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ADSORPTION AND REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF *p*-NITROBENZYL ESTERS OF MONOHYDROXY FATTY ACIDS

Z. L. BANDI* and E. S. REYNOLDS*
Department of Pathology, The University of Texas Medical Branch, Galveston, TX 77550 (U.S.A.)
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SUMMARY

To enhance the UV detectability of hydroxy fatty acids, *p*-nitrobenzyl (PNB) esters of twenty-two different monohydroxy fatty acids of various chain-lengths (C_{16}–C_{22}) and differing positional isomers were formed using O-(*p*-nitrobenzyl)-N,N-(diisopropyl)-isourea (PNBDI) as alkylating agent. Reversed-phase and adsorption high-performance liquid chromatography (HPLC) of the twenty-two monohydroxy fatty acid PNB esters were studied. The PNB group did not dominate the chromatographic properties of monohydroxy fatty acids and it did not interfere with the HPLC separation of positional isomers. PNBDI was, however, found to be less than ideal for formation of PNB derivatives of monohydroxy fatty acids because UV absorbing contaminants of PNBDI interfered with the HPLC analyses.

INTRODUCTION

Non-enzymatic oxidation (autoxidation) of unsaturated fatty acids produces rancidity, and spoilage of food[1,2] and the products of autoxidation are toxic[3,4]. *In vivo* autoxidation of biological membrane polyunsaturated fatty acids thought to cause tissue damage and cell death[3,4].

During the last decades, much information has appeared in the literature on the mechanism of *in vitro* autoxidation[5,6]. *In vivo* autoxidation, however, has only been studied with indirect and non-specific methods because in biological samples the products of autoxidation occurred only in trace amounts and because sensitive and specific methods did not exist for isolation of oxygenated fatty acids[7,8]. The results of the most direct approach to the study of *in vivo* autoxidation were reported recently by Hughes *et al.*[9] but they could isolate and identify only those oxygenated fatty acids which possessed conjugated double bonds.

Products of autoxidation that contain no conjugated double bonds need to be isolated and identified to differentiate the products of "dark" or "bulk phase" oxidation from those of the "photosensitized" or "singlet oxygen" mediated autoxida-

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tion. The "dark" oxidation has been shown to involve a free radial mechanism, to produce conjugated double bonds and to be inhibited by free radical quenchers[8,10–13]. In contrast, the "photosensitized" oxidation is believed to involve singlet oxygen produced by photosensitized excitation of triplet oxygen by chromophore impurities, to produce non-conjugated in addition to conjugated products and to be inhibited by carotene, triethylamine, nickel chelates and α-tocopherols[8,10,12,14].

Since oxygenated fatty acids without conjugated double bonds have only weak UV absorption, formation of a chemical derivative was needed to enhance detectability by high-performance liquid chromatography (HPLC) photometric detectors. A reagent for attaching a chromophore of high molar extinction coefficient to oxygenated fatty acids should meet the following criteria:

(1) The reaction of derivatization should go to completion under conditions that do not alter the oxygenated fatty acids.

(2) Side-products of the reaction, including excess reagent and reagent impurities, should not interfere with the chromatography of the derivatives. The contaminating compounds should be either non-UV absorbing or preferably they should have sufficiently different structure from the derivatives for successful separation.

(3) The derivatives should preserve chromatographic differences of the parent compounds to allow their separation in addition to their detection. The newly introduced chromophore should not dominate the chromatographic behavior of the derivative.

A number of UV visualizing reagents have been developed for HPLC analysis of non-hydroxy fatty acids[15–17] but no one seems to have applied these reagents to the detection and separation of positional isomers of monohydroxy fatty acid. Haslbeck and Grosch[19] formed phenyl esters of oleic and linoleic acids to enhance the detection of oxidation products that contained non-conjugated dienes. Their method of synthesis, however, was not applicable to analytical methods for detection of trace amounts of oxidation products because the derivatization reaction did not go to completion and because the phenyl group had relatively low molar extinction coefficient.

As the first candidate, we selected the *p*-nitrobenzyl (PNB) ester derivative (maximum absorption at 265 nm, molar extinction coefficient at 254 nm is 6200)[18] for the enhancement of HPLC detection of monohydroxy fatty acids because the PNB group was non-polar. This derivative offered hope that it would not have interfered with the difficult fractionation of positional isomers of hydroxy fatty acids.

In this communication we report the use of O-(*p*-nitrobenzyl)-N,N′-(diisopropyl)-isourea (PNBDI) for the formation of PNB esters of monohydroxy fatty acids and the HPLC analysis of the PNB derivatives on both reversed-phase and adsorption HPLC columns.

