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High-performance liquid chromatographic separation of biogenic polyamines using 2-(1-pyrenyl)ethyl chloroformate as a new fluorogenic derivatizing reagent

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ABSTRACT

The application of a new fluorogenic pre-column derivatizing reagent, 2-(1-pyrenyl)ethyl chloroformate (PEOC), is reported for the separation and detection of biogenic polyamines using column liquid chromatography. The development of the method included the optimization of excitation and emission wavelengths, efficient gradient programming, derivatization temperature, time, and pH. Minimum detection limits, linear ranges, reproducibility, and recovery from analyzed samples were determined. The procedure was applied to hydrolyzed serum samples taken from healthy individuals and cancer patients. Separation of PEOC-derivatized polyamines from the serum hydrolysis by-products was successful and detection limits were more favorable than those previously reported for 9-fluorenylmethyl chloroformate-derivatized polyamines.

INTRODUCTION

Fluorogenic 9-fluorenylmethyl chloroformate (FMOC) has been used successfully as a pre-column derivatizing agent in high-performance liquid chromatography (HPLC) for the determination of analytes containing primary amino groups. In FMOC, the chloroformate functionality reacts with primary and secondary amines to form fluorescent carbamates and the reaction has been utilized for the determination of amino acids [1–3] and polyamines [4]. Recently, 2-(9-anthryl)ethyl chloroformate (AEOC) was reported to be another effective fluorogenic HPLC pre-column reagent for polyamines [5]. Previously, polyamines have also been determined by HPLC by Seiler *et al.* as the 5-dimethylaminonaphthalene-1-sulfonyl [6,7] and monoacetyl derivatives [8,9], and in our laboratory as the *o*-phthalaldehyde derivatives [10].

The structures of the four commonly encountered biogenic polyamines putrescine (PUT), cadaverine (CAD), spermidine (SPD) and spermine (SPM) are shown in Table I. The analytical determination of these four compounds in human urine, serum, and tissue has generated much interest in recent years because elevated levels of these compounds have been associated with the rapid regeneration or regrowth of tissue [11–13]. Ion-exchange [14,15] and reversed-phase [4,5,10,16–19] chromatography, have been used extensively to determine polyamines in biological

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TABLE I

POLYAMINE STRUCTURES AND ABBREVIATIONS

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

fluids. Direct detection of polyamines is difficult, because they do not absorb in the ultraviolet (UV) region and, consequently, they do not possess native fluorescence. Therefore, HPLC procedures require either pre- or post-column derivatization with chromophoric or fluorogenic reagents to produce species that are detectable.

As far as we know, pyrenyl-substituted chloroformates have not been reported as derivatizing agents in HPLC, although both the high molar absorptivity of the pyrene chromophore around 340 nm ($\varepsilon \approx 50\,000$ l mol^{-1} cm^{-1}) and the high fluorescence quantum yield (about 0.7) are analytically attractive features. In this paper, we report 2-(1-pyrenyl)ethyl chloroformate (PEOC) as a pre-column derivatizing agent for polyamines

