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Note

Investigation of interfering products in the high-performance liquid chromatographic determination of polyamines as benzoyl derivatives

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The determination of polyamines (putrescine, spermidine and spermine) in cells, tissues and organs is necessary in order to clarify the precise physiology of living organisms and the role of cell proliferations within them. Recently, several workers have employed benzoyl derivatization procedures [1–3] in the determination of these polyamines by high-performance liquid chromatography (HPLC). These procedures are both rapid and simple. In the course of our application of these benzoylation procedures to the determination of polyamines in rat organs, we observed slow-moving peaks at retention times of 7.8 and 17.0 min in addition to those of three benzoyl polyamines which were synthesized by the previous workers [1–3]. The size of these two peaks changed with the reaction time of benzoylation and the standing time of the benzoyl polyamines in methanol before measurement. These peaks influenced the peak area of N,N'-dibenzoylbutane-1,4-diamine and made accurate measurement of the polyamines impossible. If they could be eliminated, then precise measurements could be made.

EXPERIMENTAL

Equipment

HPLC studies were performed on an LC-6A chromatograph (Shimadzu, Kyoto, Japan) equipped with a Chromatopac C-R3A data processing system and an SPD-66A UV spectrophotometric detector operated at 254 nm under isocratic conditions.

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A reversed-phase column (Cosmosil 5 C_{18}) (150 × 3 mm I.D.) was purchased from Nakalai Tesque (Kyoto, Japan).

Polyamines and benzoyl chloride were obtained from Sigma (St. Louis, MO, U.S.A.) and Wako (Osaka, Japan), respectively.

Procedure[1]

To 50 μl of a sample containing 25 nmol of polyamines were added 2.0 ml of 2 *M* sodium hydroxide followed by 50 μl of benzoyl chloride. The mixture was well shaken with a vortex mixer until the disappearance of benzoyl chloride (5.0 min), after which it was allowed to stand for 3.0 h at 40°C. Next, 2.0 ml of saturated sodium chloride solution were added, followed by 2.0 ml of diethyl ether. The solution was well mixed and centrifuged at 700 *g* for 25 min to separate the layers, and the diethyl ether phase was removed by evaporation in a stream of nitrogen. The residue was dissolved in 100 μl of hot methanol (40–50°C) and 10 μl of the solution were charged to the HPLC system.

RESULTS AND DISCUSSION

Contrary to our expectations, the HPLC of the benzoylated polyamines showed two peaks with retention times of 7.8 and 17.0 min in addition to those of N,N′-dibenzoylbutane-1,4-diamine, N,N′,4-tribenzoyl-4-azaoctane-1,8-diamine and N,N′,4,9-tetrabenzoyl-4,9-diazadodecane-1,12-diamine, as shown in Fig. 1. The peak areas of N,N′-dibenzoylbutane-1,4-diamine at 4.0 min and of the unidentified peak at

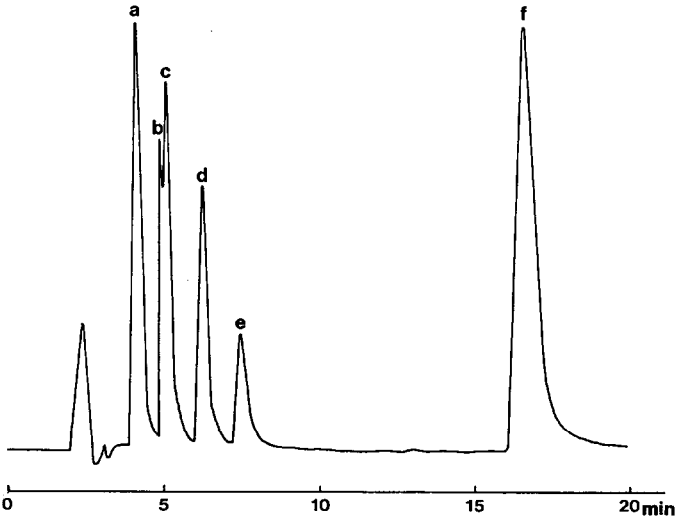

Fig. 1. Separation of benzoylated polyamine by HPLC. The reaction time was 3.0 h at 40°C. Operating conditions: column, 15 cm Cosmosil 5 C_{18}; mobile phase, water–acetonitrile–methanol (9:10:1) containing 0.2% trifluoroacetic acid; column temperature, ambient; flow-rate, 0.6 ml/min. Peaks: a = benzoylated putrescine; b = benzoylated 1,6-diaminohexane; c = benzoylated spermidine; d = benzoylated spermine; e = methyl benzoate; f = benzoic anhydride.