MATERIALS AND METHODS

Reagents

Monounsaturated fatty acid methyl esters, for synthesis of hydroxy fatty acids, were obtained from Nu Chek Prep (Elysian, MN, U.S.A.). O-(*p*-Nitrobenzyl)-N,N′-(diisopropyl)-isourea was purchased from Pierce (Rockford, IL, U.S.A.) and from Regis (Morton Grove, IL, U.S.A.). All HPLC grade solvents were obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

Synthesis of hydroxy fatty acids

Monohydroxy fatty acids were synthesized with a method similar to that of Gunstone and Inglis[20]. Briefly: mercuric acetate (2 mmol) dissolved in 5 ml water was added to monounsaturated fatty acid methyl esters (0.67 mmol) dissolved in 15 ml tetrahydrofuran and the reaction mixture was vigorously shaken at room temperature for 24–28 h. Then, in an ice bath, 2 ml 3 *M* sodium hydroxide and sodium borohydride (5.3 mmol) dissolved in 4 ml 3 *M* sodium hydroxide were added. The two-phase mixture was shaken vigorously at room temperature for 60 min. After the precipitated mercury had coagulated, aqueous sulfuric acid was added to adjust the pH to 1. The hydroxy fatty acid methyl esters were extracted with diethyl ether and were purified with thin-layer chromatography (TLC) using hexane–ether (1:1, v/v) as developing solvent.

Hydrolysis of the hydroxy fatty acid methyl esters

Benzene (1 ml) and 2.7 ml 5.8 *M* potassium hydroxide were added to 50 mg of hydroxy fatty acid methyl esters dissolved in 2 ml of ethanol. The reaction mixture was heated at 95°C for 60 min with occasional vigorous shaking. Sulfuric acid (2 *M*) was added to adjust the pH to 1 and the hydroxy fatty acids were extracted with diethyl ether.

Formation of PNB esters of hydroxy fatty acids

Into a 50-ml round bottom centrifuge tube, equipped with a PTFE-lined screw cap, 0.1 mmol of hydroxy fatty acids, 7 ml chloroform and 0.3 mmol of PNBDI dissolved in 3 ml methylene chloride were placed. The reaction mixture was heated at 80°C for 3 h with occasional shaking. The fatty acids may dissolve only after heating. Then, the centrifuge tube was placed in a ice-water bath, 5 ml of methanol and 0.2 ml of 1 *M* sulfuric acid were added to destroy the excess alkylating reagent. The one-phase mixture was left standing for 10 min at room temperature and then 4 ml of 0.1 *M* sodium hydrogen carbonate was added. The centrifuge tube was shaken for 10 sec and centrifuged. The upper (aqueous) layer was discarded and the lower layer was washed with 4 ml water. The lower layer, containing the PNB esters of hydroxy fatty acids, was evaporated on a rotary evaporator and the water was removed with repeated addition and evaporation of chloroform.

Purification of PNB esters of hydroxy fatty acids

The PNB esters of hydroxy fatty acids (3 mmol) were applied to a 1000 μm silica gel G plate containing inorganic fluorescent indicator. The plate was developed with hexane–ether (1:1, v/v) and the PNB esters were detected with short wavelength UV light. Depending on the chain-length of the hydroxy fatty acids ($R_F \approx 0.3$–0.4), the PNB esters may or may not overlap with some of the impurities of PNBDI. To check the purity, and to identify the PNB esters of hydroxy fatty acids, the compounds in question were analyzed by C_{18} (octadecyl groups bound to silica, Whatman, Clifton, NJ, U.S.A.) reversed-phase TLC, containing inorganic fluorescent indicator, using acetonitrile–tetrahydrofuran (95:5, v/v) as developing solvent. The PNBDI contaminants moved with the solvent front ($R_F \approx 0.8$–1) and the PNB esters of hydroxy fatty acids were retained by the C_{18} stationary phase ($R_F \approx 0.4$–0.6). If needed, the PNB esters were purified with C_{18} reversed-phase TLC. For purification,

the developing solvent varied, depending on the chain-length of the acids, from mixtures of acetonitrile–tetrahydrofuran to acetonitrile–water.

HPLC of PNB esters of hydroxy fatty acids

The analyses were performed on a Model 334 Beckman gradient HPLC system equipped with a 421 CRT Controller, two Model 110A pumps, Model 210 injector, mixing chamber and a Model 165 variable-wavelength UV–VIS detector. The reversed-phase analysis was carried out on two Supelcosil LC-18 (silica with octadecyldimethylsilyl coat), 3 μm particle size, 7.5 cm × 4.6 mm columns (Supelco, Bellefonte, PA, U.S.A.) linked in series. Solvent system: water content of acetonitrile was reduced from 30 to 0% in 100 min. The flow-rate was 0.5 ml/min. Temperature was ambient. The adsorption chromatography was done on a Spherisorb S5W, silica, 5 μm particle diameter, 25 cm × 10 mm reversible column (Regis, Morton Grove, IL, U.S.A.); solvent system: hexane–isopropyl alcohol (1000:4, v/v); flow-rate: 2 ml/min; detector was set at 265 nm.