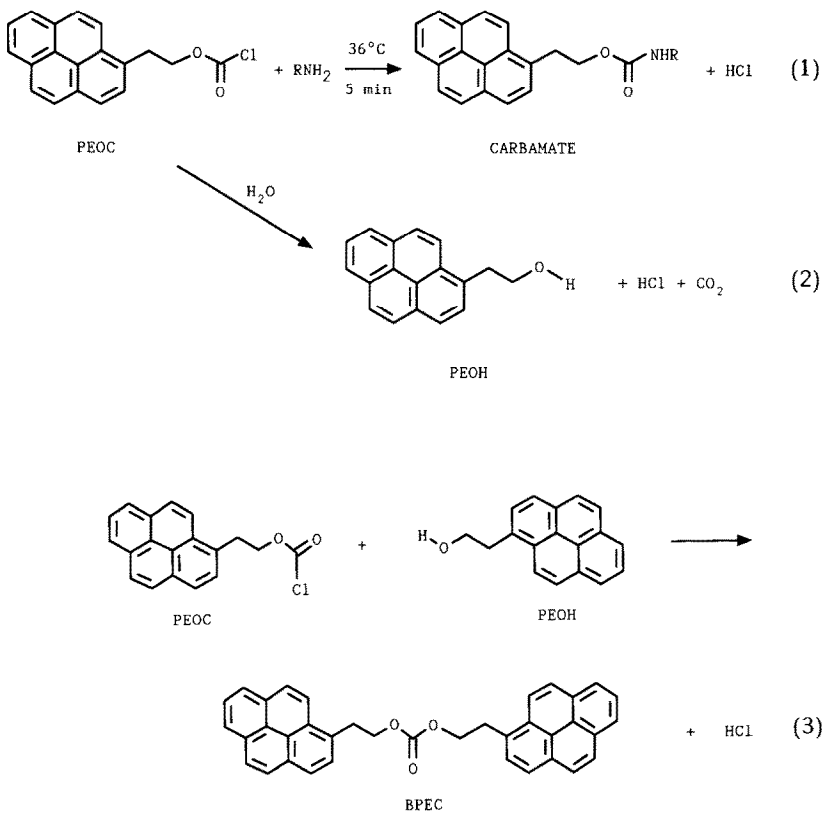

Fig. 1. Reaction of PEOC with primary amines to form fluorescent carbamates (reaction 1) and hydrolysis of PEOC in aqueous media to form PEOH (reaction 2) which may react with PEOC to give BPEC (reaction 3).

separated by HPLC. Like other chloroformates, PEOC reacts with primary and secondary amines to form fluorescent carbamates. Excess PEOC undergoes hydrolysis in aqueous media to form 2-(1-pyrenyl)ethanol (PEOH) which may react with PEOC to give bis-2-(1-pyrenylethyl) carbonate (BPEC), as shown in reactions 1–3 (Fig. 1).

EXPERIMENTAL

Apparatus

The chromatographic system included a Spectra-Physics SP-8700 ternary solvent delivery system equipped with a Model 7010 Rheodyne injection valve and a 20-μl sample loop. The fluorescence detector was a Model FS-970 (Schoeffel, Division of Kratos, Westwood, NJ, USA) with the excitation set at 275 nm and emission monitored through a 389-nm cut-off filter. The sensitivity range was set at 1.0 μA with a time constant of 1.5 s and high suppression unless noted otherwise. A Hewlett-Packard LiChrospher 100 RP-18, 125 mm × 4 mm I.D. packed column (5-μm particles) was used for analytical separations. Fluorescence spectra were obtained using a Perkin-Elmer LS-50 luminescence spectrometer. A Hewlett-Packard 3396A integrator was used for peak integration and for recording all chromatograms.

Samples were separated at a flow-rate of 1.0 ml/min with a binary gradient consisting of 10% water and 90% acetonitrile pumped isocratically for 5 min. This was followed by a step gradient to 20% ethyl acetate and 80% acetonitrile pumped isocratically for an additional 15 min. The addition of ethyl acetate was found necessary in order to prevent unreasonably long retention times for SPD and SPM. The column temperature was ambient.

Chemicals and reagents

The four polyamines, PUT, CAD, SPD, and SPM, were obtained as the hydrochloride salts from Sigma (St. Louis, MO, USA) and were used without further purification. HPLC-grade acetonitrile and ethyl acetate were obtained from J. T. Baker (Phillipsburg, NJ, USA). HPLC-grade water was generated in-house using a water purification system manufactured by Industrial Water Technology (North Attleboro, MA, USA). Reagent-grade hydrochloric acid, potassium dihydrogenphosphate, potassium hydroxide, sodium borate, and sodium bicarbonate were used to prepare buffer solutions and were obtained from Fisher Scientific (Fair Lawn, NJ, USA). The derivatizing agent PEOC (m.p. 71–73°C), and BPEC (m.p. 225–228°C) were prepared in conventional fashion from PEOH and phosgene. PEOC reagent stock solutions were kept in light-protected containers and stored under refrigeration when not in use.

Samples of physiological fluids

Individual and pooled serum samples were obtained from the Clinical Laboratory at Evangelical Community Hospital (Lewisburg, PA, USA). Cancer serum samples were obtained from the Clinical Chemistry Laboratory at Geisinger Medical Center (Danville, PA, USA). All serum samples were stored at −25°C.

Procedure

Derivatization of polyamine standards was accomplished by adding 500 μl of 0.1 m*M* PEOC in HPLC-grade acetonitrile to 400 μl of a 5.0 ng/μl working standard polyamine solution and 100 μl of pH 9.5 buffer (0.025 *M*). The derivatization solution was incubated for 5 min at 36°C to initiate the reaction. A 50-fold, post-derivatization, pre-injection dilution of the PEOC-polyamine solution with HPLC-grade acetonitrile was necessary to avoid detector overload.

