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RAPID METHOD FOR THE ANALYSIS OF RED BLOOD CELL FATTY ACIDS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method was developed for a rapid qualitative and quantitative analysis of *p*-bromophenacyl esters of red blood cell fatty acids in humans. Both free and bound fatty acids, extracted with hexane–2-propanol (3:2) from packed red blood cells were derivatized with *p*-bromophenacyl bromide and analysed. Ten identical samples taken from a mixed pool of packed red blood cells from healthy subjects were analysed on two different columns. The fatty acid *p*-bromophenacyl esters were analysed on a 10 RP-18 column with methanol–acetonitrile–0.01 *M* ammonium formate as mobile phase and also on a 10 RP-8 column with acetonitrile–0.01 *M* ammonium formate as mobile phase. The two methods gave analogous results except in total analysis time: that on a 10 RP-8 column is ca. 40% shorter. Furthermore, a quantitative analysis of a standard solution to evaluate the extraction procedure in the absence or in the presence of the red blood cell core indicated a significant difference when the core is present.

INTRODUCTION

The separation of fatty acid mixtures has a significant value in clinical and biological chemistry. It is well known that alterations in membrane lipid composition may influence cellular functions, such as transport across the cell membrane, immunological recognition, the activity of membrane-bound enzymes and the number and affinity of cell receptors [1–6]. The purpose of this work was to develop a suitable method for the analysis of long-chain fatty acids in red blood cells (RBCs).

Long-chain fatty acids have been studied extensively by gas chromatography

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[7–11]. More recently high-performance liquid chromatography (HPLC) has been introduced for such separations. Easy and efficient derivatization procedures have been developed to obtain the sensitivity required for their UV detection. The corresponding phenacyl esters or *p*-bromophenacyl esters are usually separated on 10 RP-18 columns [12–15].

In this paper a more time-saving method for the separation and quantification of *p*-bromophenacyl esters of RBC fatty acids on a 10 RP-8 column is described.

EXPERIMENTAL

Instruments and materials

HPLC was carried out with a Varian Vista 5000 liquid chromatograph equipped with a 10-μl loop injector and a Varian UV variable-wavelength detector (Palo Alto, CA, U.S.A.). Separations were done on a 10 μm particle size Cp™ Spher C_{18} and a 10 μm particle size Cp™ Spher C_8 column (25 cm × 4.6 mm I.D., both from Chrompack, Middelburg, The Netherlands). The compounds were detected at 254 nm, and the column temperature was kept constant at 40°C. HPLC-grade acetonitrile and methanol were purchased from Alltech Assoc. Applied Science Labs. (Deerfield, IL, U.S.A.).

Hexane and 2-propanol, used for the extraction of RBC lipids, were obtained from Riedel de Haën (Seelze, Hannover, F.R.G.). Ammonium formate (0.01 *M*, pH 5.8) was prepared with ultrapure all-glass tridistilled water and filtered over a Millipore filter (VCWP 04700, 0.1 μm) before use.

Butylated hydroxytoluene (BHT; 2,4-di-*tert.*-butyl-1-hydroxytoluene), 18-crown-6, *p*-bromophenacyl bromide and margaric acid (heptadecanoic acid) (C_{17}) were purchased from Janssen (Beerse, Belgium). Fatty acid standards were obtained from Serva (Heidelberg/New York) and from Sigma (St. Louis, MO, U.S.A.).

Extraction of RBC fatty acids

Heparinized blood samples (10 ml) were obtained after an overnight fast from ten healthy adults. The blood samples were centrifuged for 15 min at 1100 *g* (2500 rpm). Plasma and buffy coat were removed by gentle aspiration. The packed RBCs were washed with phosphate-buffered saline (PBS) and centrifuged again. The buffer was carefully removed by aspiration. This washing procedure was repeated twice and was necessary to obtain a relatively pure RBC suspension. The RBC contents of the different tubes were added together in order to obtain a mixed pool of packed RBCs. The extraction solvent was prepared by mixing three parts of hexane with two parts of 2-propanol and adding 5 mg/ml BHT as antioxidant. The use of antioxidant is recommended to prevent autoxidation of lipids and purified fatty acids, induced by the presence of haemoglobin derivatives [11,16]. The margaric acid standard was prepared by dissolving 209 mg (773 μmol) of margaric acid in 1.0 ml of the extraction solvent. The lipids were extracted from 1.5 ml of packed RBCs according to the method of Radin [17].