RESULTS AND DISCUSSION

Esterification of the carboxyl groups of hydroxy fatty acids was complete in 180 min if the molar ratio of alkylating agent to fatty acids was about 3:1.

Commercially available PNBDI contained UV absorbing contaminants which were more polar than the alkylating agent. Since the PNB esters of non-hydroxy fatty acids were less polar than PNBDI, the contaminants of PNBDI did not interfere with HPLC of non-hydroxy fatty acids on silica adsorption columns. The PNB esters of non-hydroxy fatty acids could be analyzed, directly after formation, on silica columns because the excess PNBDI and the contaminants remained on the silica column after the fatty acids were eluted.

The PNB esters of monohydroxy fatty acids, however, were comparable in polarity to PNBDI and to some of the contaminants of PNBDI. The excess PNBDI could be destroyed, without any damage to the PNB esters, by adding some aqueous inorganic acids to the reaction mixture (see Materials and methods). However, our efforts to remove the contaminants from PNBDI failed and HPLC analysis of PNB esters of hydroxy fatty acids was not possible without previous purification of the derivatives.

The highly polar PNBDI contaminants ($R_F \approx 0.0$–0.2) could be separated from the hydroxy fatty acid PNB esters with adsorption TLC on silica gel layers using hexane–ether (1/1, v/v). The less polar PNBDI contaminants ($R_F \approx 0.2$–0.4) overlapped with the PNB esters on silica gel but they separated from the hydroxy fatty acid PNB esters on C_{18} reversed-phase (octadecyl chains bound to silica) HPLC columns or TLC plates. On C_{18} reversed-phase plates or columns, the less polar PNBDI contaminants traveled with the solvent front ($R_F \approx 0.8$–1) but the PNB esters of monohydroxy fatty acids are retained ($R_F \approx 0.4$–0.6).

Table I shows the hydroxy fatty acids that were synthesized in reactions 1–11. From each unsaturated fatty acid methyl ester two positional isomers of hydroxy fatty acid methyl esters were formed by addition of a hydroxy group to either end of the single double bond[20].

Fig. 1 shows the reversed-phased HPLC of PNB esters of the combined prod-

TABLE I

HYDROXY FATTY ACID METHYL ESTERS SYNTHESIZED BY HYDROXY-MERCURATION–DEMERCURATION

Abbreviations: prescript denotes the position of the hydroxy group and C_x denotes the chainlength, *e.g.*, 9h-C_{16} = methyl 9-hydroxyhexadecanoate.

Reaction	*Starting material*	*Products*
1	Methyl *cis*-9-hexadecenoate	9h-C_{16} and 10h-C_{16}
2	Methyl *cis*-10-heptadecenoate	10h-C_{17} and 11h-C_{17}
3	Methyl *cis*-6-octadecenoate	6h-C_{18} and 7h-C_{18}
4	Methyl *cis*-9-octadecenoate	9h-C_{18} and 10h-C_{18}
5	Methyl *cis*-11-octadecenoate	11h-C_{18} and 12h-C_{18}
6	Methyl *cis*-7-nonadecenoate	7h-C_{19} and 8h-C_{19}
7	Methyl *cis*-8-eicosenoate	8h-C_{20} and 9h-C_{20}
8	Methyl *cis*-11-eicosenoate	11h-C_{20} and 12h-C_{20}
9	Methyl *cis*-13-eicosenoate	13h-C_{20} and 14h-C_{20}
10	Methyl *cis*-12-heneicosenoate	12h-C_{21} and 12h-C_{21}
11	Methyl *cis*-13-docosenoate	13h-C_{22} and 14h-C_{22}

ucts of reactions 1–11. These PNB esters were purified with adsorption TLC and the more polar PNBDI contaminants were removed before the reversed-phase HPLC. The less polar contaminants from PNBDI (unlabeled peaks) eluted from the reversed-phase column before the shortest (C_{16}) hydroxy fatty acid PNB ester did. On the reversed-phase columns, the PNB esters of monohydroxy fatty acids separated not only according to the chain-length but some fractionation occurred also according to the position of the hydroxy group. The separation of positional isomers interfered somewhat with the fractionation according to different chain-lengths. The

