Journal of Chromatography, 330 (1985) 307–313
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17 816

OFF-LINE LIQUID CHROMATOGRAPHIC–MASS SPECTROMETRIC STUDIES OF FLUORESCENT β-AMINOTHIOL-*o*-PHTHALALDEHYDE DERIVATIVES

PAUL A. METZ*, JOANNE GEHAS, JIMMY E. SPRIGGLE and HANS VEENING*
Department of Chemistry, Bucknell University, Lewisburg, PA 17837 (U.S.A.)
(Received April 11th, 1985)

SUMMARY

A liquid chromatographic separation and selective post-column derivatization of six β-aminothiols with *o*-phthalaldehyde is reported. The presence of both primary amine and thiol functional groups in these compounds makes it possible to derivatize with *o*-phthalaldehyde alone, eliminating the need for external thiol addition. The structures of the fluorescent derivatives formed in this procedure were determined by off-line liquid chromatography–gas chromatography–mass spectrometry. Spectral fluorescence characteristics for the β-aminothiol-*o*-phthalaldehyde derivatives are also reported.

INTRODUCTION

There are a number of reagents which are available for fluorescent labelling of primary amines. The applicability of these derivatizing agents in high-performance liquid chromatography (HPLC) has been reviewed recently[1]. One such reagent, *o*-phthalaldehyde (OPT), was first reported by Roth[2] and has since become widely used. The structure of the fluorescent product formed in the preparative reaction of a primary amine with OPT in the presence of a free thiol has been shown by Simons and Johnson[3,4] to be a 1-alkylthio-2-alkyl substituted isoindole. This structure was also confirmed under analytical post-column HPLC derivatization conditions by Simpson *et al.*[5]. OPT has also been reported as a fluorogenic derivatizing reagent for free thiols[6,7]. In the latter case, the procedure requires the use of excess primary amine.

In this paper we describe the HPLC separation and post-column derivatization of compounds containing both the primary amine and thiol functions, *i.e.*, β-aminothiols. When both of these functional groups are present in the same molecule, it is possible to derivatize these compounds with OPT alone, thus eliminating the need for external thiol addition. The fluorescent derivative structures obtained during

* Present address: Varian Instrument Group, 25 Hanover Road, Florham Park, NJ 07932, U.S.A.

0021-9673/85/$03.30

post-column HPLC detection have been characterized by gas chromatography–mass spectrometry (GC–MS) and are reported here. Their fluorescence spectral properties are also reported.

EXPERIMENTAL

Apparatus

The HPLC system used for the separation of the β-aminothiols consisted of two Model 6000A pumps controlled by a Model 660 solvent programmer, and a Model U6K injector (Waters Assoc., Milford, MA, U.S.A.). A 30 × 0.39 cm I.D. μBondapak C_{18} column (Waters Asoc.) was used for all separations. The flow-rate was 1.0 ml/min. A Model 39-650 post-column pump (Rainin, Woburn, MA, U.S.A.) was used to deliver the OPT derivatizing reagent into the column effluent at a flow-rate of 1.0 ml/min. The resulting mixture was then passed through a 165 × 0.023 cm I.D. stainless steel reaction coil maintained at 50°C. The fluorophores were detected by a Schoeffel FS-970 spectrofluoromonitor (Kratos, Westwood, NJ, U.S.A.) with the excitation monochromator set at 340 nm and equipped with a Type 440 emission filter (Kratos). A Model 056 recorder (Perkin-Elmer, Norwalk, CT, U.S.A.) was used to record all liquid chromatograms.

The gas chromatograph–mass spectrometer was a Model 4021 (Finnigan, Sunnyvale, CA, U.S.A.). The gas chromatograph was equipped with a 12 m × 0.020 mm I.D. cross-linked methyl silicone (film thickness 0.33 μm) fused-silica column (Hewlett-Packard, Palo Alto, CA, U.S.A.).