Ten aliquots of 15 ml of hexane–2-propanol (3:2) containing 5 mg/ml BHT were put into ten different extraction tubes (50 ml) and the margaric acid stan-

dard (250 μl) was added as an internal standard. Then 1.5 ml of the pool of the packed RBCs was added slowly to the solvent in each extraction tube. The samples were extracted for 30 min by continuously mixing at 4°C. The hexane–2-propanol layers were collected separately. The residues were consecutively extracted with 15 and 10 ml of the extraction solvent. The three extraction volumes of each tube were collected, and 20 ml of the total volume were dried under a stream of nitrogen in a water-bath at 37°C. Then 1 ml of a solution of 1 *M* hydrochloric acid in methanol was added in order to hydrolyse the dried residues, especially sphingomyelin where the fatty acid and sphingosine are joined by an amide linkage. Alkaline saponification will not split the amide linkage [18]. After 2 h at 80°C, 1 ml of 2 *M* potassium hydroxide (80% methanol, 20% water) was added, and the solutions were heated for another hour at 80°C. The samples were neutralized with hydrochloric acid–methanol to phenolphthalein end-point and dried under nitrogen. The fatty acid salts were obtained as white or slightly pink solids.

Derivatization procedure

The fatty acid potassium salts, obtained according to the procedure described above, were dissolved in 2 ml of acetonitrile, and 250 μl of a solution containing 0.1 m*M* *p*-bromophenacyl bromide and 0.01 m*M* 18-crown-6 in 1 ml of acetonitrile were added. The tubes were capped with a septum disk and heated with stirring at 80°C for 20 min in a Reacti-Therm (Pierce). Prior to HPLC analysis the samples were filtered with the aid of a 1-ml syringe equipped with a 0.45-μm Millipore filter (filter type: SJHV L04 NS).

Elution systems

Two different systems for the analysis of RBC fatty acids were compared in this study.

Method I. In method I the separation of RBC *p*-bromophenacyl esters was performed on a 10 RP-18 column using a procedure slightly different from that described by Halgunset et al. [14]. In the latter method [methanol–acetonitrile–water (82:9:9); flow-rate 1 ml/min] it took 60 min to complete a total fatty acid analysis, i.e. until the elution of stearic acid (C_{18}). Since the RBC fatty acid composition is characterized by the presence of nervonic acid ($C_{24:1}$), which has a higher retention time, we slightly modified this method in order to shorten the analysis time. For the first 32 min the column was eluted with 70:20:10 methanol–acetonitrile–0.01 *M* ammonium formate (pH 5.8). (The formate was used in the elution system as a buffer to improve the lifetime of the column.) The flow-rate was kept at 1.6 ml/min and the column temperature at 40°C. Then the composition of the mobile phase was changed linearly over 10 min to 70:30 methanol–acetonitrile. After 18 min of isocratic elution, the composition of the mobile phase was brought back to the starting conditions within 5 min.

Method II. In method II we analysed the same *p*-bromophenacyl esters of RBC fatty acids on a 10 RP-8 column. Acetonitrile–0.01 *M* ammonium formate (pH 5.8) (85:15) was the mobile phase, run for 15 min isocratically, followed by a linear gradient to 100% acetonitrile in 5 min. The elution with pure acetonitrile

was continued for 10 min, then the starting conditions were restored within 5 min. The flow-rate and column temperature were as in method I. The total separation time was 35 min.

RESULTS AND DISCUSSION

The most important advantage of using crown ethers to catalyse the derivatization of fatty acids with *p*-bromophenacyl bromide is that small amounts of a sample can be processed. The synthetic utility of crown ethers is derived from their ability to solvate cations in non-polar environments. The fatty acid anion accompanies the cation complex and gives a neutral ligand. The fatty acid salts are extracted from the solid crystal lattice and become strong nucleophiles that react with *p*-bromophenacyl bromide. Traces of water will not prevent quantitative derivatization ($\geq 98\%$), obviating the need for dry solvents and reagents. Furthermore, the alkylating agent is inexpensive and gives esters with a high molar absorptivity ($\epsilon = 15\,800$ at $\lambda_{max} = 254$ nm) without by-products [13,19].

More recently, phenacyl esters have been used for the determination of prostaglandins [20], quinic acid and derivatives [21].

