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Fatty acid conjugates of chlorinated phenols and their high-performance liquid chromatographic analysis

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ABSTRACT

Fatty acid (C_{16} and C_{18}) conjugates of pentachlorophenol and four other commonly used chlorinated phenols (2,4-di-, 2,4,5- and 2,4,6-tri-, and 2,3,4,6-tetra-chlorophenols) were synthesized and their structures were established by proton nuclear magnetic resonance spectroscopy and chemical ionization mass spectrometry. The high-performance liquid chromatographic (HPLC) separation was achieved on reversed-phase (C-18) column using methanol–water (39:1, v/v) at a flow-rate of 1 ml/min. Palmitoyl- and oleoyl-2,4-dichlorophenols (19.6 min), palmitoyl- and oleoyl-2,4,5-trichlorophenols and stearoyl-2,4-dichlorophenol (28.0 min), oleoyl-2,4,6-trichlorophenol and linoleoyl-2,3,4,6-tetrachlorophenol (30.8 min), palmitoyl- and oleoyl-2,3,4,6-tetrachlorophenols and stearoyl-2,4,5-trichlorophenol (43.2 min) and palmitoyl- and oleoyl-pentachlorophenols (72.0 min) were co-eluted under the HPLC conditions described above. Therefore, reversed-phase HPLC separation of a mixture of the five chlorinated phenol conjugates of each fatty acid was achieved.

INTRODUCTION

The five chlorinated phenols (CPs) namely, 2,4-dichlorophenol (DCP), 2,4,5- and 2,4,6-trichlorophenols (TCPs), 2,3,4,6-tetrachlorophenol (TeCP) and pentachlorophenol (PCP) are the common contaminants present in the environment and human body [1,2]. DCP and 2,4,5-TCP as reaction intermediates in the production of chlorophenoxy acids (herbicides) and TeCP and PCP as pesticides and wood preservatives have widely been used in the U.S.A. [3]. These CPs are also spontaneously formed when drinking or industrial waste water containing phenols is chlorinated [1,2]. Hexachlorobenzene and lindane (γ-hexachlorocyclohexane), widely used as fungicide and pesticide, respectively, and ubiquitous in the environment and human body, metabolize to CPs [4–7]. Among CPs, the most extensively used compound is PCP [8]. Contamination of PCP and other CPs in the environment and in humans and animals is well documented [3,9–14]. The environmental and biological transformation of PCP to DCP, TCPs and TeCP has also been demonstrated [6].

Once absorbed in the body, a significant amount of the CPs may be retained due to the formation of lipophilic conjugates of fatty acids. Surprisingly, very little is known about such conjugates and their toxicity [15,16]. A palmitic acid ester of PCP (palmitoylpentachlorophenol or PPCP) shown to be formed *in vitro* using the rat liver microsomes fortified with coenzyme A and ATP [17] has also been detected in human

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fat [18]. An accumulation of such conjugates in membranes of tissues and their further metabolism may have deleterious consequences. Recently, we have found a selective toxicity of PPCP to exocrine pancreas in rats [19]. The presence and formation in the body tissues, and toxicity of other fatty acid conjugates of PCP as well as other four CPs mentioned earlier have not been studied. Therefore, the fatty acid (C_{16} and C_{18}) conjugates of DCP, TCPs, TeCP and PCP were synthesized, characterized by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy, chemical ionization mass spectrometry (CIMS) and reversed-phase high-performance liquid chromatography (HPLC). The conjugates synthesized in this study will be used as standards to study their formation under *in vitro* and *in vivo* conditions and toxicity.

MATERIALS AND METHODS

Chemicals and reagents

Acyl chlorides of palmitic, stearic, oleic and linoleic acids and linolenic anhydride (purity 98–99%) were obtained from Sigma (St. Louis, MO, U.S.A.). Pyridine (anhydrous), 2,4-DCP, 2,4,5- and 2,4,6-TCPs and PCP (purity 98–99%) purchased from Aldrich (Milwaukee, WI, U.S.A.) and 2,3,4,6-TeCP (purity 93.6%) from American Tokyo Kasei (Portland, OR, U.S.A.) were used. Silica gel (Bio-Sil A, 200–325 mesh) for column chromatography obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.) and HPLC grade solvents from Fisher Scientific (Fairlawn, NJ, U.S.A.) were used in the present study.