A Model J4-6981 Aminco-Bowman spectrophotofluorometer, a Model J10-280 photomultiplier microphotometer (American Instruments, Silver Springs, MD, U.S.A.), and an Omnigraph 2000 X-Y recorder (Houston Instruments, Austin, TX, U.S.A.) were used to obtain all fluorescence spectra.

Reagents

L-Cysteine (L-CYS) was obtained as the hydrochloride salt from Eastman Kodak (Rochester, NY, U.S.A.). L-Cysteine ethyl ester hydrochloride (L-CEE), "gold" labeled D-penicillamine (D-PEN) and 2-cyclohexylaminoethanethiol (CAET) were obtained from Aldrich (Milwaukee, WI, U.S.A.). *o*-Aminobenzenethiol (o-ABT) and β-mercaptoethylamine (BMEA) were purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.). o-ABT, BMEA, and CAET were purchased in the free base form; they were precipitated as their hydrochloride salts with gaseous hydrogen chloride in anhydrous ether and recrystallized from absolute ethanol. OPT, glacial acetic acid, phosphoric acid and anhydrous sodium carbonate were purchased from Sigma (St. Louis, MO, U.S.A.). HPLC grade methanol, acetonitrile, and methylene chloride were obtained from Burdick & Jackson (Muskegon, MI, U.S.A.). Ultrapure water was produced in-house by a Milli-Q reagent grade water system (Millipore, Bedford, MA, U.S.A.). All other incidental chemicals were of reagent grade purity.

The OPT derivatizing reagent solution was prepared by dissolving 200 mg of OPT in 1 ml of methanol; this mixture was then diluted to 250.0 ml with 0.50 *M* potassium borate buffer at pH 10.50.

Procedure

Aqueous 100-μl samples containing mixtures of β-aminothiols were injected into the HPLC system and eluted through the column by using a gradient with mobile phase A (0.50 *M* aqueous sodium acetate, pH 4.50) and mobile phase B (88% methanol, 10% 0.50 *M* sodium acetate, pH 4.50, and 2% acetonitrile) at a flow-rate of 1.00 ml/min. A concave gradient (No. 9) was used to program the mobile phase composition from 0 to 80% B in 20 min. The OPT reagent was added to the column effluent at a rate of 1.00 ml/min. The detector effluent was collected for the duration of full-scale detector response. The collected fraction was then extracted with two 2-ml portions of methylene chloride. The methylene chloride layer was removed and rendered anhydrous by being passed through a short Pasteur pipet containing a plug of glass wool and solid anhydrous sodium sulfate. The resulting methylene chloride fraction was collected and evaporated under a stream of dry nitrogen to a volume of *ca.* 100 μl. In the case of D-PEN, the presence of a carboxylic acid functionality necessitated careful adjustment of the pH of the aqueous fraction to 5.50 with 10% hydrochloric acid, prior to extraction with methylene chloride. In addition it was necessary to derivatize the D-PEN extract further with bis(trimethylsilyl)trifluoroacetamide (BSTFA) in order to render the sample sufficiently volatile for GC–MS analysis.

A 1-μl aliquot of the methylene chloride concentrate (plus BSTFA in the case of D-PEN) was injected into the GC–MS at an injector temperature of 305°C using a 15:1 split ratio and a helium carrier flow-rate of 0.80 ml/min. The column temperature was initially held at 100°C for 2 min, then increased to 300°C at a rate of 20°C/min. The mass spectrometer was used in the electron-impact (EI) mode with the electron energy set at 70 eV. The scan time was 1 sec over a mass range of *m/z* 33 to *m/z* 500.

Spectrofluorescence spectra were obtained for each β-aminothiol-OPT derivative by adding 1.0 ml of 0.0060 *M* OPT (in borate buffer, pH 10.50) to 3.0 ml of aqueous β-aminothiol (0.10 μg/ml) in a fused-silica fluorescence cell. After mixing in an ultrasonic bath for 10 sec, the excitation and fluorescence spectra were recorded from 200 to 800 nm.