We used five different standard series of *p*-bromophenacyl esters to identify the RBC fatty acids in both methods:

(1) $C_{18:3}$, $C_{18:2}$, $C_{18:1}$, C_{18};
(2) C_{14}, C_{16}, C_{18}, C_{20}, C_{22};
(3) $C_{20:5}$, $C_{20:4}$, $C_{20:3}$, $C_{20:2}$, $C_{20:1}$;
(4) $C_{18:4}$, $C_{20:5}$, $C_{22:6}$, $C_{20:4}$;
(5) $C_{18:2}$, $C_{18:1}$, $C_{24:1}$.

In all systems, residual alkylating reagent, antioxidant (BHT), oxidized fatty acids, phenolphthalein and apolar amino acids that were extracted from erythrocytes eluted during the first 7 min, thus obviating the need for purification prior to analysis.

As illustrated in Fig. 1, method I allows the separation of all RBC fatty acids in 65 min. Although this procedure was satisfactory, we looked further for a procedure allowing a still higher sample input rate.

The chromatogram obtained by method II is given in Fig. 2. The retention times (t_R) and capacity factors (k') of the compounds in the two methods are listed in Table I. The detection limit in both systems (at signal-to-noise ratio of 4) for margaric acid is ca. 5 ng at a detector attenuation of 0.05 a.u.f.s.

From these data, it is obvious that method II requires a shorter analysis time (35 min) than method I. The resolution (R_s) between the fatty acid pairs is shown in Table II. Method I gives baseline-separated fatty acids, except for C_{16} and $C_{18:1}$ ($R_s = 1.25$). Method II shows a loss in resolution between all fatty acid pairs except for $C_{16:0}$ and $C_{18:1}$ ($R_s = 1.25$ for Method I and 1.35 for Method II). In method II all fatty acids are baseline-separated except for the pairs $C_{20:4}$–$C_{18:2}$ and $C_{16:0}$–$C_{18:1}$ ($R_s = 1.13$ and 1.35, respectively). In order to evaluate the influence of the loss of resolution in method II on the quantitation of RBC fatty acids, ten identical samples were analysed. Table III gives the means and the standard deviation

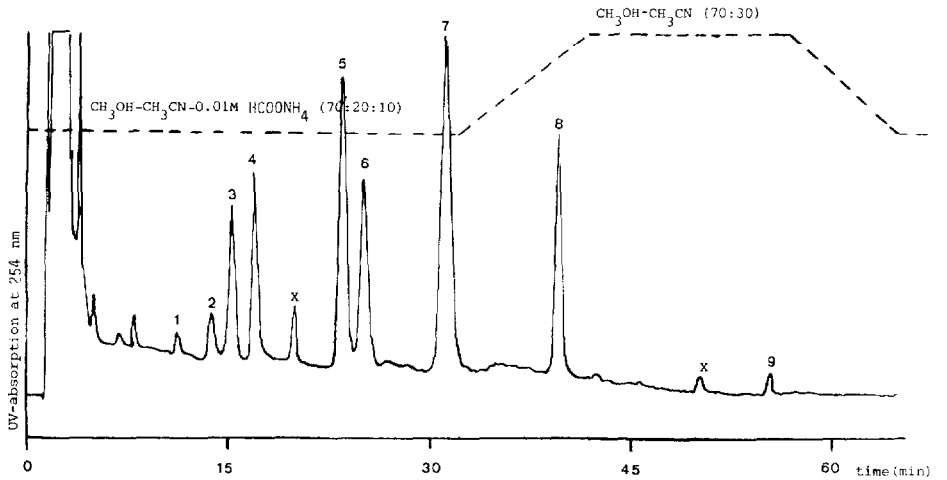

Fig. 1. Separation of RBC *p*-bromophenacyl esters performed on a 10 RP-18 column by using method I. The column was eluted with methanol–acetonitrile–0.01 *M* ammonium formate at a flow-rate of 1.6 ml/min; detection was at 254 nm and the temperature 40 °C. For peak identification see Table I. X = unidentified compound.