Synthesis of fatty acid conjugates of CPs

The fatty acid (C_{16} and C_{18}) conjugates of the five CPs were synthesized according to the method described earlier by Ansari *et al.* [18]. Chlorinated phenol was dissolved in dry pyridine and to this fatty acid chloride or anhydride was added dropwise, in a conical flask. The contents were mixed well. The flask was screw capped and kept in a water bath at 50–60°C. The reaction was monitored periodically and after 24 h, the mixture was transferred to a separating funnel and extracted twice with diethyl ether. The extract was washed several times with 0.1 *M* hydrochloric acid and followed by 10% Na_2CO_3 in order to remove the impurities of pyridine and fatty acid residues, respectively. The solvent was evaporated under nitrogen. The synthesized products were crystallized in hexane–methanol (for the CP conjugates of palmitic and stearic acids) or purified by silica gel column chromatography (for the CP conjugates of oleic, linoleic and linolenic acids) on a silica gel packed column (30 × 2 cm I.D.) to a height of 20 cm using hexane–chloroform (10:1, v/v) as eluting solvent. The elution flow-rate was 7 ml/min. A small aliquot from each fraction collected at every minute was analyzed by thin-layer chromatography (TLC) on silica gel coated glass plates (Analtech, Newark, DE, U.S.A.; 250 μm thick) using hexane–ethyl acetate (9:1, v/v) as the solvent system. Fractions between 21 and 53 showing single spot at same R_F by TLC were pooled and the solvent was evaporated under a reduced pressure. The high-resolution ^{1}H NMR spectra were acquired by using a WB Fourier transform NMR spectrometer (6.3 T) on JEOL GX 270. The samples were dissolved in deuterated chloroform and tetramethylsilane was used as an internal standard. Nermag, R10-10C, quadrupole mass spectrometer equipped with PDP 11/73 data system was operated under positive chemical ionization mode with ammonia

as a reagent gas (gas pressure 10^{-1} Torr). The sample was dissolved in *n*-hexane (5 mg/ml) and 1–2 μl was applied on tungsten filament probe tip. The solvent was allowed to evaporate and the probe was inserted into the ion source, set at 1.1 A. The temperature of the probe was programmed from 0 to 500 mA at 20 mA/s.

HPLC analysis of fatty acid conjugates of CPs

The fatty acid conjugates of CPs were separated on a reversed-phase (C-18) column (25 × 0.46 cm I.D., 5 μm particle size) using 334 Beckman liquid chromatograph equipped with 165 variable-wavelength UV detector. The effluent was monitored at 210 nm. A mixture of all the 25 chlorinated phenol conjugates of the five fatty acids were analyzed by reversed-phase HPLC using methanol–water (39:1, v/v) solvent system at a flow-rate of 1 ml/min. Several conjugates were co-eluted under the reversed-phase HPLC conditions described above. Therefore, a reversed-phase HPLC separation was achieved for a mixture of five chlorinated phenol conjugates of each fatty acid. The five chlorinated phenol conjugates of each palmitic, stearic and oleic acids were separated by using methanol–hexane (39:1, v/v) at a flow-rate of 1 ml/min. The five chlorinated phenol conjugates of linoleic (flow-rate 1.5 ml/min) and linolenic (flow-rate 1.0 ml/min) acids were resolved by using methanol–water (39:1, v/v) solvent system.

