Journal of Chromatography, 500 (1990) 443–452
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 935

AUTOMATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PLASMA FREE FATTY ACIDS USING ON-LINE DERIVATIZATION WITH 9-BROMOMETHYLACRIDINE BASED ON MICELLAR PHASE-TRANSFER CATALYSIS

F. A. L. VAN DER HORST*[a] and M. H. POST
Department of Pharmaceutical Analysis, University of Utrecht, Catharijnesingel 60, 3511 GH Utrecht (The Netherlands)
J. J. M. HOLTHUIS
EuroCetus BV, Paasheuvelweg 30, 1105 BJ Amsterdam (The Netherlands)
and
U. A. Th. BRINKMAN
Department of Analytical Chemistry, Free University, de Boelelaan 1083, 1081 HV Amsterdam (The Netherlands)
(Received June 26th, 1989)

SUMMARY

The on-line use of micellar phase-transfer catalysis is described for the automated reversed-phase high-performance liquid chromatographic (RP-HPLC) determination of free fatty acids in plasma; minimum manual sample handling is involved. After diluting plasma ten-fold with the aqueous micellar system, which contains 25 m*M* of the non-ionic surfactant Arkopal N-130 and 6 m*M* of the ion-pair agent tetrakis(decyl)ammonium bromide, the reaction of the fatty acids with the fluorophore 9-bromomethylacridine is complete within 5 min at 60°C. Prior to RP-HPLC separation, interfering proteins are removed using an on-line filter and a column-switching unit. More than 100 samples can be injected onto a single pre-column. The detection limit is *ca.* 300 n*M*; the precision is better than 3% using an internal standard.

INTRODUCTION

The trace-level determination of drugs by high-performance liquid chromatography (HPLC) often requires derivatization of the analyte in order to attain the desired sensitivity and selectivity. Unfortunately, derivatization reactions involving a carboyxlic group are often extremely slow in aqueous solution, because of the solvation of this moiety by water molecules. Therefore, the derivatization procedures often include prior extraction of the analyte from the aqueous matrix using, *e.g.*, liquid–

[a] Present address: Department of Clinical Chemistry, University Hospital of Leiden, Rijnsburgerweg 10, 2333 AA Leiden, The Netherlands.

0021-9673/90/$03.50

liquid or solid-phase extraction. After the extraction solvent has been evaporated, the derivatization reaction can be performed in a suitable organic medium. Advantages of both procedures are that interfering compounds are removed and that the analyte can be concentrated; however, such sample clean-up and/or concentration steps are not always required. Further, the various steps in the extraction procedures, and the compatibility with the subsequent derivarization procedure, often seriously complicate the set-up of an automated analytical system.

For the development of an automated system for the determination of carboxylic acids it would be highly advantageous if laborious extraction procedures could be circumvented. This can be accomplished by using, *e.g.*, 2-nitrophenylhydrazine, because this reagent has been reported to derivatize carboxylic acids directly in the aqueous matrix[1]. Unfortunately, the on-line use of this chromophore is virtually impossible, because the time required for complete conversion of the acids is *ca.* 40 min (at 60°C); also, the derivatization procedure involves two separate reaction steps[1].

An interesting method for the on-line derivatization of carboxylic acids is the use of phase-transfer catalysis (PTC)[2]. PTC is based on the principle that a charged analyte is extracted with a lipophilic ion-pair agent, *i.e.*, as a neutral ion-pair complex, into a suitable organic solvent, in which the analyte can be directly derivatized[3,4]. Unfortunately, because the derivatization product dissolves in the organic phase, the on-line application of PTC with HPLC will be restricted to a combination with normal-phase HPLC.

Recently, we have demonstrated that carboxylic acids can be derivatized in an aqueous matrix using micellar phase-transfer catalysis (MPTC)[5–8]. The mechanism of MPTC (Fig. 1) is similar to that of PTC; however, instead of a separate organic phase we use non-ionic micelles as an organic pseudo-phase. A significant advantage of MPTC over conventional PTC is that the aqueous derivatization mixture can directly be injected into a reversed-phase HPLC (RP-HPLC) system without encountering the problem of peak deterioration[5–9]. The MPTC derivatization procedure for carboxylic acids is relatively easy[8]. After the addition of a micellar system that contains the non-ionic surfactant Arkopal N-130 and the quaternary ammonium salt tetrakis(decyl)ammonium bromide, the reaction with the fluorescence label 9-bromomethylacridine (BrMAC) is complete within 5 min at 60°C and pH 7.0 for, *e.g.*, decanoic acid. The reaction rate obviously meets the requirements for an on-line combination with HPLC.