Serum samples required a modified procedure, consisting of a pre-derivatization acidic hydrolysis to release the polyamines from the conjugates that they form with proteins and other components in serum [14]. The procedure consisted of a 10-h reflux of a 5.0-ml aliquot of serum with 5.0 ml of 6 *M* hydrochloric acid. For recovery studies, spiking of the serum with known amounts of polyamine standards was performed after the hydrolysis step. The resulting hydrolysate was evaporated to dryness at reduced pres-

sure and a temperature of 60°C. The residue was neutralized with dilute potassium hydroxide to pH 6, re-evaporated, and quantitatively diluted to a volume of 10 ml. The resulting solution was then centrifuged for 30 min at 1380 *g* and filtered through a 0.45-μm Millex-HV filter unit (Millipore). Hydrolyzed serum (400 μl), 300 μl of pH 9.0 buffer (0.025 *M*), and 300 μl of 5.6 m*M* PEOC stock solution were mixed and incubated for 5 min at 36°C. It was necessary to dilute the post-derivatized serum solution 400-fold prior to HPLC injection.

RESULTS AND DISCUSSION

The fluorescence excitation and emission spectra for 0.025 m*M* PEOC in 75% acetonitrile are shown in Fig. 2. The emission spectrum was scanned at λ_{exc} = 240 nm and the excitation spectrum at λ_{em} = 379 nm. Prominent excitation and emission maxima were observed at 240, 275, and 340 nm and 379 and 395 nm, respectively. Similar spectra were recorded for eluted fractions of the PEOC derivative of putrescine. These spectra were identical to those shown in Fig. 2. On the basis of the spectra obtained, HPLC detection of PEOC-polyamine was optimized by using an excitation wavelength of 275 nm and a low-end cut-off emission filter of 389 nm.

Derivatization of polyamines with PEOC at ambient temperature was found to proceed slowly without reaching completion. At 36.0°C, however, a reaction time of only 5 min was needed in order for quantitative conversion to occur. Higher temperatures and longer incubation times had no apparent effect on the PEOC-polyamine derivative peak size, but did enlarge the PEOH peak size.

The reaction of PEOC with polyamines was found to be pH-dependent. The optimum reaction pH was determined by derivatizing each of the four polyamines at pH values ranging from 7.00 to 10.50 and measuring the fluorescence response (λ_{exc} = 275 nm; λ_{em} > 389 nm) for each eluted analyte as a function of pH. From these results shown in Fig. 3, an optimum derivatization pH of 9.50 was selected for most of the experimental work; it was found that pH 9.0 was more effective for derivatization of hydrolyzed serum samples. At lower pH values, it is possible

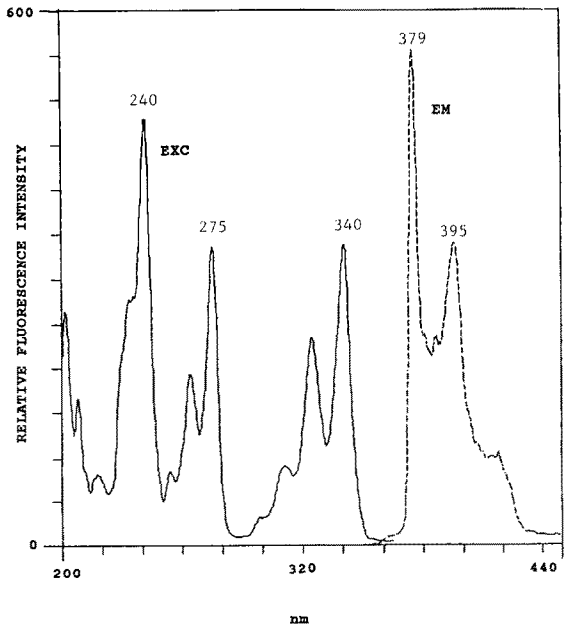

Fig. 2. Fluorescence excitation and emission spectra of 0.025 m*M* PEOC.

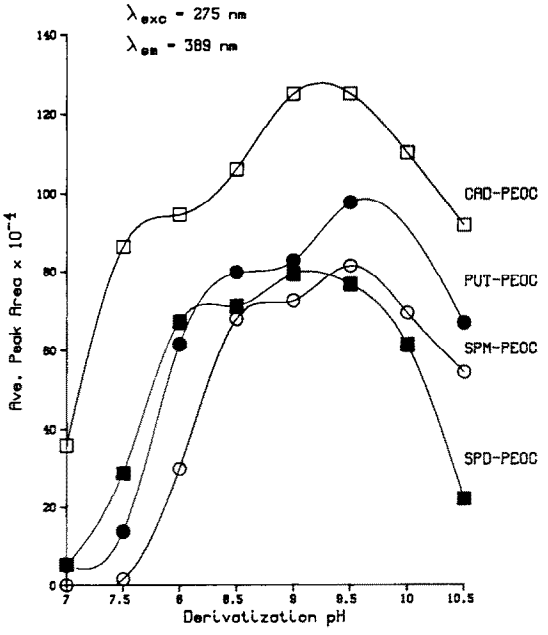

Fig. 3. Detector response *versus* derivatization pH for eluted PEOC-polyamine derivatives.