RESULTS AND DISCUSSION

The palmitic and stearic acid conjugates of all the five CPs were solids and their melting points (uncorrected) were recorded to be 49, 78, 55, 55, 77, 54, 83, 59, 62 and 85°C for the conjugates I to X (Fig. 1), respectively. Oleic, linoleic and linolenic acid conjugates of the CPs were liquids at room temperature. The ^{1}H NMR spectra of all the conjugates synthesized in the present study gave signals at δ 0.88–2.66 for palmitoyl, δ 0.87–2.67 for stearoyl and δ 0.87–5.37 for oleoyl, linoleoyl and linolenoyl conjugates of CPs. Similarly, the signals for olefinic and allylic protons were detected from δ 5.34–5.37 and δ 2.77–2.81, respectively, for the CP conjugates of unsaturated C_{18} fatty acids depending upon the number of double bonds. The signals for the aromatic protons were observed at δ 7.04–7.46 for 2,4-dichlorophenyl, δ 7.26–7.55 for 2,4,5-trichlorophenyl, δ 7.26–7.37 for 2,4,6-trichlorophenyl and δ 7.51 for 2,3,4,6-tetrachlorophenyl conjugates of all the fatty acids (Table I). The chemical ionization mass spectral analysis of all the conjugates is summarized in Table II. The pseudomolecular ion peaks ($M + NH_4^+$) of all the compounds exhibited a ratio characteristic to the number of chlorines present in dichlorophenyl, trichlorophenyls, tetrachlorophenyl, and pentachlorophenyl fatty acid conjugates, respectively. These spectral patterns of the pseucomolecular ion peaks were found similar to those reported for the di-, tri-, tetra- and penta-chlorophenols and other equivalent number of chlorine containing compounds, respectively [20]. Relatively low abundance of pseudomolecular ion peaks were observed for the fatty acid conjugates of TeCP and PCP than those of the conjugates of DCP and TCPs. Loss of one chlorine was also observed in most of the spectra of CP conjugates of unsaturated fatty acids. Other major peaks of $RCOO^- + NH_4^+$, RCOOH, $RCO{\equiv}O^+$ (R = fatty acid carbon chain) were also detected in the spectra of the CP conjugates of fatty acids (Table II).

When a mixture of the 25 conjugates (I–XXV) was analyzed by reversed-phase

TABLE I

270 MHz PROTON NMR SPECTRAL DATA FOR THE FATTY ACID CONJUGATES OF CPs

Chemical shifts expressed in ppm, J values are given in Hz.

