Journal of Chromatography, 363 (1986) 402–406
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 810

Note

High-performance liquid chromatographic analysis of saturated monohydroxy fatty acid mixtures containing positional isomers of various chain-lengths

Z. L. BANDI* and G. A. S. ANSARI
Department of Pathology, The University of Texas Medical Branch, Galveston, TX 77550 (U.S.A.)
(Received May 21st, 1986)

Complete resolution and quantitation by high-performance liquid chromatography (HPLC) of mixtures of *p*-nitrobenzyl (PNB) esters of monohydroxy fatty acids containing positional isomers of various chain-lengths is a technical problem because their separation occurs according to both chain-length and position of the hydroxyl group on both reversed-phase and silica adsorption HPLC columns[1].

In a previous communication we reported 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 derivatives on both reversed-phase and adsorption HPLC columns[1]. We found that separation of positional isomers of PNB esters of monohydroxy fatty acids could be carried out successfully on adsorption (silica) HPLC columns but resolution according to chain-length by reversed-phase HPLC was only partially achieved because separation of positional isomers occurred in addition to the separation according to chain-length. In addition, UV-absorbing components of the PNBDI reagents interfered with adsorption chromatography because the polarities of the hydroxy fatty acid PNB esters were similar to those of the PNBDI components.

To improve the purification by adsorption thin-layer chromatography (TLC) and to reduce the separation of positional isomers by reversed-phase HPLC, we reduced the polarity of the hydroxyl groups. In this communication we report the formation, purification and reversed-phase HPLC of *tert.*-butyldimethylsiloxy derivatives of PNB esters of monohydroxy fatty acids (PNB-TBDMS-OHFA) using two different solvent systems.

MATERIALS AND METHODS

Reagents

Mono-unsaturated fatty acid methyl esters, for synthesis of hydroxy fatty acids, were obtained from Nu Chek Prep (Elysian, MN, U.S.A.). The synthesis of saturated monohydroxy fatty acids, formation and purification of the PNB derivatives were described previously[1]. Methyl 12-hydroxydodecanoate and methyl 2-hydroxytetradecanoate were obtained from Aldrich (Milwaukee, WI, U.S.A.) and from Foxboro/Analabs (North Haven, CT, U.S.A.), respectively. PNBDI and *tert.*-

0021-9673/86/$03.50

butyldimethylsilylimidazole–dimethylformamide were purchased from Pierce (Rockford, IL, U.S.A.) and from Supelco (Bellefonte, PA, U.S.A.), respectively. All HPLC grade solvents were obtained from Burdick & Jackson Lab. (Muskegon, MI, U.S.A.).

Formation and purification of PNB-TBDMS-OHFA

Hydroxy fatty acid PNB esters (150–200 μmol) were placed in a 13 × 100 mm round-bottom tube equipped with a PTFE-lined screw cap and the solvents were evaporated. Traces of water were removed from the PNB derivatives by repeated addition and evaporation of chloroform. Then, *tert.*-butyldimethylsilylimidazol–dimethylformamide (1 ml) was added and the mixture was heated at 95°C for 120 min. After cooling in an ice-bath, hexane (4 ml) was added and the tube was shaken vigorously. Aqueous hydrochloric acid (3 ml, 0.33 mol/l) was added and the tube was shaken and centrifuged (5 min, 3 *g*). The hexane (upper phase) was removed and the aqueous layer was extracted twice with hexane and the combined extracts were evaporated to dryness. The formation of siloxy derivatives was about 99% completed in 1 h.

Purification of PNB-TBDMS-OHFA

In a fume hood, the PNB-TBDMS-OHFA (about 200 μmol) were applied in the narrowest possible band to three 500-μm-thick silica gel G plates (20 × 20 cm) containing inorganic fluorescent indicator, using a TLC sample streaker (Applied Science, State College, PA, U.S.A.). The plates were developed twice in a lined chamber with hexane–diethyl ether (90:10, v/v). The PNB-TBDMS-OHFA were detected by short-wavelength UV light ($R_F \approx 0.6$–0.7), scraped off the plate and were eluted from the silica gel with chloroform or diethyl ether.

Reversed-phase high-performance liquid chromatography of PNB-TBDMS-OHFA

The analyses were performed on a Model 334 Beckman gradient HPLC system equipped with a 421 CRT controller, two 110A Model pumps, Model 210 injector, and a Model 165 variable-wavelength UV–VIS detector (Beckman Instruments, Irvine, CA, U.S.A.). The reversed-phase analysis was carried out on a Supelcosil LC-18 (octadecyldimethylsilyl bonded silica), 3 μm particle size, 15 × 4.6 mm I.D. column (Supelco). Solvent systems used are described in Fig. 2 and 3. To prevent formation of UV-absorbing products in the solvent mixtures and drifting of the baseline, acetonitrile was mixed with the other solvents immediately before use and a new solvent mixture was prepared every eight 8 h.

