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Liquid chromatography–mass spectrometry studies of 9-fluorenylmethyl chloroformate derivatives of polyamines

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ABSTRACT

Liquid chromatography–mass spectrometry experiments were carried out to elucidate the structure of the carbamate derivatives formed when polyamines, such as ethylenediamine and putrescine, react with the precolumn fluorophore forming reagent, 9-fluorenylmethyl chloroformate. It has been shown that both primary amine functionalities derivatize with the reagent for polyamines.

INTRODUCTION

The precolumn derivatizing reagent, 9-fluorenylmethyl chloroformate (FMOC), has been used successfully for high-performance liquid chromatographic (HPLC) separations of amino acids [1–3] and polyamines [4,5]. The reagent reacts rapidly with the primary amine functionality of each of these classes of compounds to form a highly fluorescent and stable carbamate derivative. A general reaction of FMOC with a primary amine is shown in eqn. 1.

FMOC $\qquad$ CARBAMATE

FMOC also undergoes hydrolysis in aqueous solution to form 9-fluorenylmethanol (FMOH) as shown in eqn. 2.

$$+ \; H_2O \; \longrightarrow \qquad\qquad + \; HCl \; + \; CO_2 \qquad (2)$$

FMOC   FMOH

The structures for the four commonly encountered biogenic polyamines, putrescine (PUT), cadaverine (CAD), spermidine (SPD) and spermine (SPM), are shown in Table I. All four compounds have two primary amine groups; in addition, SPD and SPM each have one and two secondary amine groups, respectively. It is currently not known whether FMOC derivatization occurs at one primary amine end of these molecules, or both. The purpose of this study was to establish the extent of derivatization of polyamines with FMOC. We have carried out thermospray (TSP) liquid chromatography (LC)–mass spectrometry (MS) experiments to elucidate the structure of the carbamate derivatives of three of the polyamines. The results of these investigations are presented in this paper.

EXPERIMENTAL

Apparatus
    Liquid chromatography. A Spectra-Physics SP-8700 solvent delivery system equipped with a Model 7120 Rheodyne injection valve and a 20-μl sample loop were used for liquid chromatography. The fluorescence detector was a Model FS-970 (Schoeffel, Division of Kratos, Westwood, NJ, U.S.A.) with the excitation set at 265 nm and emission monitored through a 440 nm cut-off filter. Separations were achieved on a 150 mm × 4.6 mm I.D. Macrosphere 300, C_4 (Alltech) column using a mobile phase of 0.1 M aqueous ammonium acetate–acetonitrile programmed in a linear gradient (10 min) from 40% to 50% acetonitrile at 1.0 ml/min.
    Liquid chromatography–mass spectrometry. The HPLC system used for LC–MS consisted of a 600-MS multi-solvent delivery system (Waters, Milford, MA, U.S.A.) specifically designed to minimize flow pulsations. Manual injections (20 μl) were done using a Waters UK6 injector. A Waters 490-MS multi-wavelength detector set at 284 nm was installed in series between the column and the TSP vaporizer to allow on-line monitoring of the UV chromatogram. A solution of 0.5 M aqueous ammonium acetate

TABLE I

POLYAMINE STRUCTURES

Name	Abbreviation	Structure
Putrescine	PUT	$H_2N(CH_2)_4NH_2$
Cadaverine	CAD	$H_2N(CH_2)_5NH_2$
Spermidine	SPD	$H_2N(CH_2)_3NH(CH_2)_4NH_2$
Spermine	SPM	$H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$

was added using a Waters 590-MS pump at a flow-rate of 0.2 ml/min through a mixing tee located between the UV detector and the TSP vaporizer to minimize solvent changes during the gradient run and to enhance TSP ionization.

The TSP-MS apparatus consisted of a Hewlett-Packard (Palo Alto, CA, U.S.A.) 5790 mass selective detector mounted in a Vestec (Houston, TX, U.S.A.) Model 101 thermospray interface consisting of both the TSP ion source and associated vacuum systems. Data were acquired and the instrument was controlled by a Hewlett-Packard 59970 ChemStation. The optimum control temperature for the thermospray vaporizor was determined to be 142°C. The ion source block temperature (desolvation chamber) was maintained at 260°C. The same column (Alltech C_4) and gradient mobile phase conditions, as described above for the HPLC with fluorescence, were used.