Compounds[a]	Aromatic	Olefinic	Allylic	C_2–CH_2–	–CH_2–	C_3–CH_2–	Other–CH_2–	–CH_3(terminal)
I	7.04–7.45(3H)	–	–	2.60 t(2H,J=7.51)	–	1.77 m(2H)	1.26 b(24H)	0.88 t(3H,J=6.77)
II	7.27–7.55(2H)	–	–	2.60 t(2H,J=7.51)	–	1.77 m(2H)	1.26 b(24H)	0.88 t(3H,J=6.59)
III	7.37 (2H)	–	–	2.65 t(2H,J=7.51)	–	1.78 m(2H)	1.26 b(24H)	0.88 t(3H,J=6.59)
IV	7.51 (H)	–	–	2.66 t(2H,J=7.51)	–	1.77 m(2H)	1.26 b(24H)	0.88 t(3H,J=6.59)
V	–	–	–	2.66 t(2H,J=7.51)	–	1.77 m(2H)	1.26 b(24H)	0.88 t(3H,J=6.59)
VI	7.04–7.45(3H)	–	–	2.60 t(2H,J=7.33)	–	1.77 m(2H)	1.25 b(28H)	0.87 t(3H,J=6.59)
VII	7.26–7.55(2H)	–	–	2.60 t(2H,J=7.33)	–	1.77 m(2H)	1.25 b(28H)	0.87 t(3H,J=6.59)
VIII	7.36 (2H)	–	–	2.65 t(2H,J=7.33)	–	1.77 m(2H)	1.25 b(28H)	0.87 t(3H,J=6.59)
IX	7.51 (H)	–	–	2.66 t(2H,J=7.33)	–	1.77 m(2H)	1.26 b(28H)	0.88 t(3H,J=6.59)
X	–	–	–	2.67 t(2H,J=7.33)	–	1.81 m(2H)	1.26 b(28H)	0.88 t(3H,J=6.59)
XI	7.05–7.46(3H)	5.36 b(2H)	–	2.60 t(2H,J=7.70)	2.03 b(4H,$C_{8,11}$)	1.78 m(2H)	1.45 b(20H)	0.88 t(3H,J=6.22)
XII	7.26–7.55(2H)	5.35 b(2H)	–	2.60 t(2H,J=7.33)	2.02 b(4H,$C_{8,11}$)	1.77 m(2H)	1.45 b(20H)	0.88 t(3H,J=6.59)
XIII	7.26–7.35(2H)	5.35 b(2H)	–	2.65 t(2H,J=7.33)	2.02 b(4H,$C_{8,11}$)	1.77 m(2H)	1.45 b(20H)	0.88 t(3H,J=6.59)
XIV	7.51 (H)	5.35 b(2H)	–	2.65 t(2H,J=7.48)	2.02 b(4H,$C_{8,11}$)	1.80 m(2H)	1.30 b(20H)	0.87 t(3H,J=6.59)
XV	–	5.34 b(2H)	–	2.66 t(2H,J=7.48)	2.02 b(4H,$C_{8,11}$)	1.80 m(2H)	1.32 b(20H)	0.87 t(3H,J=6.59)
XVI	7.04–7.45(3H)	5.35 b(4H)	2.77 t(2H,J=5.72)	2.60 t(2H,J=7.33)	2.03 b(4H,$C_{8,14}$)	1.77 m(2H)	1.31 b(14H)	0.88 t(3H,J=6.59)
XVII	7.26–7.54(2H)	5.36 b(4H)	2.77 t(2H,J=5.72)	2.60 t(2H,J=7.48)	2.03 b(4H,$C_{8,14}$)	1.77 m(2H)	1.31 b(14H)	0.88 t(3H,J=6.59)
XVIII	7.36 (2H)	5.36 b(4H)	2.77 t(2H,J=5.72)	2.64 t(2H,J=7.48)	2.03 b(4H,$C_{8,14}$)	1.77 m(2H)	1.34 b(14H)	0.88 t(3H,J=6.59)
XIX	7.51 (H)	5.36 b(4H)	2.77 t(2H,J=5.72)	2.66 t(2H,J=7.48)	2.03 b(4H,$C_{8,14}$)	1.77 m(2H)	1.35 b(14H)	0.88 t(3H,J=6.59)
XX	–	5.36 b(4H)	2.77 t(2H,J=5.72)	2.67 t(2H,J=7.48)	2.03 b(4H,$C_{8,14}$)	1.77 m(2H)	1.35 b(14H)	0.88 t(3H,J=6.80)
XXI	7.04–7.45(3H)	5.37 b(6H)	2.81 t(4H,J=5.72)	2.60 t(2H,J=7.48)	2.08 b(4H,$C_{8,17}$)	1.76 m(2H)	1.35 b(8H)	0.97 t(3H,J=7.48)
XXII	7.26–7.55(2H)	5.37 b(6H)	2.81 t(4H,J=5.72)	2.60 t(2H,J=7.48)	2.08 b(4H,$C_{8,17}$)	1.76 m(2H)	1.35 b(8H)	0.97 t(3H,J=7.48)
XXIII	7.26–7.37(2H)	5.37 b(6H)	2.81 t(4H,J=5.87)	2.64 t(2H,J=7.33)	2.08 b(4H,$C_{8,17}$)	1.76 m(2H)	1.36 b(8H)	0.97 t(3H,J=7.33)
XXIV	7.51 (H)	5.37 b(6H)	2.81 t(4H,J=5.30)	2.66 t(2H,J=7.48)	2.07 b(4H,$C_{8,17}$)	1.77 m(2H)	1.35 b(8H)	0.97 t(3H,J=7.48)
XXV	–	5.37 b(6H)	2.80 t(4H,J=5.72)	2.66 t(2H,J=7.48)	2.07 b(4H,$C_{8,17}$)	1.77 m(2H)	1.35 b(8H)	0.97 t(3H,J=7.68)

[a] See Fig. 1.

