *Nature (London)*, 233 (1971) 144.
2 D. H. Russell, C. C. Levy, S. C. Schimpff and I. A. Hawk, *Cancer Res.*, 31 (1971) 1555.
3 L. T. Kremzner, *Anal. Biochem.*, 15 (1966) 270.
4 B. Elliot and I. A. Michaelson, *Anal. Biochem.*, 19 (1967) 184.
5 H. Veening, W. W. Pitt and G. Jones, *J. Chromatogr.*, 90 (1974) 129.
6 U. Bachrach and B. Reches, *Anal. Biochem.*, 17 (1966) 38.
7 J. E. Hammond and E. J. Herbst, *Anal. Biochem.*, 22 (1968) 474.
8 N. Seiler and A. Askar, *J. Chromatogr.*, 62 (1971) 121.
9 P. Capella and E. C. Horning, *Anal. Chem.*, 38 (1966) 316.
10 M. D. Denton, H. S. Glazer, D. C. Zellner and F. G. Smith, *Clin. Chem.*, 19 (1973) 904.
11 D. R. Morris, K. L. Koffron and C. J. Okstein, *Anal. Biochem.*, 30 (1969) 449.
12 H. Hatano, K. Sumizo, S. Rokushika and F. Murakami, *Anal. Biochem.*, 35 (1970) 377.
13 H. J. Bremer, E. J. Kohne and W. Endres, *Clin. Chim. Acta*, 32 (1971) 407.
14 L. J. Marton, D. H. Russell and C. C. Levy, *Clin. Chem.*, 19 (1973) 923.
15 C. W. Gehrke, K. C. Kuo, R. W. Zumwalt and T. P. Waalkes, *J. Chromatogr.*, 89 (1974) 231.