Because the LC–MS system described above has an upper mass limit of 800 daltons, additional LC–MS experiments were done using a Finnigan MAT (San Jose, CA, U.S.A.) TSQ-46 triple quad mass spectrometer operated as a single stage mass spectrometer using Q1 with a mass range of 1900 daltons. The TSP ion source was a Vestec Model 701 which is similar in construction to the ion source used on the Model 101 system described above. The thermospray vaporizor used on this ion source was of a new design with replaceable orifices (75 μm) which resulted in a lower control temperature of 105°C. The ion source block temperature was 250°C. The HPLC system used was identical to that described above. The only change was the substitution of a Vydac C_4 column (250 mm × 4.6 mm I.D.) for the 150 mm Alltech C_4 column.

Reagents and solutions

The 9-fluorenylmethyl chloroformate (FMOC) derivatizing reagent was purchased from Fluka (Buchs, Switzerland). FMOC reagent solution was prepared by dissolving 200 mg of FMOC in 100 ml of dry, HPLC grade acetonitrile. This solution was refrigerated when not in use. Borate buffer solution was prepared by adjusting the pH of 50 ml of 0.25 M sodium borate solution with 0.1 M HCl to pH 8.5 and diluting with HPLC-grade water to 100 ml. The buffer was refrigerated when not in use. Stock solutions of putrescine, ethylenediamine, and spermidine (Sigma) were prepared by dissolving weighed amounts of the respective dihydrochloride salts, such that the final concentration of each was 500 ng/μl.

Procedure

The diamine samples were derivatized by adding 100 μl of the buffer and 500 μl of FMOC solution to 100 μl of each diamine solution. The mixture was swirled gently for 30 s prior to injection into the LC systems. Separation of diamine-FMOC derivatives was achieved with 0.1 M aqueous ammonium acetate–acetonitrile gradients. These chromatographic conditions were chosen because they gave the most satisfactory separation and they were the most compatible with the LC/MS techniques. Full scan mass spectra were obtained by scanning from 120 to 600 daltons (850 daltons for the high mass experiments) in 2 s throughout the LC–MS run.

RESULTS AND DISCUSSION

The purpose of this study was to expose two FMOC-derivatized diamines, EN
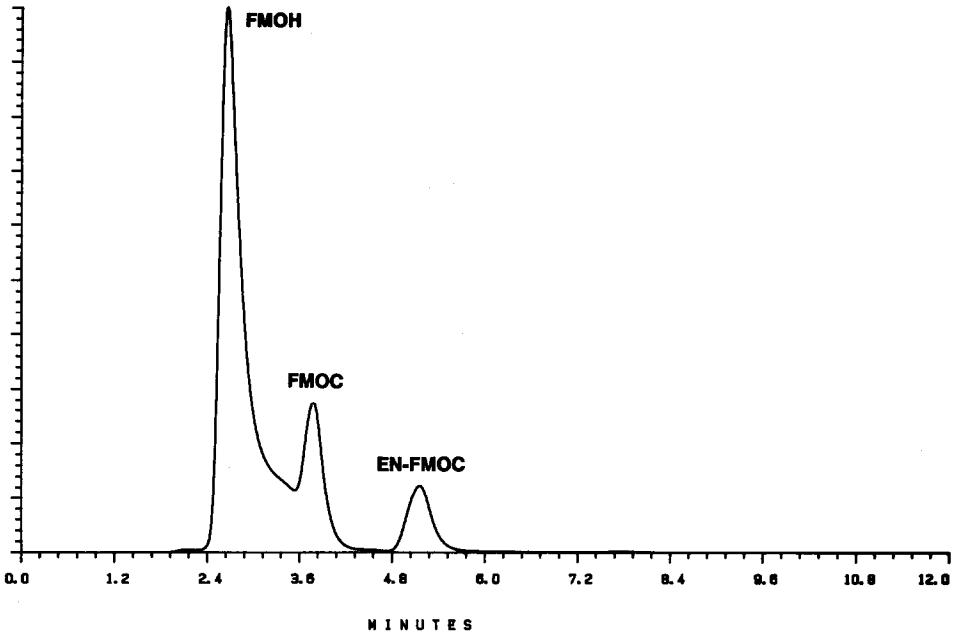
Fig. 1. Chromatogram for hydrolyzed FMOC (FMOH), unreacted FMOC and ethylenediamine-FMOC (EN-FMOC) using UV detection.

and PUT, to conditions actually encountered in typical HPLC separations and to determine the structure of the resulting derivatives by LC–MS experiments.