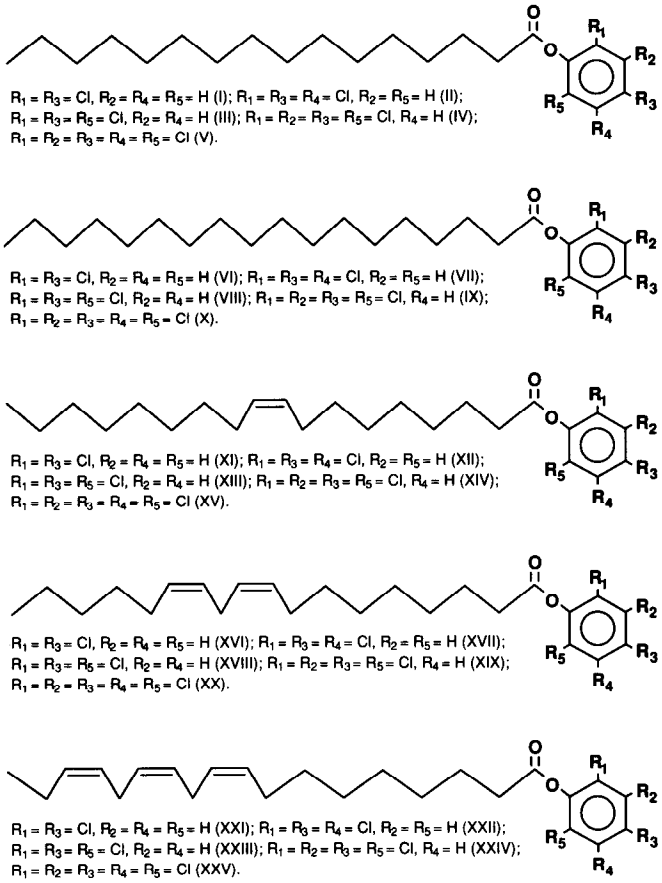

Fig. 1. Structure of palmitic (I–V), stearic (VI–X), oleic (XI–XV), linoleic (XVI–XX) and linolenic (XXI–XXV) acid conjugates of CPs.

HPLC using methanol–water (39:1, v/v) at a flow-rate of 1 ml/ml, I and XI (19.6 min), II, VI and XII (28.0 min) XIII and XIX (30.8 min), IV, VII and XIV (43.2 min), and V and XV (72.0 min) co-eluted (Fig. 2). Therefore, a mixture of DCP, TCPs, TeCP and PCP conjugate each fatty acid was separated by reversed-phase HPLC. The five chlorinated phenol conjugates of each palmitic, stearic and oleic acids could be separated by methanol–hexane (39:1, v/v) at a flow-rate of 1 ml/min. Methanol–water (39:1, v/v) was found to be a suitable solvent system to separate the five chlorinated phenol conjugates of linoleic (flow-rate 1.5 ml/min) and linolenic (flow-rate 1.0 ml/min) acids (Fig. 3). The minimum detectability of the fatty acid conjugates of CPs ranges 0.05–0.10 μg. Among the five CP conjugates of a fatty acid, the 2,4-DCP conjugate was eluted first followed by the conjugates of 2,4,5-TCP, 2,4,6-TCP and 2,3,4,6-TeCP, and the PCP conjugate was eluted in the last. The elution time of these conjugates from the reversed-phase HPLC column depended on the number of chlorines in phenyl ring, degree of unsaturation and number of alkyl residues in the fatty

TABLE II

CHEMICAL IONIZATION MASS SPECTRAL ANALYSIS USING NH_3 AS A REAGENT GAS OF FATTY ACID CONJUGATES OF CPs

RCOOR'; R = fatty acid carbon chain, R' = chlorinated phenyl ring.

