Journal of Chromatography, 260 (1983) 517–521.
Elsevier Science Publishers B.V., Amsterdam — Printed in the Netherlands

CHROM. 15,670

Note

Separation and determination of saturated fatty acids by reversed-phase high-performance thin-layer chromatography

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(Received January 5th, 1983)

The present work is part of a more general study on the effects of ionizing radiation on biological matter in order to evaluate the radioprotective or radiosensitizing properties of some drugs. One of the effects of ionizing radiation, induced by hydroxyl radicals[1], is lipid peroxidation in cell membranes. Moreover, the peroxide anion, a species produced under aerobic irradiation, could induce lipid hydrolysis resulting in release of fatty acids[2]. Such a release could be representative of the damage to cell membranes induced by radiation, and might be useful for estimating the radioprotective or radiosensitizing efficiency of a drug.

This paper describes a reversed-phase high-performance thin-layer chromatographic (HPTLC) procedure for the separation and quantitation of saturated fatty acids which is especially suitable when isotopic labelling is used. Since most fatty acids show no appreciable UV absorption, UV-absorbing derivatives must be prepared for determination on TLC plates by densitometry[3]. The most widely used derivatives have been phenacyl and substituted phenacyl esters[4] and their separation has been performed especially with gradient elution liquid chromatography[5]. Phenacyl derivatives are usually prepared in a quantitative manner using crown ethers as catalysts[6]. We have used triethylamine as the catalyst according to Engelhardt and Elgass[5], because it is inexpensive and gives quantitative yields. By employing this simple method for the preparation of the esters and HPTLC RP-18 bonded-phase layers for the separation, we were able to analyze rapidly mixtures of representative saturated straight-chain fatty acids from C_{10} to C_{24}.

EXPERIMENTAL

Chemicals

All the fatty acids used for standards were quantitative grade with a purity greater than 99% and were obtained from NU-CHEK-PREP (Copenhagen, Denmark), except for arachidic acid which was obtained from Aldrich Europe (Beerse, Belgium). The UV tag (ω-bromoacetophenone) was obtained from Carlo Erba

0021-9673/83/$03.00

(Milan, Italy) and was recrystallized from ethanol, unless obtained as a white crystalline material in which case it was used without further purification. All organic solvents and the catalyst (E. Merck, Darmstadt, G.F.R.) were commercial A.R. solvents and were used without purification).

Derivatization procedure

To obtain the phenacyl derivative, 0.2 mmoles of the fatty acid were dissolved in about 10 ml of solvent and 0.5 mmoles of ω-bromoacetophenone and 0.8 mmoles of triethylamine were added. The solvent was isopropanol for C_{10} acid, acetone for C_{12}–C_{18} acids and acetone–chloroform mixtures for C_{20}–C_{24} acids. The reaction mixture was refluxed with stirring for a time depending on the acids involved (low-molecular-weight acids require about 20 min, high-molecular-weight acids about 90 min). The course of the reaction was followed by TLC, spotting 1-μl aliquots on silica gel layers and developing with hexane–diethyl ether (9:1, v/v). The reaction was considered complete when the absorbance of the spot of the phenacyl derivative was constant. The solvent was then evaporated from the cooled reaction mixture. The pure phenacyl derivative was obtrained by preparative TLC on 1.5 mm thick silica gel layers.

Chromatographic procedure

Standard solutions in chloroform of each tagged fatty acid were prepared at various concentrations up to 10^{-4} *M* to establish the linear working range and UV absorption spectra were recorded. 1-μl aliquots of the standard solutions were spotted quantitatively with a Camag micro-applicator (Camag, Muttenz, Switzerland) on a HPTLC RP-18 bonded-phase layer (Merck) pre-washed with hexane–diethyl ether (1:1, v/v). The layer was developed at room temperature in a twin-trough chamber after 15 min of preconditioning with the eluent. The best developing solvent was acetonitrile–isopropanol (2:1, v/v). The development time was about 15–20 min and the run distance was 8 cm. The layers were air-dried and the spots were quantitated by a Camag TLC/HPTLC 76500 scanner using the absorbance of the phenacyl derivatives at 254 nm. Peak areas or heights were used to obtain the calibration graphs.

RESULTS AND DISCUSSION

The triethylamine-catalyzed derivatization of fatty acids proves to be very effective for the rapid preparation of the esters. Furthermore, the reaction gives quantitative yields with no by-products. Consequently, large excesses of tagging reagent are not needed. Another advantage is that the catalyst and excess of ω-bromoacetophenone do not interfere with subsequent TLC analysis. Triethylamine does not absorb UV radiation at 254 nm, and ω-bromoacetophenone is close to the solvent front on the chromatogram.

The chromatogram in Fig. 1 illustrates the excellent separation achieved with the eluent acetonitrile–isopropanol (2:1). This is a typical reversed-phase separation, that is the more polar is the compound (the shorter the chain length) the greater is the R_F value. Under the conditions described the R_F values were quite reproducible as demonstrated by the results in Table I. The optimal mobile phase was selected by performing preliminary chromatographic runs with some commonly used solvents mixtures.