The MPTC system described can easily be used for the automated determination of drugs in purely aqueous matrices. However, the processing of plasma samples is still hampered by the presence of proteins, because the proteins form small aggregates during derivatization and, therefore, will prevent the direct injection of the reaction mixture into an HPLC system. The solution of this problem is the main theme of this paper. The determination of plasma free fatty acid (FFA) was used as a model system.

EXPERIMENTAL

Materials

The non-ionic surfactant Arkopal N-130 [a polyoxyethylene(13)nonylphenyl

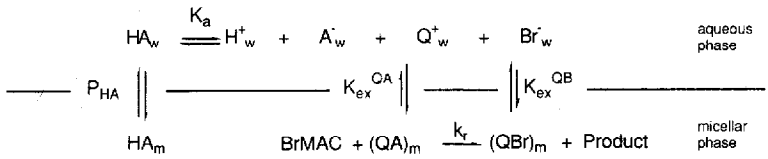

Fig. 1. Schematic diagram of micellar phase-transfer catalysis for the derivatization of carboyxlic acids. With the use of a cationic ion-pair agent Q^+, the conjugate base A^- is extracted as a neutral ion-pair complex (QA), from the aqueous phase into the micelle. In the micelle A^- reacts with the BrMAC reagent. K_a is the acidity constant of the acid in the aqueous phase and P_{HA} is the partition coefficient of the undissociated acid. K_{ex}^{QA} and K_{ex}^{QB} are the extraction constants of the conjugate base and the counter ion of the ion-pair agent with Q^+, respectively, and k_r is the rate constant for the derivatization of A^- with BrMAC in the micellar core.

ether] was a gift from Hoechst Holland (Amsterdam, The Netherlands). Tetrakis-(decyl)ammonium bromide (TDeABr) was obtained from Fluka (Buchs, Switzerland) and methanol, acetonitrile and acetone from Merck (Darmstadt, F.R.G.). Even-numbered saturated fatty acids were purchased from Merck, and unsaturated acids were a gift from the Department of Bio-organic Chemisty of the University of Utrecht. The cellulose acetate and nylon membranes (pore size 0.45–8 μm) and the glass-fibre filters (pore size *ca.* 100 μm) were a gift from Schleicher & Schüll (Dassel, F.R.G.). The 2-μm stainless-steel screens were a gift from Chrompack (Middelburg, The Netherlands).

Acetonitrile and acetone were distilled prior to use. The synthesis of 9-bromomethylacridine (BrMAC) has been reported previously[8,10]. BrMAC was dissolved in acetone (28 mg/ml) and stored at 4°C. The reference fatty acids were dissolved at a concentration of 5 m*M* in acetone and stored at 4°C. In a 10 m*M* phosphate buffer (pH 7.0), 25 m*M* Arkopal N-130 and 6 m*M* TDeABr were dissolved by sonication.

Sample pretreatment

To a 1-ml stoppered glass-walled reaction vial (Chrompack) a volume of 450 μl of the micelle solution, 50 μl of plasma and 10 μl of an internal standard (heptadecanoic acid) were added. After vortexing for 10 s, the vials were placed in a Gilson (Villiers-le-Bel, France) Model 232 autosampler.

Automated MPTC procedure

The samples were processed fully automatically (Fig. 2), using a Model 401 low-pressure syringe pump (Gilson). A multi-purpose stream-switch unit (MUST; Spark, Emmen, The Netherlands) that was connected to the inlet of the Gilson 401 pump served as a solvent selector.