Compounds[a]		Pseudomolecular ions[b]	$RCOO^-$-NH_4^+	RCOOH	$RC\equiv O^+$
I	(400)	418/420/422(a)	273	256	239
II	(434)	452/454/456(b)	273	256	239
III	(434)	452/454/456(b)	273	256	239
IV	(468)	486/488/490/492(c)	273	256	239
V	(502)	Reported earlier [18]			
VI	(428)	446/448/450(a)	301	284	267
VII	(462)	480/482/484(b)	301	284	267
VIII	(462)	480/482/484(b)	301	284	267
IX	(496)	514/516/518/520(c)	301	284	267
X	(530)	548/550/552/554(d)	301	284	267
XI	(426)	444/446/448(a)	–	282	265
XII	(460)	478/480/482(b)	–	282	265
XIII	(460)	478/480/482(b)	–	282	265
XIV	(494)	512/514/516/518(c)	299	282	265
XV	(528)	546/548/550/552(d)	299	282	265
XVI	(424)	442/446/446(a)	–	–	263
XVII	(458)	476/478/480(b)	–	–	263
XVIII	(458)	476/478/480(b)	–	280	263
XIX	(492)	510/512/514/516(c)	–	280	263
XX	(526)	544/546/548/550(d)	–	280	263
XXI	(422)	440/442/444(a)	–	–	261
XXII	(456)	474/476/478(b)	–	–	261
XXIII	(456)	474/476/478(b)	–	–	261
XXIV	(490)	508/510/512/514(c)	–	–	261
XXV	(524)	542/544/546/548(d)	–	–	261

[a] See Fig. 1. Digits in parentheses are the molecular weights.

[b] a, b, c and d are the m/z values for the fatty acid conjugates of di-, tri-, tetra- and pentachlorophenols, respectively.

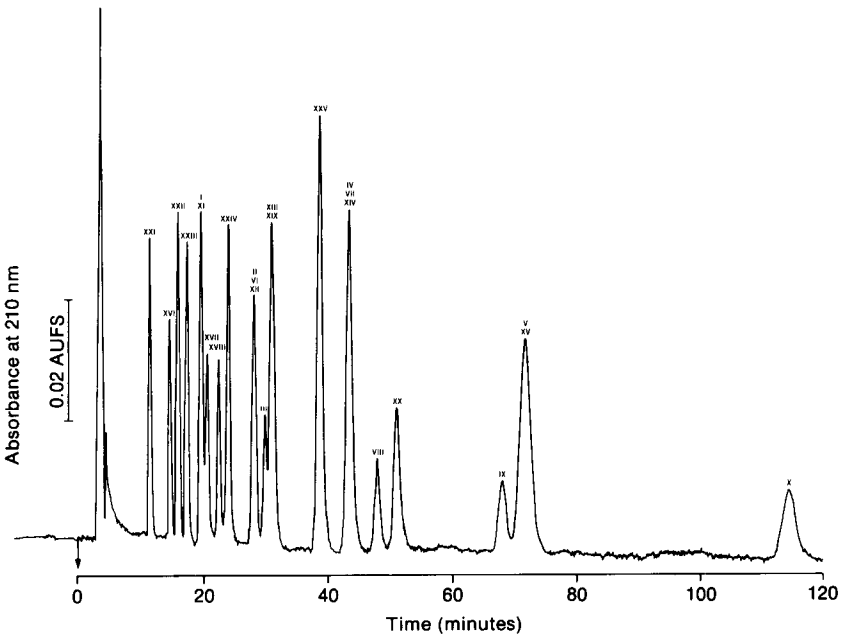

Fig. 2. Reversed-phase HPLC analysis of a mixture of the 25 conjugates (I–XXV) on C-18 column (25 × 0.46 cm I.D., 5 μm particle size) using methanol–water (39:1, v/v) solvent system at a flow-rate of 1 ml/min.

