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Note

Determination of fatty acids by high-performance liquid chromatography of Dns-ethanolamine derivatives

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Analysis of long-chain fatty acids, of importance for the understanding of lipid metabolism, is difficult owing to the chemical nature of these substances and the small amount present in biological samples. Two factors contribute to this difficulty; the small changes in structure in the series inhibits separation and the lack of a naturally occuring reporter group hinders detection. Currently the most used technique for fatty acid analysis is formation of the volatile methyl esters for gas chromatographic analysis with flame ionization detection[1,2]. More recently increased sensitivity and decreased sample through put time have been sought through derivatization to yield UV absorbing or fluorescent compounds and high-performance liquid chromatography (HPLC) analysis[3–8]. The present report concerns a fluorescent derivatization technique for reversed-phase HPLC analysis which appears to offer distinct advantages over previously reported techniques.

MATERIALS AND METHODS

Standards of the saturated fatty acids palmitic (C_{16}), heptadecanoic (C_{17}), stearic (C_{18}), arachidic (C_{20}) and the unsaturated fatty acids palmitoleic ($C_{16:1}$), oleic ($C_{18:1\ cis}$), elaidic ($C_{18:1\ trans}$), linoleic ($C_{18:2}$), linolenic ($C_{18:3}$) and arachidonic ($C_{20:4}$) were obtained from Sigma (St. Louis, MO, U.S.A.). N,N'-Dicyclohexylcarbodiimide, ethanolamine and 5-dimethylamino-1-naphthalene sulfonyl chloride (Dns-Cl) were obtained from Aldridge (Milwaukee, WI, U.S.A.) and used as supplied. HPLC grade solvents were supplied by J. T. Baker (Phillipsburg, NJ, U.S.A.). Glass-distilled water was further purified by passage over ion-exchange resin and a C_{18} Sep-Pak (Waters Instruments, Milford, MA, U.S.A.).

Formation of Dns-ethanolamine

Dns-ethanolamine was prepared by adding Dns-Cl to a large excess of stirred ethanolamine. Dns-ethanolamine precipitated and was collected by filtration. Re-

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crystallization from methanol yielded an amorphous solid. A stock solution was prepared (40 mg Dns-ethanolamine per ml chloroform) and stored refrigerated.

Formation of fatty acid derivatives

The esterification between the hydroxyl group of Dns-ethanolamine and the carboxylic acid group of fatty acids was accomplished using dicyclohexylcarbodiimide. Amounts of 4 mg fatty acid and 3 mg dicyclohexylcarbodiimide were combined with 120 μl Dns-ethanolamine solution in chloroform. The reaction was allowed to proceed overnight at room temperature protected from light, forming N-Dns-ethanolamine esters of various fatty acids.

As the Dns derivatives emit at visible wavelengths of light, the reaction could be conveniently monitored by spotting a small sample on reversed-phase thin layer plates (Baker Si-C_{18}, Philipsburg, NJ, U.S.A.) and developed in methanol or acetonitrile (see Table I).

For HPLC analysis, water was added to the reaction mixture, the resulting dicyclohexyl urea precipitant was removed by filtration, and the fatty acid derivatives diluted with chloroform. Alternatively the fatty acid derivatives were isolated by reversed-phase thin-layer chromatography (TLC) and eluted with chloroform.

HPLC analysis

Chromatographic analysis was performed on a Varian Model 5000 high-performance liquid chromatograph (Varian, Palo Alto, CA, U.S.A.). A Varian Fluorochrome (excitation at 360 nm, emission above 420 nm) was used for detection.

The analysis was performed on a 250 × 4.6 mm I.D. Ultrasphere ODS reversed-phase 5-μm column (Beckman, Berkeley, CA, U.S.A.). Separations were conducted at room temperature.

RESULTS

Fluorescent fatty acyl derivatives were readily formed at room temperature. Reaction progress was monitored by reversed-phase chromatography on TLC plates. The reaction was essentially complete in 2–3 h. Table I presents the R_F values for the

TABLE I

R_F VALUES FOR FATTY ACYL DERIVATIVES ON REVERSED-PHASE TLC

Fatty acid	*Mobile phase*	
	Acetonitrile	*Methanol*
$C_{18:3}$	0.59*	0.69
$C_{16:1}$	0.57	0.65
$C_{18:2}$	0.57	0.64
$C_{18:1\ cis}$	0.47	0.61
$C_{18:1\ trans}$	0.47	0.61
C_{16}	0.47	0.61
C_{17}	0.40	0.55
C_{20}	0.25	0.42

* R_F value with indicated solvent.