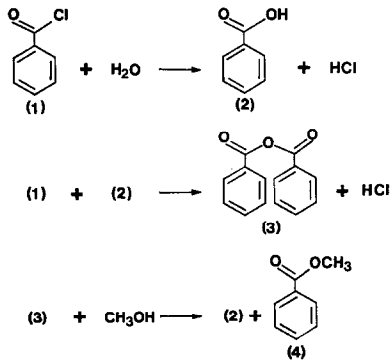

Fig. 2. Reactions producing benzoic acid, benzoic anhydride and methyl benzoate.

7.8 min increased with extended storage time in methanol, whereas the peak area at 17.0 min decreased. Therefore, these three peaks seem to be closely related to each other as shown in reactions poducing benzoic acid, benzoic anhydride and methyl benzoate (Fig. 2). Benzoyl chloride (1) reacted partly with polyamines and was hydrolysed to benzoic acid (2), then benzoyl chloride and benzoic acid condensed to give benzoic anhydride (3) which was extracted efficiently by diethyl ether. After evaporation of the diethyl ether, the benzoic anhydride immediately began to undergo solvolysis in methanol and produced methyl benzoate (4) and benzoic acid during storage, as shown in Fig. 3. The peaks with retention times of 4.0, 7.8 and 17.0 min were thus identified as benzoic acid, methyl benzoate and benzoic anhydride, respectively, by comparison with authentic samples.

Unfortunately, the peak of benzoic acid was superimposed on that of N,N′-dibenzoylbutane-1,4-diamine under our experimental conditions regardless of the application of different solvent systems. Therefore, care should be taken in using

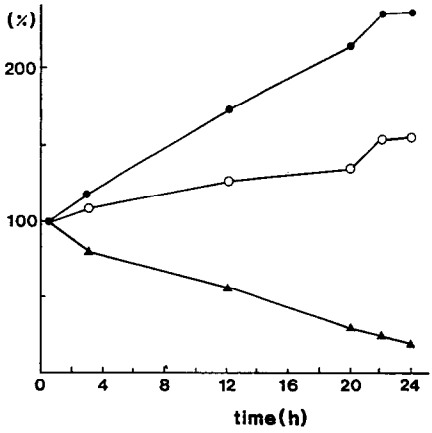

Fig. 3. Time course of the solvolysis of benzoic anhydride (▲) in methanol to benzoic acid (○) and methyl benzoate (●) at 25°C, as determined by HPLC. The ordinate represents the percentages of benzoic acid, methyl benzoate and benzoic anhydride *versus* the respective concentrations at 30 min after starting the reaction.

the benzoylation method for the determination of polyamines, as peaks of products occurring as a result of the reaction might be superimposed over those of the benzoylated polyamines in HPLC, leading to incorrect deductions. To eliminate this benzoic acid, it is necessary to decompose the benzoic anhydride produced in the solution by the benzoylation reaction before diethyl ether extraction of the benzoylated polyamines, as the benzoic anhydride solvolyses to benzoic acid and methyl benzoate in methanol. The decomposition of the benzoic anhydride produced in the reaction mixture seems to be dependent on the temperature and time of the benzoylation. When benzoylation was carried out overnight at 40°C with shaking of the mixture of benzoyl chloride and polyamines, benzoic anhydride was still observed. However, it was not present in the mixture when it was shaken vigorously overnight at 60°C. There were no differences in the concentrations of N,N′-dibenzoylbutane-1,4-diamine, N,N′,4-tribenzoyl-4-azaoctane-1,8-diamine and N,N′,4,9-tetrabenzoyl-4,9-diazadodecane-1,2-diamine due to the variations in temperature.

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