The automated procedure involves the following steps. The reagent solution (typically 25 μl) is pipetted into the vial and, after mixing, an aliquot of 50 μl of the reaction mixture is transferred to a reaction coil (PTFE tubing, 0.5 mm I.D.; Omnifit, Cambridge, U.K.) that is thermostated at 60°C using a water-bath. After a 6-min residence time in the reaction coil, in which the sample is repetitively moved forward and backward, it is transferred, through a 2-μm stainless-steel screen, mounted in a laboratory built PTFE housing (*ca.* 30 μl), onto a 10 × 2.1 mm I.D. precolumn, containing 40 μm Chromsep C_{18} (Chrompack). Next, the precolumn is washed with

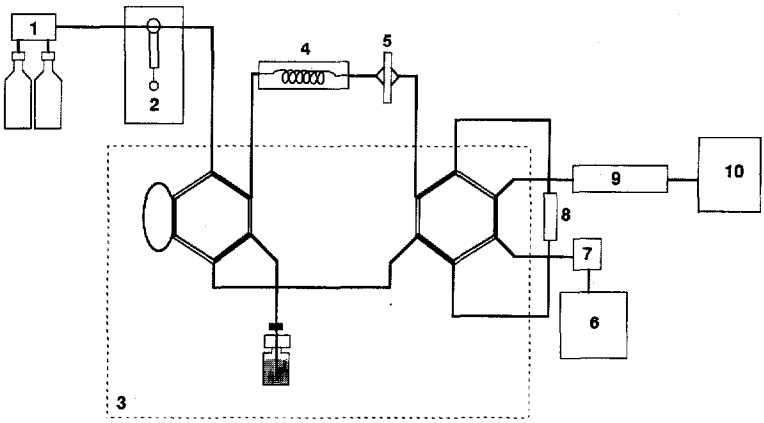

Fig. 2. Schematic diagram of the automated MPTC–HPLC system for the determination of the BrMAC-derivatized plasma free fatty acids. (1) Low-pressure solvent selector; (2) Gilson 401 low-pressure syringe pump; (3) Gilson 232 autosampler; (4) 100 × 0.5 mm I.D. PTFE reaction coil (60°C); (5) 2-μm filtration unit; (6) high-pressure solvent delivery system; (7) additional injection valve; (8) 10 × 2.1 mm I.D. pre-column; (9) 100 × 3 mm I.D. analytical column; (10) detector.

400 μl of 10 m*M* phosphate buffer (pH 7.0) and is switched on-line into the HPLC system in the back-flush mode. During the HPLC analysis the low-pressure PTFE tubing (0.5 mm I.D.; Omnifit) is rinsed with, subsequently, 1 ml of water and 2 ml of methanol. For reasons of comparison, samples were also derivatized batchwise in the micellar system described above, and were directly injected into the RP-HPLC system using an additional U6K injection valve (Waters Assoc., Milford, MA, U.S.A.).

Chromatography

A 100 × 3 mm I.D. analytical column (5-μm Chromspher C_{18}; Chrompack) was used for separation. Generally, after an initial elution with methanol–water (75:25, v/v) for 3 min, a concave gradient to 100% methanol was run in 12 min using two Model M 6000 A pumps which were controlled by an automated gradient controller (all from Water Assoc.). The column effluent was monitored at 254 nm with a Model 440 absorbance detector (Waters Assoc.) and a Model 650 fluorescence detector (Perkin-Elmer/Hitachi, Tokyo, Japan) with optimized excitation and emission wavelengths of 362 and 418 nm, respectively. Retention times and peak areas were determined using a Model 4290 integrator (Spectra-Physics, Santa Clara, CA, U.S.A.) and a Model BD 41 recorder (Kipp & Zonen, Delft, The Netherlands). The plasma free fatty acids were identified by comparing their retention times with those of the reference fatty acids.

RESULTS AND DISCUSSION

The automated determination of plasma samples involves various processing steps such as sample transport, column switching and circuit rinsing, which pose stringent demands on the autosampler. We selected the Gilson Model 232/401 autosampler for the development of the automated system, because this autosampler, which is equipped with two Rheodyne 7010 valves, meets most of these demands.

Sample loading

The present automated HPLC monitoring is hampered by the presence of proteins in plasma samples. First, the proteins form small aggregrates during derivatization reactions, which complicates proper sample handling within the low-pressure circuit. As a consequence, it was necessary to dilute the plasma sample 10-fold with the aqueous micellar solution prior to its automated processing. Second, if after derivatization the diluted plasma is injected directly into the HPLC system, then the back-pressure of the system increases steeply and only a limited number of injections ($n \leq 15$) can be preformed. The back-pressure build-up is caused by the protein aggregates in the sample and by the gradual precipitation of the non-aggregated proteins, effected by the presence of an organic modifier.