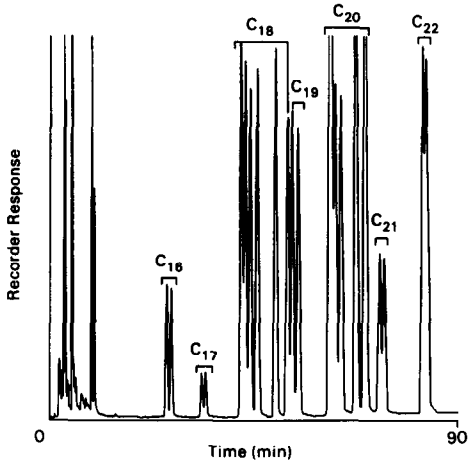

Fig. 1. Reversed-phase HPLC of PNB esters of monohydroxy fatty acids. Two Supelcosil LC-18, 3 μm particle size, 7.5 cm × 4.6 mm columns were linked in series. Solvent system: the water content of acetonitrile was reduced from 30 to 0% in 100 min. Flow-rate, 0.5 ml/min. Temperature was ambient. Detector was set at 265 nm. Abbreviations denote the chainlength of the positional isomers of PNB esters of monohydroxy fatty acids.

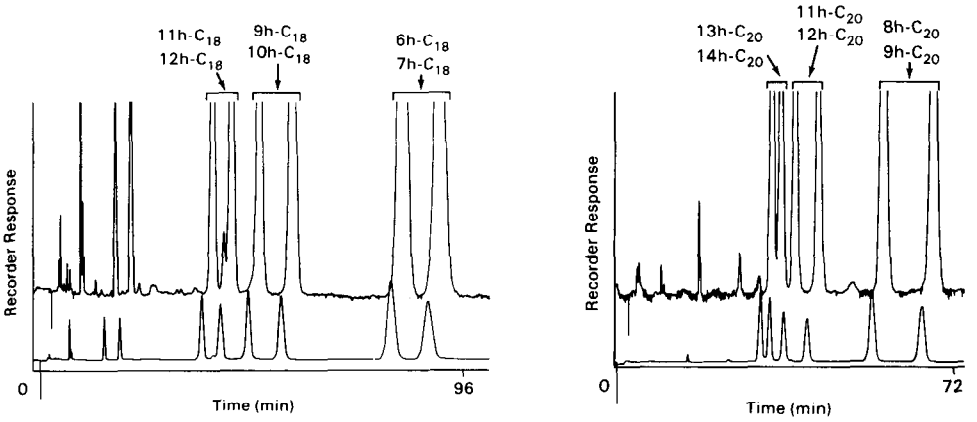

Fig. 2. Adsorption HPLC of PNB hydroxyoctadecanoate positional isomers. Spherisorb S5W, 5 μm silica particle diameter, 25 cm × 10 mm column. Solvent system: hexane–isopropyl alcohol (1000:4, v/v). Flow-rate, 2 ml/min. Temperature was ambient. Detector was set at 265 nm. Abbreviations: prescript denotes the position of the hydroxy group, *e.g.*, 11h-C_{18} = *p*-nitrobenzyl 11-hydroxyoctadecanoate.

Fig. 3. Adsorption HPLC of PNB hydroxyeicosanoate positional isomers. Spherisorb S5W, 5 μm silica particle diameter, 25 cm × 10 mm column. Solvent system: hexane-isopropyl alcohol (1000:4, v/v). Flow-rate, 2 ml/min. Temperature was ambient. Detector was set at 265 nm. Abbreviations: prescript denotes the position of the hydroxy group, *e.g.*, 14h-C_{20} = *p*-nitrobenzyl 14-hydroxyeicosanoate.

C_{19} hydroxy fatty acids, for example, were not completely separated from a positional isomer of C_{18} hydroxy fatty acids.

Figs. 2 and 3 show the adsorption HPLC of positional isomers of C_{18} (reactions 3–5) and C_{20} (reactions 7–9) monohydroxy fatty acid PNB esters. The PNB group did not seem to interfere with adsorption HPLC of positional isomers of monohydroxy fatty acids. With optimized conditions very good separation of positional isomers of hydroxy fatty acids could be achieved. However, the PNBDI impurities (unlabeled peaks) did interfere with the HPLC analysis.

The information presented in this report shows that PNB esters of monohydroxy fatty acids could be fractionated on both reversed-phase and adsorption HPLC columns; the PNB group did not interfere with the separation of positional isomers. PNBDI was less than ideal for the formation of PNB derivatives because the UV absorbing contaminants interfered with the chromatography. If PNBDI was used for detection of non-conjugated hydroxy fatty acids, then the PNB derivatives would need to be purified with adsorption TLC before reversed-phase HPLC could be performed. For maximal resolution of naturally occurring mixtures of hydroxy fatty acids, that may contain positional isomers of various different chain-lengths, combined use of reversed-phase HPLC followed by adsorption HPLC might be used.

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