RESULTS AND DISCUSSION

Fig. 1 shows a 16-min HPLC separation of a standard mixture of six β-aminothiols employing post-column derivatization with OPT, followed by fluorescence detection. The optimum post-column reaction pH and temperatures were found to be 10.50 and 50°C, respectively. The unique feature of this post-column derivatization technique is that the β-aminothiols examined all react to form fluorophores with OPT alone (without externally added free thiol). Thus, it was of interest to determine the structures of the fluorescent derivatives formed in this HPLC procedure. This was achieved by GC–MS analysis of extracts of the post-column derivatization effluent for five of the compounds studied.

The EI mass spectra for the BMEA-OPT and the silylated D-PEN-OPT derivatives are shown in Figs. 2 and 3. In the latter case, a pH-controlled extraction at 5.5, as well as further derivatization of the methylene chloride extract with BSTFA, were required in order to achieve recovery of the D-PEN-OPT derivative from the

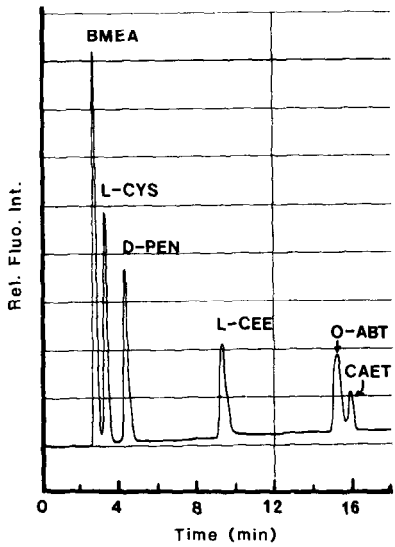


Fig. 1. Chromatogram for 93.0 μg of BMEA, 76.4 μg of L-CYS, 103 μg of D-PEN, 184 μg of L-CEE, 210 μg of o-ABT, and 242 μg of CEAT. Flow-rate, 1.0 ml/min; column, μBondapak C_{18}; Mobile phase A, 0.50 *M* acetate buffer (pH 4.50); mobile phase B, 88.0% methanol, 10.0% acetate buffer, 2.0% acetonitrile; gradient, concave, 0 to 80.0% B in 20 min; fluorescence detection, λ_{exc} = 340 nm, λ_{em} = 440 nm.

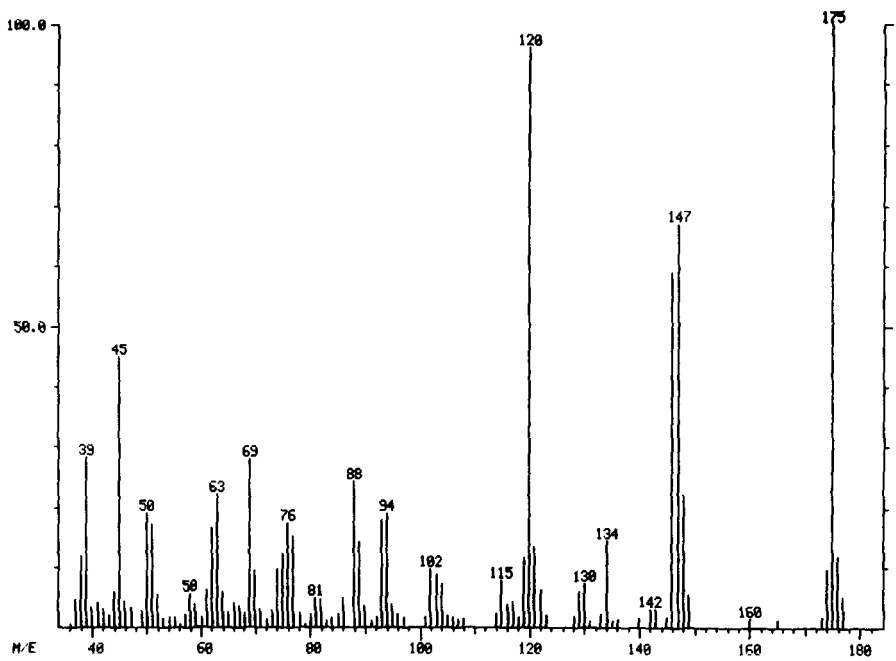


Fig. 2. EI mass spectrum for the BMEA-OPT derivative.