The back-pressure problem was solved as follows. The aggregated proteins are retained by an in-line filter that is mounted between the reaction coil and the injection valve, while the non-precipitated proteins are removed using a column-switching step. As regards the in-line filter, it appeared that neither cellulose acetate nor nylon membranes could be used, because of the protein-binding properties of these materials. With a multi-layer glass-fibre filter the retention of the protein aggregates was insufficient. We finally selected a 2-μm stainless-steel screen, which was mounted into a laboratory-built housing. The effective filtration area was *ca.* 8 mm^2. The trapped aggregates were removed by back-flush rinsing of the screen with 1 ml of buffer solution after each injection of a sample into the HPLC system.

For the removal of the non-precipitated proteins, a 10 × 2.1 mm I.D. precolumn was used, which was inserted between the low-pressure circuit and the HPLC system. During loading, the precolumn offers the opportunity to retain the free fatty acid derivatives, while the proteins go to waste and are therefore efficiently removed. Next, the precolumn is switched on-line with the HPLC system and, subsequently, desorption and separation occur. We selected a relatively short precolumn, because in this case the sample loading can be performed using the low-pressure (< 1.3 HPa) Gilson 401 syringe pump. With the use of the in-line filter unit and the column-switching step, 100–150 samples can be injected into the HPLC system and analysed without any problem. Such large series of samples were processed repeatedly to illustrate the potential of the system. With a larger number of injections, there is still back-pressure build-up at the top of the precolumn. To ensure a good performance, we replaced the top layer of the precolumn after 100 injections.

It is important to note that, if no solvent is flushed through the precolumn for a longer period of time, its performance deteriorates considerably. It is therefore advisable to flush the precolumn continuously with the mobile phase at a low flow-rate of, *e.g.*, 0.1 ml/min between two series of experiments.

Derivatization reaction

Fig. 3 shows the influence of the hold-up time of the derivatization mixture in the reaction coil (60°C) on the fluorescence yield of the MAC derivative of 50 μM tetradecanoic acid in plasma which was processed with the automated system. This figure demonstrates that the reaction is complete within 4 min, which agrees well with the derivatization rate obtained from the batchwise experiments[8]. To be on the safe side with all fatty acids, a reaction time of 6 min was selected for all further experiments.

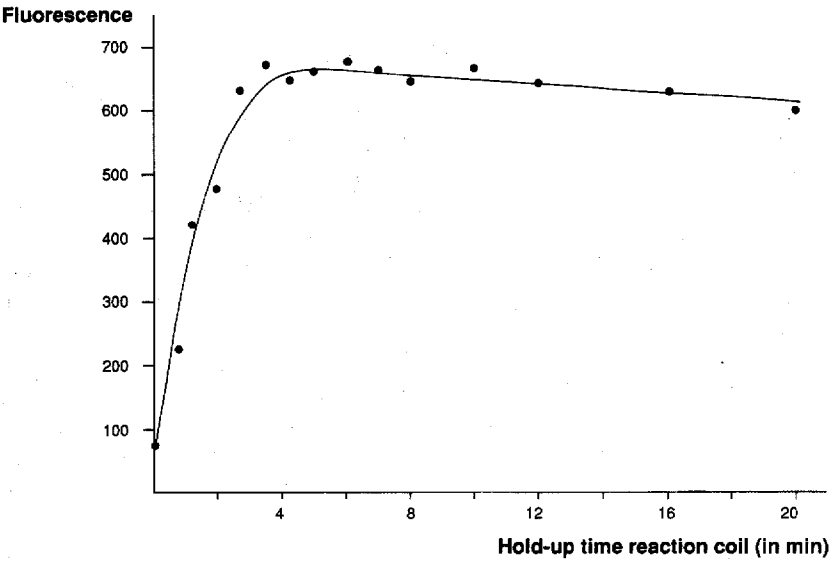

Fig. 3. Dependence of the fluorescence yield of 50 μM tetradecanoic acid derivatized with BrMAC in the micellar system at 60°C on the hold-up time in the reaction coil.

Fig. 4 demonstrates that a BrMAC concentration of at least 3 mM required for the complete conversion of the fatty acids in plasma. To ensure the presence of a sufficient excess of the label, a concentration of 5 mM BrMAC was used for all further experiments.