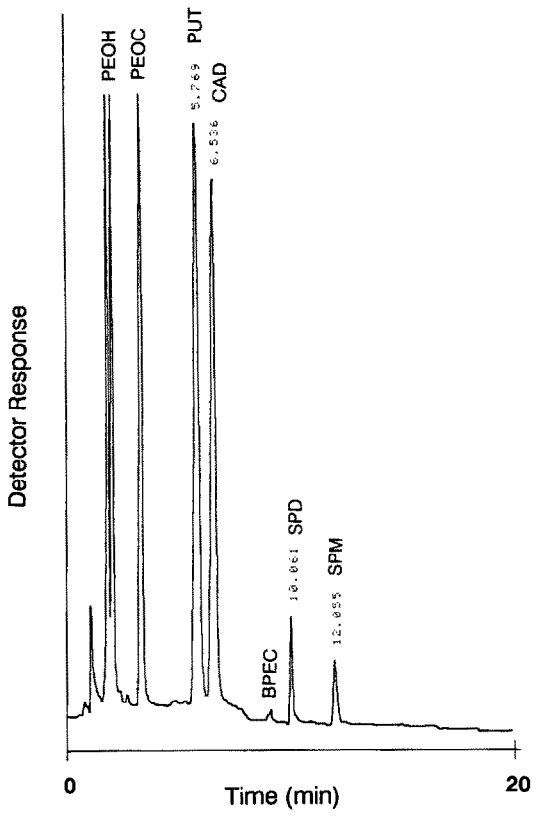

Fig. 4. Chromatogram for the separation of a standard mixture of four PEOC-derivatized polyamines (0.4 ng of each polyamine injected).

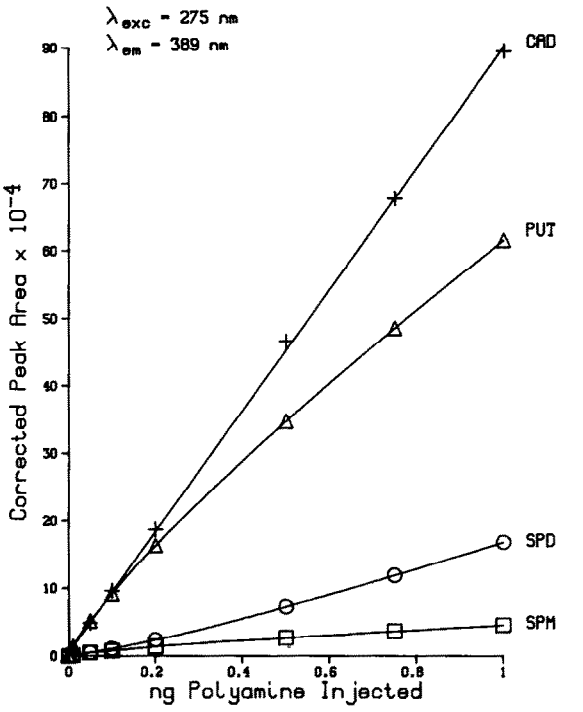

Fig. 5. Corrected peak area *versus* nanograms of polyamine injected. (Experimental data replotted according to ref. 20.)

that excessive polyamine protonation prevents complete derivatization, whereas at pH > 9, the hydrolysis of PEOC appears to be favored.

A chromatogram for the separation of a standard mixture of the four polyamines (0.4 ng of each polyamine) is shown in Fig. 4. It can be seen that the four PEOC-polyamine derivatives were completely resolved from each other and from excess reagent and reaction by-products.

Fig. 5 shows a plot of corrected peak area *versus* nanograms of polyamine injected. Data points were obtained from 0 to 1 ng by derivatizing solutions containing various amounts of polyamines prior to chromatography. The original plots showed significant curvature due to nonlinear detector behavior. The data were replotted according to the method of Dorschel *et al.* [20] in order to show the actual response more clearly.