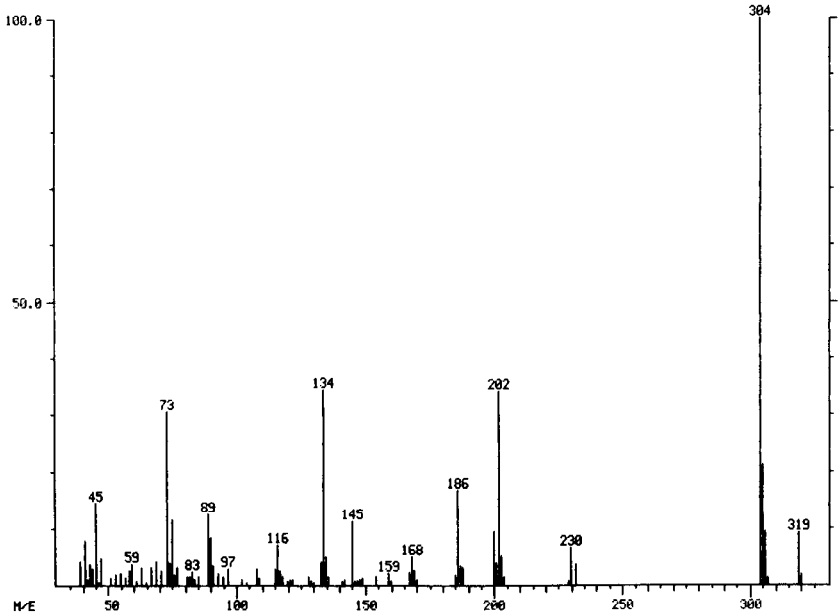
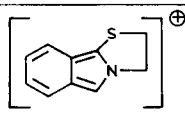
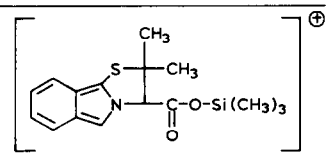
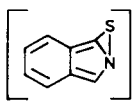
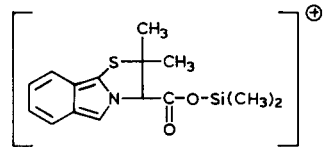
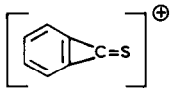
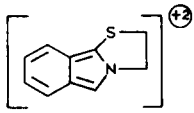
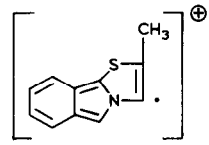
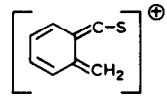
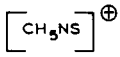
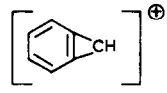
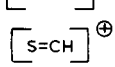


Fig. 3. EI mass spectrum for the trimethylsilylated D-PEN-OPT derivative.

TABLE I

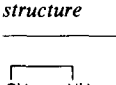
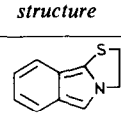
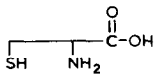
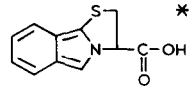
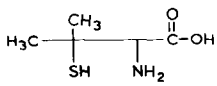
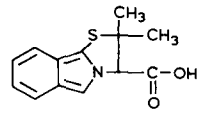
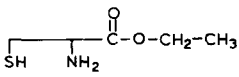
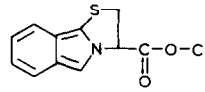
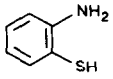
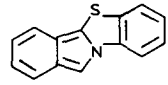
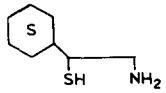
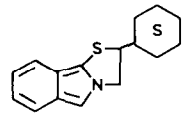
EI MASS SPECTRAL FRAGMENTATION PATTERNS