Because the free fatty acids are strongly bound to proteins, we investigated the effect of this protein binding on the recovery of the derivatives. For this purpose, plasma and buffer samples to which 50 μM tetradecanoic acid had been added were derivatized batchwise for 6 min at 60°C. After derivatization, 20 μl of the incubation mixtures were injected directly, via the U6K valve, into the HPLC system and the peak area for tetradecanoic acid in plasma was compared with that for the buffer solution. The recovery in plasma, relative to that in buffer, was 97 ± 2% (n = 6). The endogenous tetradecanoic acid concentration in the plasma used was negligible; its contribution was not taken into account. The high recovery indicates that, during the incubation, the analyte is almost completely released from the proteins. The coefficient of variation (C.V.) of the peak area measurements in plasma was 2.4% (n = 6) and in buffer the C.V. was 2.1 % (n = 6). The combined results indicate that the recovery and the precision of the batchwise derivatization are satisfactory.

The same series of buffer and plasma samples was also derivatized and processed using the automated system. The recovery for the buffer samples was 99 ± 3% (n = 6) and that of the plasma samples was 92 ± 4% (n = 6), both compared with directly injected buffer samples. The lower recovery in plasma can at least partially be explained by a *ca.* 2% loss of the tetradecanoyl–MAC derivative, which was recovered in the precolumn effluent. The recovery could be slightly increased by decreasing the total flush volume from 600 to 200 μl (Fig. 5); however, this led to a rapid back-pressure build-up on top of the precolumn. A rinsing volume of 400 μl was finally selected to circumvent back-pressure build-up and to ensure a sufficiently fast flush time.

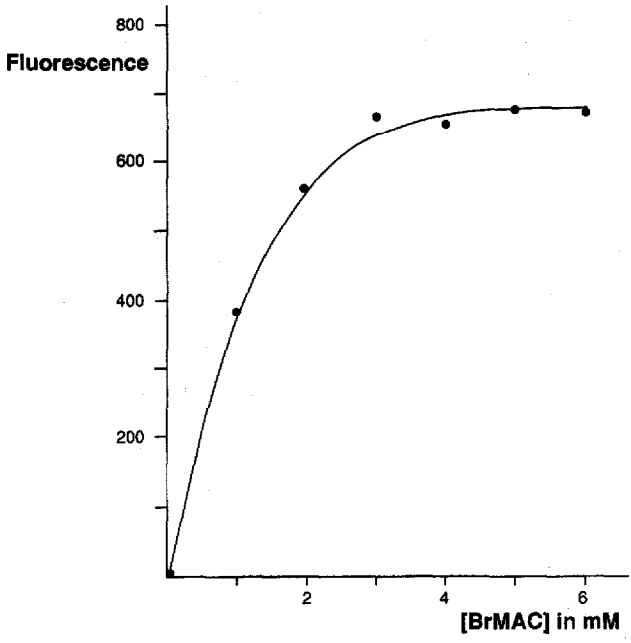

Fig. 4. Dependence of the fluorescence yield of 50 μM tetradecanoic acid added to plasma on the BrMAC concentration using the automated system. Temperature, 60°C.

The C.V. of the automated procedure was 5.6% ($n = 6$) for the buffer and 5.5% ($n = 6$) for the plasma sample. In view of the recovery and precision of the automated analysis of plasma samples, it is advisable to use an internal standard, for which heptadecanoic acid was selected for all further experiments. When using the internal standard, the C.V. at a spiked tetradecanoic acid level of 5 μM was 3.4% and at 50 μM is was 3.5%. The linearity of the calibration graph for the on-line derivatization

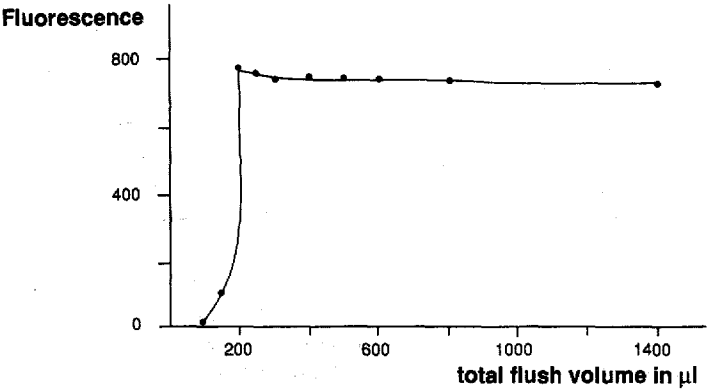

Fig. 5. Dependence of the fluorescence yield of 50 μM tetradecanoic acid added to plasma after derivatization with BrMAC and processing using the automated system, on the overall flush volume of the precolumn.

of tetradecanoic acid added to plasma was satisfactory ($r = 0.9992$, $n = 15$) from 2 to 200 μM, which covers the normal concentration range of plasma free fatty acids (Table I). The minimum concentration of heptadecanoic acid that could be determined in plasma was about 300 nM; it was limited by the presence of unknown endogenous compounds in plasma. In buffer the detection limit was ten times lower.