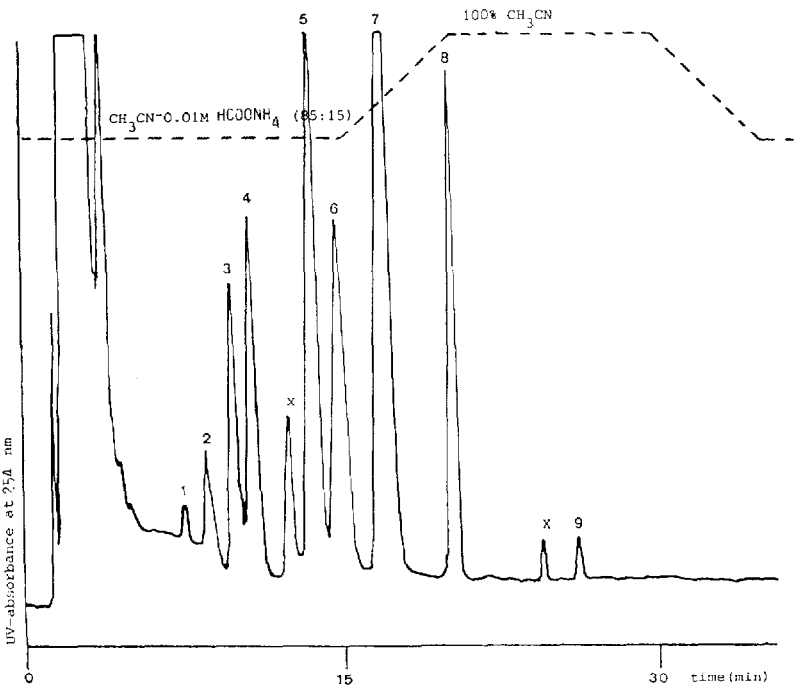

Fig. 2. Separation of RBC *p*-bromophenacyl esters on a 10 RP-8 column. Mobile phase, acetonitrile–0.01 *M* ammonium formate; flow-rate, 1.6 ml/min; detection, 254 nm; temperature, 40 °C. For peak identification see Table I. X = unidentified compound.

TABLE I

RETENTION TIMES AND CAPACITY FACTORS OF RBC FATTY ACIDS ANALYSED ON A 10 RP-18 AND ON A 10 RP-8 COLUMN

Retention times (t_R) and capacity factors (k') of RBC fatty acid *p*-bromophenacyl esters, analysed on a 10 RP-18 column (method I) and on a 10 RP-8 column (method II).

Peak	Compound	Method I		Method II	
		t_R (min)	k'	t_R (min)	k'
1	$C_{20:5}$	11.21	6.84	7.54	4.17
2	$C_{22:6}$	13.01	8.10	8.63	4.92
3	$C_{20:4}$	15.33	9.72	9.87	5.77
4	$C_{18:2}$	16.98	10.88	10.75	6.37
5	$C_{16:0}$	23.52	15.45	13.62	8.34
6	$C_{18:1}$	25.12	16.56	14.89	9.21
7	$C_{17:0}$	31.19	20.81	17.03	10.68
8	$C_{18:0}$	39.48	26.61	20.29	12.91
9	$C_{24:1}$	54.26	36.95	26.20	16.97

TABLE II

BAND WIDTH AT HALF HEIGHT AND RESOLUTION BETWEEN RBC FATTY ACIDS ANALYSED ON A 10 RP-18 AND ON A 10 RP-8 COLUMN

The band widths at half height ($W_{0.5}$) and the retention times (t_R) listed in Table I were used to calculate the resolution (R_s) for the analysis of RBC fatty acids on a 10 RP-18 column (method I) and on a 10 RP-8 column (method II).

$$R_s = \frac{t_{R2} - t_{R1}}{0.85\ (W_{0.5(1)} + W_{0.5(2)})}$$

Peak	Compound	Method I		Method II	
		$W_{0.5}$ (s)	R_s	$W_{0.5}$ (s)	R_s
1	$C_{20:5}$	24.05		15.80	
			2.06		1.80
2	$C_{22:6}$	28.55		20.50	
			2.51		1.68
3	$C_{20:4}$	26.85		22.60	
			1.82		1.13
4	$C_{18:2}$	27.55		24.85	
			6.08		3.00
5	$C_{16:0}$	36.95		30.60	
			1.25		1.35
6	$C_{18:1}$	39.85		34.40	
			4.84		2.04
7	$C_{17:0}$	48.75		39.65	
			7.41		3.37
8	$C_{18:0}$	30.25		28.70	
			15.76		6.92
9	$C_{24:1}$	35.95		31.55	

of ten blood samples extracted and determined by both methods. There are no significant differences between the two methods.