RESULTS AND DISCUSSION

We chose the TBDMS derivatives for reducing the polarity of hydroxyl groups of hydroxy fatty acid PNB esters because the TBDMS derivatives were (i) resistant to hydrolysis[2,3], (ii) stable in common organic solvents (excluding those solvents that can be silylated), (iii) changed the adsorption TLC and reversed-phase HPLC retention of PNB hydroxy fatty acids more than smaller siloxy groups, and (iv) the TBDMS groups could easily be removed by fluoride ions (sodium fluoride or tetra-*n*-butylammonium fluoride) to free the hydroxyl groups for separation of positional isomers by adsorption HPLC[4,5].

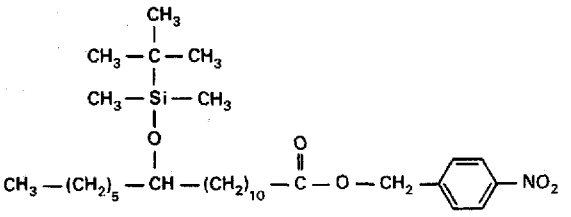

Fig. 1. *p*-Nitrobenzyl 12-*tert.*-butyldimethylsiloxyoctadecanoate (12h-C_{18} PNB-TBDMS-PHFA).

To keep the UV-absorbing reagents to a minimum we explored the formation of PNB-TBDMS-OHFA by N-methyl-N-butylsilyltrifluoroacetamide (MTBSTFA), but the reaction failed to go to completion at 95°C in 48 h in the presence of large excess of MTBSFTFA, with or without the use of 1% *tert.*-butyldimethylchlorosilane as catalyst. On the other hand, the formation of PNB-TBDMS-OHFA by *tert.*-butyldimethylimidazole in dimethylformamide was completed in about 1 h at 95°C without the formation of detectable (TLC and HPLC) amounts of side products.

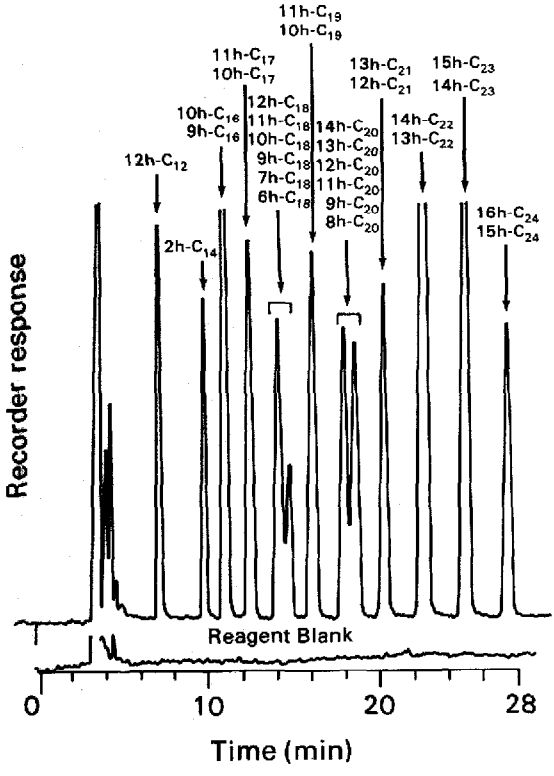

Fig. 2. Reversed-phase HPLC of PNB-TBDMS-OHFA. A Supelcosil LC-18, 3 μm particle size, 15 cm × 4.6 mm I.D. column was used. Solvent system: A, acetonitrile, B, acetonitrile–tetrahydrofuran–chloroform (50:27.5:22.5). A linear gradient from 30% to 100% of B, in 70 min, was used. Flow-rate was 0.5 ml/min at ambient temperature. Detector was set at 265 nm, 0.005 a.u.f.s. Abbreviations denote the chain-length (*e.g.* C_{12}) and positional isomers (*e.g.* 12h).

Fig. 1 depicts the structure of *p*-nitrobenzyl 12-*tert*.-butyldimethylsiloxyoctadecanoate (12h-C_{18} PNB-TBDMS-OHFA). Mixtures of TBDMS derivatives of various chain-lengths (C_{12} to C_{24}) and positional isomers of PNB esters of monohydroxy fatty acids migrated in a narrow band on layers of silica gel, which made purification of these compounds more complete. Furthermore, the polarity and the adsorption TLC properties of PNB-TBDMS-OHFA were such that these compounds migrated ahead of most of the UV-absorbing reagent components. The trace amounts of UV-absorbing reagent components that remained in the adsorption TLC purified PNB-TBDMS-OHFA were not retained on reversed-phase HPLC columns (see Fig. 2, reagent blank) and were eluted in the void volume under the conditions used. The reagent blank contained all of the reagents that were used for formation of 200 μmol of PNB-TBDMS-OHFA and it was purified the same way as the hydroxy fatty acid derivatives were, using a mixture of C_{12} and C_{24} PNB-TBDMS-OHFA as TLC standards for preparative TLC. The upper part of Fig. 2 shows that various different chain-lengths (C_{12} to C_{24} with one carbon increment) of PNB-TBDMS-OHFA separated well enough in 30 min for effective recovery of the components by an absorbance slope detecting fraction collector–detector combination. The separation of the positional isomers present in the used mixture was only minor and it did not interfere with the fractionation according to chain-length. From the retention time of the PNB-TBDMS derivative of 2-hydroxytetradecanoate, it may be concluded that some positional isomers of PNB-TBDMS-OHFA may overlap with PNB-TBDMS-OHFA