Fig. 1 shows the conventional chromatogram for the ethylenediamine-FMOC (EN-FMOC) derivative. It can be seen that the derivative elutes at 5.2 min and is
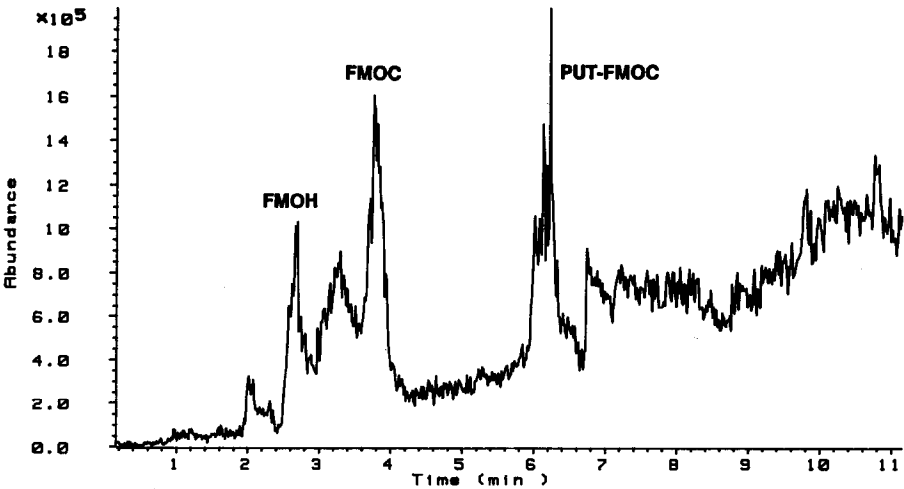

Fig. 2. LC–MS total ion chromatogram for putrescine-FMOC (PUT-FMOC).

cleanly separated from hydrolyzed FMOC (FMOH) and unreacted FMOC. A control sample of FMOC and water showed no peak at 5.2 min. Fig. 2 shows the total ion chromatogram for PUT-FMOC obtained by LC–MS. It can be seen that a corresponding peak for PUT-FMOC occurs at 6.3 min. Similar results were observed for EN-FMOC.

The mass spectrum for the EN-FMOC derivative obtained by LC–MS is shown in Fig. 3. The data clearly show an $(M + H)^+$ ion at m/z 505 indicating that both amine groups of EN were derivatized with FMOC. An intense ion at m/z 283 is a fragment which represents the loss of one FMOC with a proton transfer to the amine. Additionally, adduct ions were observed at m/z 522 for $(M + NH_4)^+$ and at m/z 527 for $(M + Na)^+$.

The mass spectral data for the PUT derivative are shown in Fig. 4. The protonated molecule, $(M + H)^+$, is located at m/z 533 and adduct ions due to $(M + NH_4)^+$ at m/z 550 and $(M + Na)^+$ at m/z 555 are observed. Again, an intense fragment ion for the loss of one FMOC moiety from $(M + H)^+$ was found at m/z 311. A TSP mass spectrum of PUT-FMOC, was obtained on the high-mass LC–MS system with a smaller vaporizer. This spectrum was similar with the exception of a more intense protonated molecule (m/z 533) and an adduct ion ($[M + NH_4]^+$ at m/z 550). In addition, in this mass spectrum, the absence of an adduct ion due to the addition of Na^+ is probably due to the use of an HPLC system and column that had not been contaminated with sodium containing buffers.