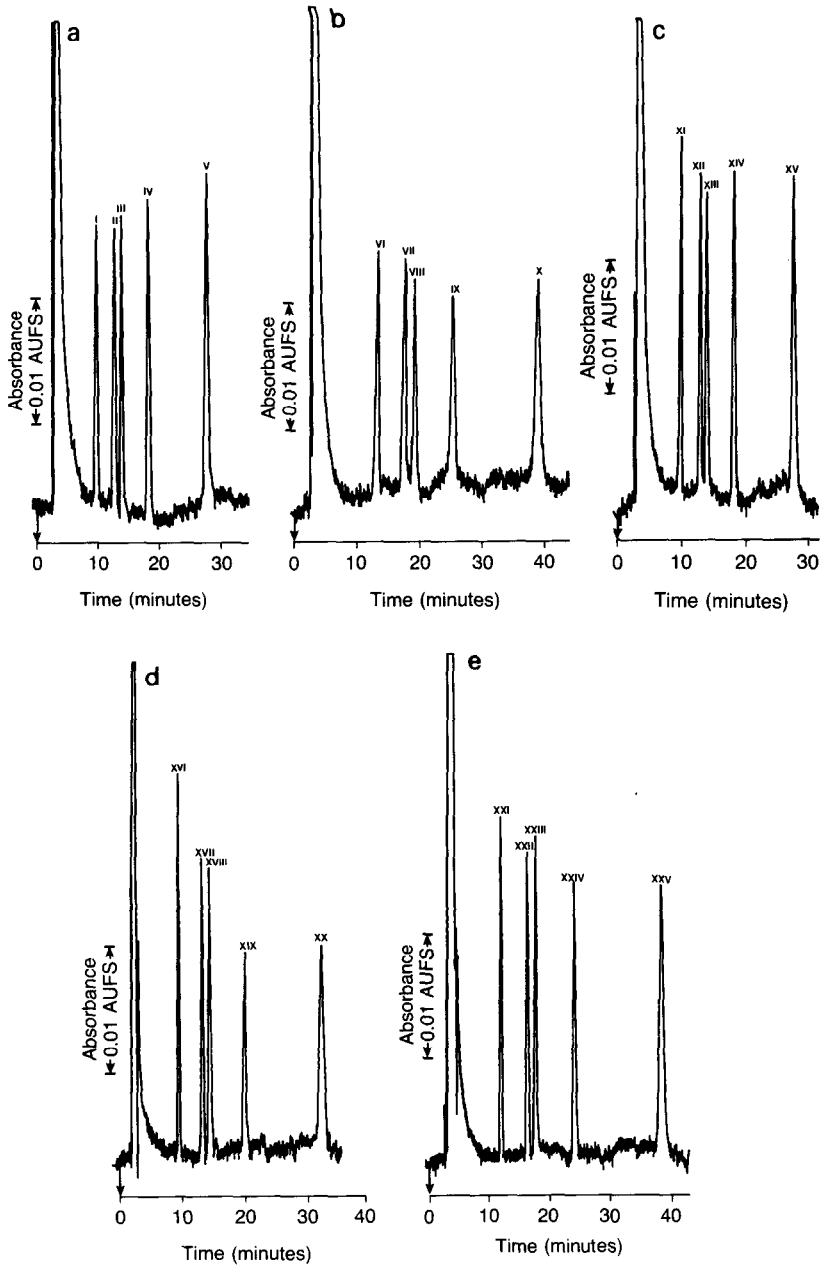

Fig. 3. Reversed-phase HPLC separation of the five CP conjugates of (a) palmitic (I–V), (b) stearic (VI–X), (c) oleic (XI–XV), (d) linoleic (XVI–XX) and (e) linolenic (XXI–XXV) acids. Conjugates I–XV were separated by methanol–hexane (39:1, v/v) at a flow-rate of 1 ml/min, XVI–XX by methanol–water (39:1, v/v) at a flow-rate of 1.5 ml/min and XXI–XXV by methanol–water (39:1, v/v) at a flow-rate of 1.0 ml/min.

acid chain. The results of ^1H NMR, mass spectral and HPLC analysis of all the synthesized compounds (I–XXV) together support the synthesis of chlorinated phenol conjugates of C_{16} and C_{18} fatty acids (Fig. 1). Thus, the CP conjugates of fatty acids synthesized in the present study will be used to study their toxicity, structure activity relationship and formation under *in vitro* and *in vivo* conditions.

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