BMEA-OPT derivative			*D-PEN-OPT derivative (silylated)*		
m/z	*Rel. int. (%)*	*Assigned fragment*	*m/z*	*Rel. int. (%)*	*Assigned fragment*
175 (M^+)	100		319 (M^+)	10	
147	68		304	100	
120	97				
88	25		186	17	
69	28		134	34	
63	22		89	13	
45	45				

aqueous effluent, and to improve its volatility for GC–MS analysis. The molecular ions (M^+) for the BMEA-OPT and the silylated D-PEN-OPT derivatives were observed at *m/z* 175 and *m/z* 319, respectively, as seen in Figs. 2 and 3. Table I lists the major *m/z* values, relative peak intensities and assigned molecular fragments for the BMEA-OPT and silylated D-PEN-OPT derivatives. For the three other β-aminothiols, L-CEE, o-ABT and CAET, neither pH-controlled extraction nor trimethylsilylation was required. Similar mass spectral patterns consistent with their respective molecular weights were observed for these compounds. Repeated attempts to isolate the L-CYS-OPT derivative for GC–MS analysis were unsuccessful, apparently owing to rapid decomposition of the compound, as verified by the observed loss of extract fluorescence and changes in color within 3 min after collection from the column. It was, however, possible to record excitation and emission fluorescence spectra for all of the other derivatives.

TABLE II

SUMMARY OF MS AND SPECTROFLUORESCENCE DATA FOR β-AMINOTHIOL-OPT DERIVATIVES

Parent β-Aminothiol structure	*Abbrev.*	*β-Aminothiol-OPT structure*	M^+ *(m/z)*	λ_{ex} *(max) (nm)*	λ_{em} *(max) (nm)*
	BMEA		175	338	438
	L-CYS		–	356	438
	D-PEN		319**	331	446
	L-CEE		247	348	448
	o-ABT		223	372	440
	CAET		191***	346	446

* Proposed structure.

** Trimethylsilylated derivative.

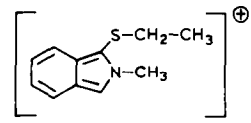
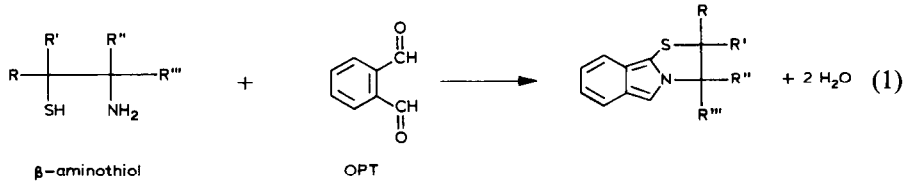
*** *m/z* value is for the ion of highest mass: 

Table II summarizes the principal mass spectral and spectrofluorometric characteristics which were observed for the six aminothiols studied in this investigation. The structures for the derivatives formed in this off-line HPLC–GC–MS study are also listed.

This investigation has demonstrated that OPT reacts selectively with β-aminothiols to produce fluorescent 2,3-dihydrothiazo[2,3*a*]-isoindole derivatives, as confirmed by off-line HPLC–GC–MS analysis. Eqn. 1 summarizes the reaction.



(1)

The highly selective nature of this reaction, combined with its rapidity, makes it ideally suited for detecting β-aminothiols in HPLC procedures with on-line post-column derivatization.

ACKNOWLEDGEMENTS

We thank the donors of The Petroleum Research Fund, administered by the American Chemical Society, and the Camille and Henry Dreyfus Foundation for financial support.

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