The loss of resolution in method II compared with method I was not crucial for the determination and quantification of the RBC fatty acids. Therefore, method II is preferable to method I because of its shorter analysis time.

TABLE III

COMPOSITION OF RBC FATTY ACIDS ANALYSED ON A 10 RP-18 AND ON A 10 RP-8 COLUMN

Composition of RBC fatty acids (means ± S.D.) calculated from ten samples of a mixed pool of packed RBCs. The ten samples were analysed on a 10 RP-18 column (method I) and a 10 RP-8 column (method II).

Peak	Compound	RBC fatty acid composition (%)	
		Method I	Method II
1	$C_{20:5}$	1.41 ± 0.07	1.46 ± 0.04
2	$C_{22:6}$	3.93 ± 0.09	3.91 ± 0.16
3	$C_{20:4}$	12.93 ± 0.57	12.42 ± 0.51
4	$C_{18:2}$	15.44 ± 0.52	15.49 ± 0.54
5	$C_{16:0}$	28.81 ± 0.91	28.53 ± 0.78
6	$C_{18:1}$	18.40 ± 0.68	18.48 ± 0.53
7	$C_{17:0}$	–	–
8	$C_{18:0}$	19.51 ± 0.97	19.49 ± 0.73
9	$C_{24:1}$	1.31 ± 0.22	1.31 ± 0.05

TABLE IV

INFLUENCE OF THE PRESENCE OF RBCs ON THE EXTRACTION OF A STANDARD SOLUTION

Comparison of ten samples of a standard solution extracted in the absence of RBCs (2) or in the presence of RBCs (3), and a non-extracted sample of the standard solution (1).

Compound	Standard solution (1) (μmol)	Extracted standard solution (2) (μmol)	Extracted standard solution in the presence of RBCs (3) (μmol)
$C_{20:5}$	0.98	0.95 ± 0.03	0.94 ± 0.01
$C_{18:2}$	1.04	0.98 ± 0.03	0.97 ± 0.01
$C_{18:1}$	1.17	1.10 ± 0.06	1.02 ± 0.06
$C_{17:0}$	1.93	1.89 ± 0.03	1.86 ± 0.04
$C_{18:0}$	1.03	0.98 ± 0.04	0.97 ± 0.03

Quantification and evaluation of the extraction procedure

In order to evaluate the extraction procedures, a standard solution containing known concentrations of $C_{20:5}$, $C_{18:2}$, $C_{18:1}$, $C_{17:0}$ and $C_{18:0}$ was made. This solution contained, respectively, 49, 52, 58.5, 96.5 and 51.5 μmol of these fatty acids, dissolved in 50 ml of hexane–2-propanol (3:2). From this standard solution, ten aliquots of 1.0 ml were extracted and analysed on a 10 RP-8 column as described in the extraction procedure and in *Elution systems, Method II*. We analysed the influence of the presence of RBCs on the extraction procedure by means of the standard addition method. Theoretically, the fatty acids could adhere with their hydrophilic side to the RBC core, constituted of proteins and sugars and so be-

come unavailable for extraction. Ten determinations of equal amounts of the standard solution were done without and with RBCs in the extraction solvent. The RBCs, necessary for this purpose, were taken from the mixed pool. Table IV gives the amounts of the fatty acid standards that were analysed without any extraction (1), after extraction without RBCs (2) and after extraction in presence of RBCs (3). It can be seen that the extraction is indeed influenced by the RBC core. In the presence of RBCs, the yield is somewhat lower (without RBCs $95.6 \pm 1.7\%$ extraction of all fatty acids; in the presence of RBCs $93.4 \pm 3.7\%$; $p \leqslant 0.04$ Wilcoxon test).

CONCLUSION

It can be concluded that the analysis of fatty acids from human RBCs can be done satisfactorily on a 10 RP-8 column with acetonitrile–0.01 *M* ammonium formate as mobile phase. The recovery rates of the internal standard solution were higher than 93% in the presence of RBCs and higher than 95% in absence of RBCs. The procedure described here has several advantages. The chromatographic procedure requires only 35 min, which is faster than the previously published methods [12–15] without any significant loss of resolution. It can easily be adapted for automatic injection. This simple and sensitive method is especially suitable for the separation of RBC fatty acids.

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