The limits of detection for this procedure were determined for each of the four polyamines. This was accomplished by setting the detector sensitivity at the maximum range of 0.01 μA and injecting successively diminishing quantities of each of the derivatized polyamines (*i.e.*, for PUT the solutions injected contained 20.0, 6.7, 2.2, and 1.1 ± 0.1 pg of free base, respectively). Each detection limit was obtained by recording the smallest amount of polyamine that still produced a peak at a signal-to-noise ratio of 3:1. Minimum detection limits were found to be 1.1, 0.9, 1.3, and 2.9 pg for PUT, CAD, SPD, and SPM, respectively. These limits are similar to those measured earlier for AEOC-derivatized polyamines [5]. Recoveries for synthetically prepared polyamine solutions were: PUT, 128%; CAD, 97%; SPD, 100%; SPM 135%.

The method was applied to several hydrolyzed normal and cancer serum samples. The pre-column derivatization procedure was modified for hydrolyzed serum samples by using a higher con-

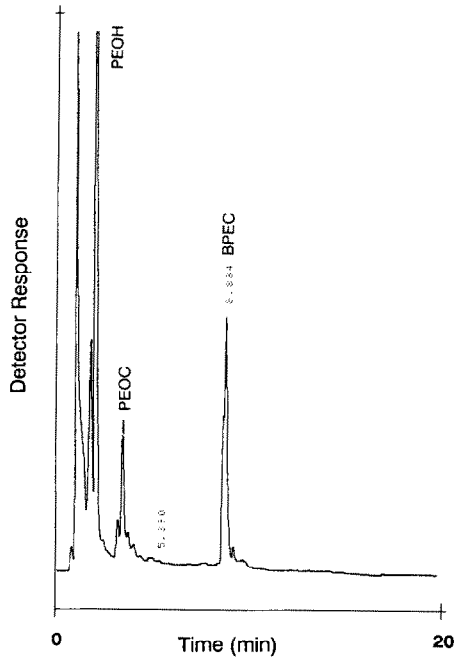

Fig. 6. Chromatogram for hydrolyzed PEOC-derivatized normal blood serum.

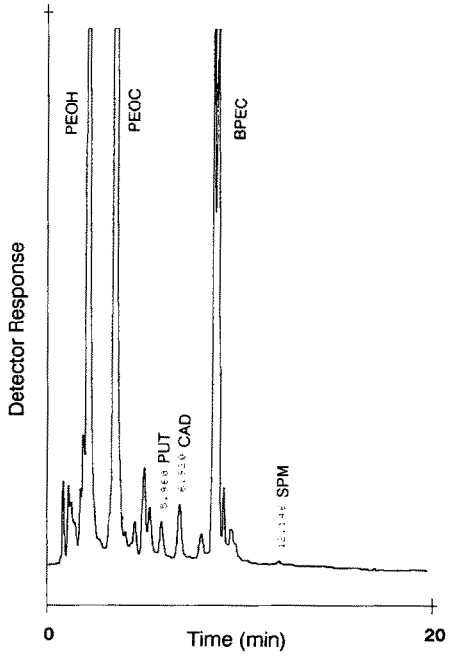

Fig. 7. Chromatogram for hydrolyzed PEOC-derivatized breast cancer blood serum. PUT, 2 ng/μl; SPD, 7.0 ng/μl; SPM, 65 ng/μl.

centration of PEOC (5.6 m*M*) and a pH of 9.0 for the derivatization step. The increased PEOC concentration compensated for derivatization with other chloroformate-reactive components in serum and the slightly lower pH resulted in more satisfactory derivatization in a blood serum medium. Figs. 6 and 7 illustrate chromatograms for pooled samples of normal and breast cancer sera, respectively. No polyamines were found in normal serum; however, the cancer serum showed substantial levels of three polyamines. The concentrations found represented the average of three runs and are given with Fig. 7.

The peak eluting at 8.9 min in Fig. 6 was identified as BPEC. Its identity was confirmed by comparing the retention time to that of an authentic sample. The retention time of this peak was observed to depend on reaction pH. For example, BPEC eluted at 9.4 min in the chromatogram for the standard mixture (Fig. 4), run at a derivatization pH of 9.5. BPEC is the product that forms when PEOC reacts with PEOH in aqueous solution, as shown in reaction 3 (Fig. 1). This reaction is analogous to that observed previously between AEOC and 2-(9-anthryl)ethanol [5].

CONCLUSIONS

This investigation has demonstrated that PEOC is an effective and sensitive pre-column derivatizating reagent for polyamines determined by HPLC with fluorescence detection. Resolution and total time of analysis were found to be considerably improved compared to AEOC-derivatized polyamines. The limits of detection are similar to those found previously for AEOC-derivatized polyamines and substantially better than for FMOC-derivatized polyamines. The derivatization procedure is rapid and simple, making the method attractive for biomedical studies.

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