The data shown in Table II represent a summary of the major ions found for each of the two diamine derivatives. It can be concluded from these data that diamines derivatize with FMOC at *both* primary amine functionalities. It is also clear from the presence of a single chromatographic peak for each derivative, that only the di-derivative forms. In each case, the intense peak that occurs at m/z 283 for
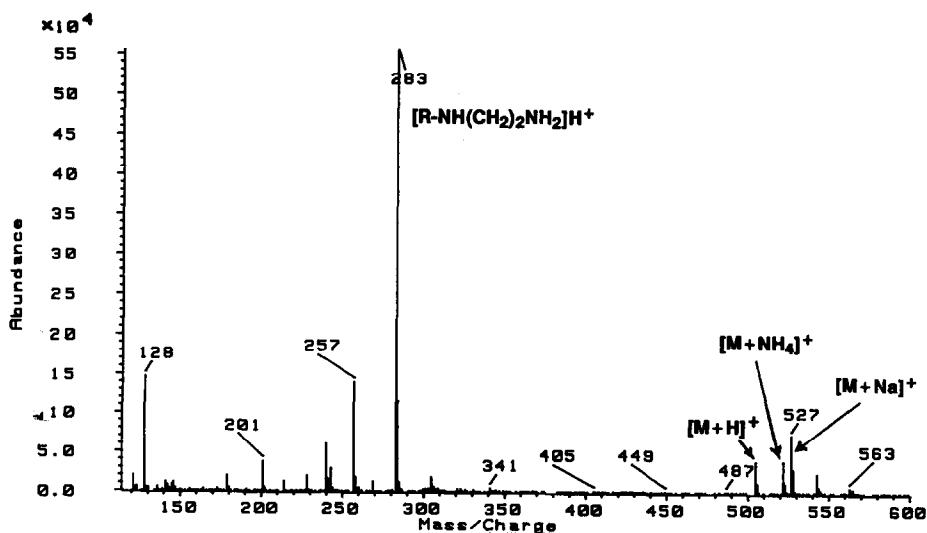

Fig. 3. Mass spectrum for ethylenediamine-FMOC (EN-FMOC) (130–600 a.m.u.).

TABLE II

LC-MS FRAGMENTATION DATA FOR ELUTED FMOC DERIVATIVES OF EN AND PUT

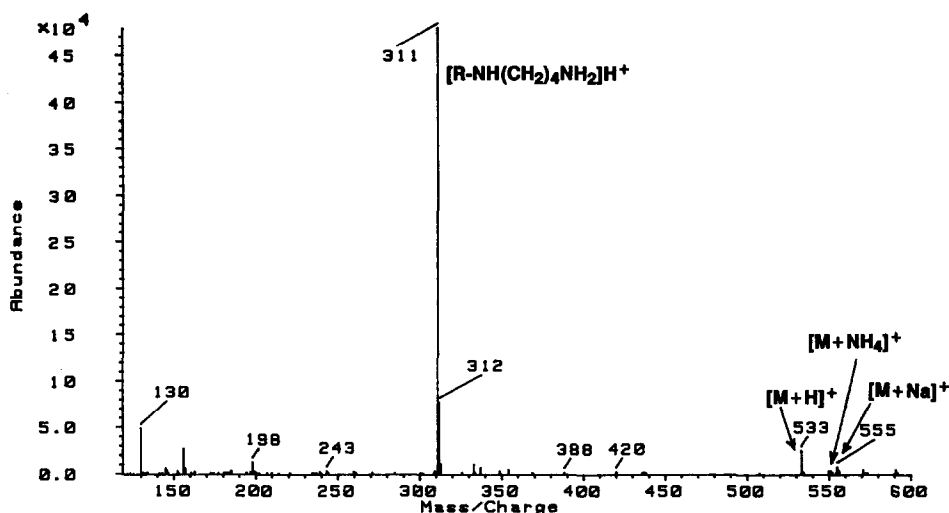| EN-FMOC derivative				PUT-FMOC derivative			
m/z	Ion	Assigned fragment[a]	Relative abundance (% of Base Peak)	m/z	Ion	Assigned fragment[a]	Relative abundance (% of Base Peak)
527	$[M+Na]^+$	$\left[\begin{array}{c} R-N(CH_2)_2N-R \\ \mid \quad \mid \\ H \quad H \end{array}\right]Na^+$	13.6	555	$[M+Na]^+$	$\left[\begin{array}{c} R-N(CH_2)_4N-R \\ \mid \quad \mid \\ H \quad H \end{array}\right]Na^+$	2.2
522	$[M+NH_4]^+$	$\left[\begin{array}{c} R-N(CH_2)_2N-R \\ \mid \quad \mid \\ H \quad H \end{array}\right]NH_4^+$	7.5	550	$[M+NH_4]^+$	$\left[\begin{array}{c} R-N(CH_2)_4N-R \\ \mid \quad \mid \\ H \quad H \end{array}\right]NH_4^+$	1.1
505	$[M+H]^+$	$\left[\begin{array}{c} R-N(CH_2)_2N-R \\ \mid \quad \mid \\ H \quad H \end{array}\right]H^+$	7.5	533	$[M+H]^+$	$\left[\begin{array}{c} R-N(CH_2)_4N-R \\ \mid \quad \mid \\ H \quad H \end{array}\right]H^+$	5.1
283	$[M-R+2H]^+$	$\left[\begin{array}{c} R-N(CH_2)_2N-R \\ \mid \quad \mid \\ H \quad H \end{array}\right]H^+$	100	311	$[M-R+2H]^+$	$\left[\begin{array}{c} R-N(CH_2)_4N-R \\ \mid \quad \mid \\ H \quad H \end{array}\right]H^+$	100
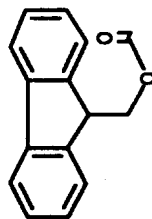
[a] R =