TABLE II

RETENTION TIMES (min) OF FATTY ACYL DERIVATIVES

Mobile phase: acetonitrile with varying amounts of aqueous silver nitrate. ND = Not determined.

Fatty acid	*% 20 mM aqueous silver nitrate*		
	0	*5*	*10*
$C_{18:3}$	3.1	4.8	9.8
$C_{20:4}$	3.5	5.8	11.2
$C_{16\ 1}$	3.9	6.8	12.4
$C_{18\ 2}$	4.0	7.1	13.3
C_{16}	6.0	10.9	21.8
$C_{18\cdot1\ cis}$	5.8	10.8	22.0
$C_{18\cdot1\ trans}$	6.1	11.9	24.5
C_{17}	7.1	14.6	28.0
C_{18}	8.6	19.1	ND
C_{20}	15.3	ND	ND

various fatty acids derivatives employed in this study. Satisfactory separations of many fatty acids could be obtained on TLC, although the C_{16}–$C_{18:1}$ and $C_{18:1\ cis}$–$C_{18:1\ trans}$ couples were not completely resolved. Detection limit on the TLC plate was on the order of 10^{-8} moles.

Reversed-phase HPLC separations were performed using acetonitrile or methanol as the major solvent with varying amounts of aqueous silver nitrate (20 m*M*). Tables II and III depict the results of these studies. Silver nitrate was included as it was observed to limit tailing of peaks but failed to alter retention times when compared to an equivalent mobile phase containing water. With acetonitrile the polyunsaturates are readily resolved but the C_{16}–$C_{18:1\ cis}$ couple was incompletely separated. On the other hand this couple was resolved with methanol based mobile phases but these conditions failed to resolve palmitoleic acid and arachidonic acid.

TABLE III

RETENTION TIMES (min) OF FATTY ACYL DERIVATIVES

Mobile phase: methanol with varying amounts of aqueous silver nitrate. ND = Not determined.

Fatty acid	*% 20 mM aqueous silver nitrate*	
	5	*10*
$C_{18\ 3}$	4.3	10.8
$C_{16\ 1}$	4.8	12.3
$C_{20:4}$	5.3	13.7
$C_{18.2}$	5.4	15.0
C_{16}	6.8	19.5
$C_{18.1\ cis}$	7.3	21.6
$C_{18:1\ trans}$	7.7	23.8
C_{17}	8.7	27.0
C_{18}	11 3	37.0
C_{20}	17.4	ND

TABLE IV

RETENTION TIMES OF FATTY ACYL DERIVATIVES

Mobile phase: acetonitrile–methanol–20 m*M* aqueous silver nitrate (45:45:10, v/v/v).

Fatty acid	*Retention time (min)*
$C_{18:3}$	9.3
$C_{16:1}$	10.2
$C_{20:2}$	13.2
$C_{18:2}$	15.0
C_{16}	23.7
$C_{18:1\ cis}$	24.9
$C_{18:1\ trans}$	27.0
C_{17}	32.1

The data assembled in Tables II and III suggested that adequate resolution of all fatty acid derivatives could be obtained with a mixture of acetonitrile, menthanol and aqueous silver nitrate. Indeed, this proved to be the case. The retention times using methanol–acetonitrile–aqueous silver nitrate (45:45:10) are shown in Table IV. Fig. 1 shows a representative chromatogram using this mobile phase. The insert in Fig. 1 shows that with this system exhibits good resolution even when one species is present in marked excess over another species with similar retention time.