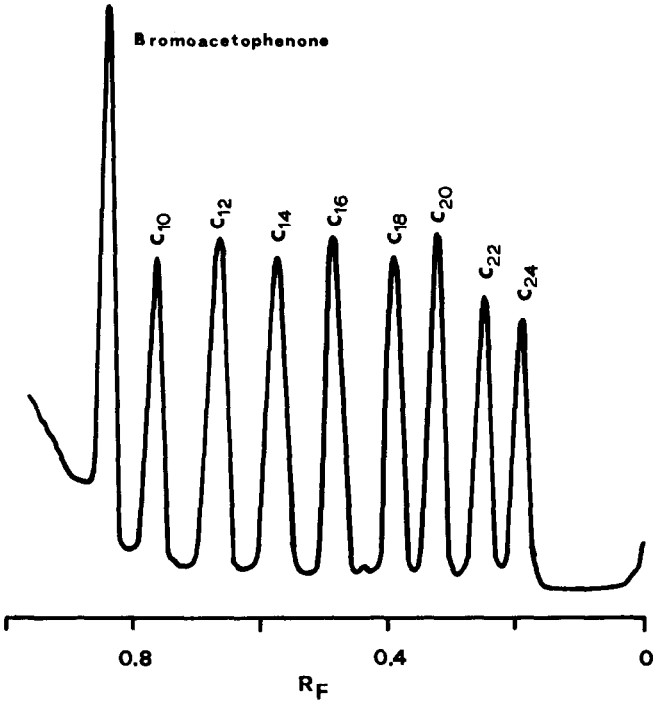

Fig. 1. Chromatogram of the phenacyl derivatives of a standard mixture of saturated fatty acids from C_{10} to C_{24}.

The R_F values obtained are reported in Table II. Since solutions of the phenacyl derivatives have a single absorption peak in their UV spectra, the position and intensity of the absorption being independent of the chain length of the fatty acid derivative, the peak heights or areas can be compared directly without introducing response factors. All calibration graphs were linear up to about 800 ng applied to the layer; the correlation coefficients were all greater than 0.99 using peak areas or

TABLE I

CHROMATOGRAPHIC PROPERTIES OF FATTY ACID DERIVATIVES

Each value is the mean (± S.D.) of five determinations.

Phenacyl derivative	$R_F \times 100$	*Resolution factor**
C_{10}	79 ± 2	
C_{12}	70 ± 2	1.10 ± 0.09
C_{14}	61 ± 2	1.13 ± 0.08
C_{16}	51 ± 2	1.21 ± 0.16
C_{18}	42 ± 1	1.08 ± 0.07
C_{20}	34 ± 1	1.14 ± 0.05
C_{22}	26 ± 1	1.06 ± 0.04
C_{24}	20 ± 1	1.10 ± 0.03

* Defined as $2\Delta x/(w_1 + w_2)$ where Δx is the distance between the two peaks and w_1 and w_2 are peak widths.

TABLE II

R_F × 100 VALUES IN SOME SOLVENT SYSTEMS

Solvent systems: S_1 = acetonitrile–isopropanol (1:1); S_2 = acetonitrile–methanol (1:1); S_3 = acetonitrile–95% ethanol (1:2); S_4 = acetonitrile; S_5 = acetone–acetonitrile (2:3); S_6 = acetone–methanol (1:4); S_7 = methanol; S_8 = 95% ethanol; S_9 = methanol–95% ethanol (1:1).

Phenacyl derivative	*R_F × 100*								
	S_1	*S_2*	*S_3*	*S_4*	*S_5*	*S_6*	*S_7*	*S_8*	*S_9*
C_{10}	85	68	72	59	72	64	59	77	62
C_{12}	79	54	67	45	63	57	51	73	54
C_{14}	71	41	59	32	54	49	44	67	47
C_{16}	65	28	50	21	43	42	35	60	39
C_{18}	55	20	38*	13	34	34	27	55	30
C_{20}	50	12	31	8	27	26*	19	49	25
C_{22}	40	8	23*	4	19	20*	13	44	20*
C_{24}	35	5	17*	3	15	16*	10	40	17*
Development time (min)	20	25	15	15	15	30	30	30	40

* Tailing.

TABLE III

PRECISION AND REPRODUCIBILITY OF THE TLC METHOD

Each value is the mean from four determinations.

Fatty acid	*Actual weight (ng)*	*Recovered weight (ng)*	*Coefficient of variation (%)*
C_{10}	314	316	5.1
	471	459	4.8
C_{12}	355	370	4.6
	532	550	3.1
C_{14}	361	377	5.2
	541	545	3.8
C_{16}	398	415	3.7
	597	602	3.2
C_{18}	387	404	5.9
	580	570	4.3
C_{20}	436	460	4.9
	654	631	2.9
C_{22}	419	402	4.6
	628	610	3.9
C_{24}	372	388	4.7
	558	539	4.4

peak heights. The precision of the TLC method was determined by weighing exact amounts of each of the eight fatty acid standards. The resulting mixture was then esterified and assayed as described above. The precision and reproducibility of the method were very satisfactory as is seen from Table III. The limits of detection were calculated on an response of three times the noise level and ranged from 7 to 10 ng. This sensitivity is comparable to that of HPLC.

The time required for the chromatographic separation is about 30 min. Taking into account that ten samples can be spotted on the same layer, the average analysis time for each sample is about 3 min. This is a significant improvement over the 2–3 h required for HPLC employing RP-18 packing[7] or the 45 min required when RP-8 packing is used[8]. Furthermore, after a development, the layer can be washed with hexane–diethyl ether (1:1 v/v) to obtain a complete elution of the samples and then re-utilized for new measurements until the baseline is no longer sufficiently uniform.

Thus, it appears that the TLC procedure described can be used as an alternative approach to the separtion and quantitation of saturated fatty acids and has an accuracy and precision comparable to those of gas–liquid chromatography (GLC) and HPLC, but with a shorter analysis time. This procedure would be the preferred approach when isotopic labelling is employed.

ACKNOWLEDGEMENTS

This study was supported in part by the Italian National Research Council (C.N.R.), Finalized Project "Controllo della Crescita Neoplastica", Grant No. 80.01486.96, and is published with the permission of C.N.R.

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