Fig. 4. Mass spectrum for putrescine-FMOC (PUT-FMOC).

EN-FMOC and at m/z 311 for PUT-FMOC, is a fragment ion and not the mono-derivative.

As these data have shown, both primary amine functionalities of polyamines are derivatized with FMOC. The question of the reactivity of the secondary amine in
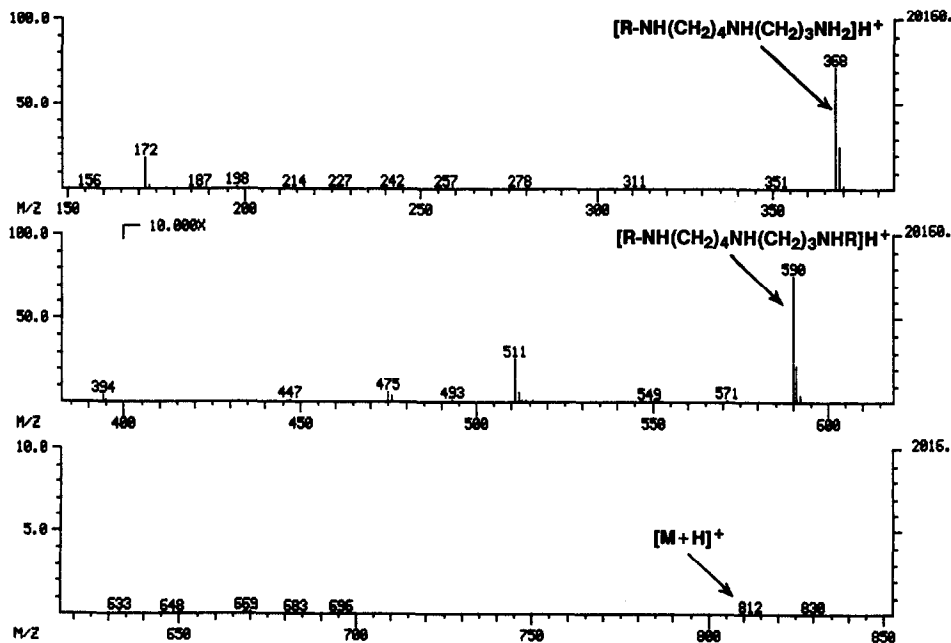

Fig. 5. Mass spectrum for spermidine-FMOC (SPD-FMOC) using a high-mass LC–MS system.

a polyamine such as spermidine, was raised as a result of this work. FMOC has been used as a precolumn label for the fluorescence detection of secondary amino acids [6] and would be expected to form the derivative of the secondary amine in polyamines if steric factors are not important. The mass spectrum for the SPD-FMOC derivative is shown in Fig. 5. The protonated molecule for the di-derivative would be m/z 590 and the tri-derivative would be m/z 812. There are intense peaks in the mass spectrum of SPD-FMOC for ions corresponding to spermidine with one and two FMOC moieties attached. The peak at m/z 812 is extremely small but only occurs in the LC–MS run during the elution of the SPD derivative. Also, the adduct ion for the addition of NH_4^+ to the molecule of the di-derivative, expected at m/z 607, is absent. There is a small peak at m/z 829.6 (marked as m/z 830) that could be the NH_4^+ adduct of the tri-derivative (molecular weight 811). While not conclusive, the evidence suggests that the FMOC derivative of spermidine is the tri-derivative.

ACKNOWLEDGEMENTS

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