DISCUSSION

The present results indicate that formation of Dns-ethanolamine fatty acyl esters provides a convenient system for analysis of fatty acids. The varying elution times observed with different mobile phases provides a versatile system whereby analysis time can be appreciably decreased depending upon the nature of the fatty acids to be analysed. For example saturated fatty acids could readily separated with 100% acetonitrile or methanol. Two pairs of fatty acids were found to be difficult to completely separate, $C_{16:1}$ and $C_{20:4}$ and C_{16} and $C_{18:1\ cis}$. Although these couples could be readily resolved by increasing the polarity of the mobile phase with additional aqueous silver nitrate, this markedly increases overall retention and analysis time. Altering the polarity of the mobile phase by mixing methanol and acetonitrile however, provided complete separation without increasing analysis time. Mixtures of mobile phases with more marked differences in polarity, as have been used for separation of triglycerides[9], may also prove useful for separation of fatty acid derivatives.

Analyses of fatty acids according to chain length and degree of unsaturation are important for characterizing changes in fatty acid metabolism and for identifying molecular species of cellular phospho- and sphingolipids. Compared to the more generally used gas chromatography with flame ionization detection, HPLC with fluorescent detection is faster, more sensitive and allows sample recovery. Recently, HPLC methods have been reported with various UV absorbing or fluorescent derivatives[3–8]. The generally more sensitive fluorescent procedures (*i.e.* Ikeda *et al.*[8] with 9-aminophenanthrene and Hayashi *et al.*[7] with 4-bromomethyl-7-methoxycoumarin)

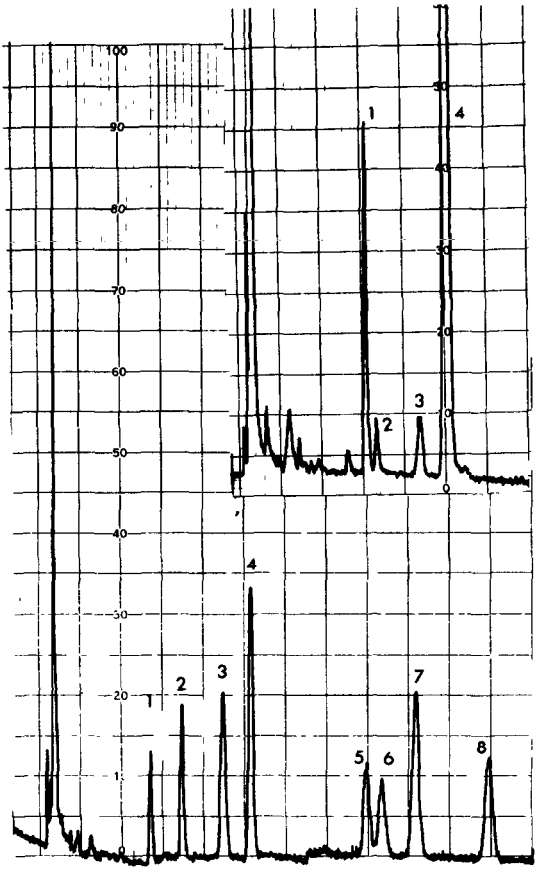

Fig. 1. Representative chromatograms for separation of acyl Dns-ethanolamine derivatives by reversed-phase HPLC. Fluorescence intensity in arbitrary units. Each major grid is 3 min. Mobile phase: acetonitrile–methanol–20 m*M* aqueous silver nitrate (45:45:10, v/v/v); flow-rate 2 ml/min. Peaks: 1 = $C_{18\,3}$; 2 = $C_{16\,1}$, 3 = $C_{20\,4}$; 4 = $C_{18\,2}$; 5 = C_{16}, 6 = $C_{18.1\ cis}$; 7 = $C_{18\,1\ trans}$; 8 = C_{17}. Insert: chromatogram of mixture of unequal amounts of $C_{18\,3}$, $C_{16\,1}$, $C_{20\,4}$, $C_{18\,2}$ resolved under identical conditions.

have employed relatively harsh derivatization conditions (*ca.* 70°C). The present method offers mild reaction conditions, superior resolution and a fluorophor which emits at visible wavelengths. As with all fluorescence methods the sensitivity is quite high although adequate care must be taken to ensure the fluorescence intensity is not markedly altered by the concentration of substance and the nature of the mobile phase. The best manner to correct for this is to include an internal standard and express fluorescence intensity of the unknown relative to the standard. In this regard C_{17} would be ideal as it is not commonly found in biological samples and is adequately resolved by HPLC.

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