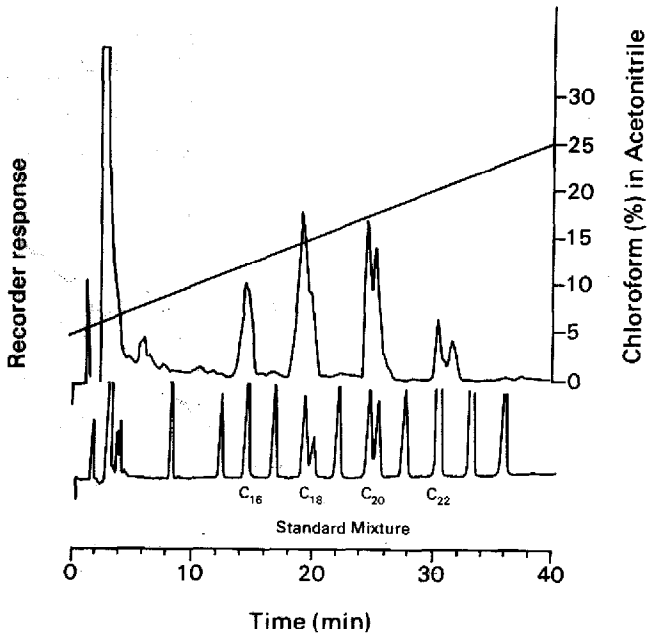

Fig. 3. Reversed-phase HPLC of PNB-TBDMS-OHFA derived from peroxidized rat liver glycerophosphatides. A Supelcosil LC-18, 3 μm particle size, 15 cm × 4.6 mm I.D. column was used. Solvent system: A, acetonitrile; B, acetonitrile–chloroform (50:50). A linear gradient from 10% to 100% of B, in 90 min, was used. Flow-rate was 0.5 ml/min at ambient temperature. Detector was set at 265 nm, 0.005 a.u.f.s. Abbreviations denote chain-length (*e.g.* C_{20}) of hydroxy fatty acids.

of different chain-lengths. If that occurs, a more polar solvent system may be used to improve the resolution.

Fig. 3 demonstrates the use of a binary solvent system for fractionation of naturally occurring mixtures of hydroxy fatty acids. The upper part of Fig. 3 depicts the fractionation of hydroxy fatty acids derived from peroxidized rat liver glycerophosphatides (experimental details will be described elsewhere). The lower part of Fig. 3 shows the separation of the same standard mixture of hydroxy fatty acids as is shown in Fig. 2. Since the derivatization reagents do not interfere with the analysis, this method can be used for quantitation in addition to chain-length fractionation of saturated monohydroxy fatty acids.

For complete resolution of naturally occurring mixtures of hydroxy fatty acids, which may contain positional isomers of various chain-lengths, a combination of reversed-phase HPLC followed by adsorption HPLC need to be used. The TBDMS groups of homologous PNB-TBDMS-OHFA isolated by reversed-phase HPLC may be removed by 3 molar excess of fluoride ions using tetra-*n*-butylammonium fluoride[4,5]. Positional isomers of homologous PNB esters of monohydroxy fatty acids can then be resolved by adsorption HPLC as was described earlier[1].

ACKNOWLEDGEMENTS

This research was supported in parts by U.S. PHS Grant AM-27135 awarded by the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases and by Biomedical Research Support Grant BRSG NO S07 RR07205, Division of Resrarch Resources, National Institute of Health.

REFERENCES

1 Z. L. Bandi and E. S. Reynolds, *J. Chromatogr.*, 329 (1985) 57.
2 E. J. Corey and R. K. Varna, *J. Am. Oil. Chem. Soc.*, 93 (1971) 7319.
3 G. Stork and P. F. Huorlik, *J. Am. Oil. Chem. Soc.*, 90 (1968) 4462.
4 E. J. Corey and A. Venkateswarlu, *J. Am. Oil. Chem. Soc.*, 94 (1972) 6190.
5 K. K. Ogilvie, *Can. J. Chem.*, 51 (1973) 3790.