Chromatography

Fig. 6A shows gradient HPLC of plasma free fatty acids, Fig. 6B shows the chromatogram of plasma that was processed without the fluorescence label and Fig. 6C is the reagent blank. Fig. 6A demonstrates that the plasma free fatty acids are satisfactorily separated. From Fig. 6B and C, it can be seen that no compounds that originate from the plasma and the derivatization system interfere with the determination of the plasma fatty acids, that is, the fluorescent peaks are MAC derivatives of carboxylic acids.

The calculated concentrations of identified free fatty acids, using heptadecanoic acid as an internal standard, are given in Table I, which additionally shows several results reported in the literature[1,11–13]. Despite the marked differences in the total fatty acid concentration, *e.g.* from 64 to 331 μM, the present fatty acid pattern is similar to those reported by the various groups of workers, the differences found for $C_{14:0}$ and $C_{18:0}$ probably originating from inter-individual variations.

It should be noted that the fluorescence yield of the MAC derivatives is solvent dependent[8]. Because in the current procedure no corrections have been made for this effect, the concentration of the early eluted fatty acids is overestimated by up to about 10%. As an alternative, one can consider using UV instead of fluorescence detection. UV absorption measurement has the advantage that it is not dependent on the methanol concentration in the mobile phase[8]. In addition, the MAC derivatives have large molar absorptivities of about $2 \cdot 10^5$ at 254 nm, which limits the interference of endogeneous plasma compounds in the present case.

TABLE I

FREE FATTY ACID LEVELS IN NORMAL SUBJECTS

Results are given in μM or percentage of the total FFA concentration (%). Data taken from (A) this work, (B) ref. 11, (C) ref. 12, (D) ref. 13 and (E) ref. 1.

Fatty acid	*A*		*B*		*C*		*D*		*E*	
	μM	%	μM	%	μM	%	μM	%	μM	%
$C_{14:0}$	30	13	4	1	5	8	5	2		3
$C_{16:0}$	72	31	91	28	21	33	51	25		27
$C_{16:1}$	12	5	21	6	3	5	12	6		4
$C_{18:0}$	5	2	34	10	8	13	25	13		8
$C_{18:1}$	47	20	135	41	16	25	73	37		31
$C_{18:2}$	47	19	42	13	9	14	21	10		19
$C_{18:3}$	7	3	4	1	1	2	2	1		2
$C_{20:4}$	2	1	–	–	1	2	11	5		2
Total	222		331		64		200		321	

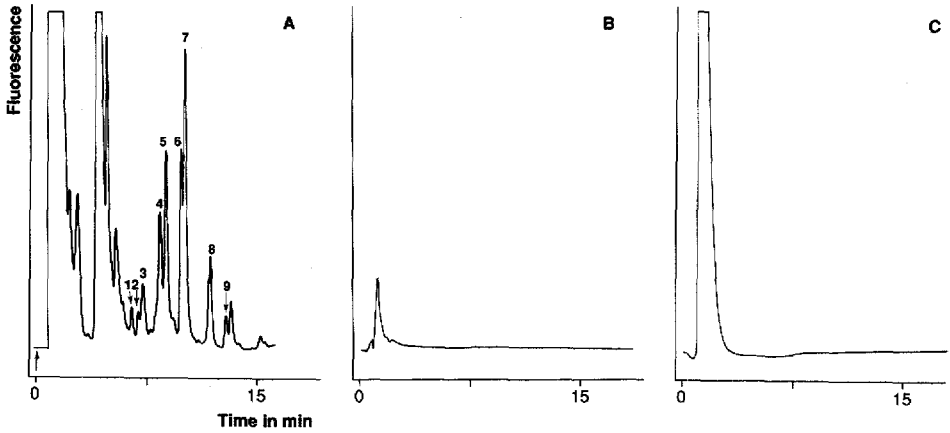

Fig. 6. (A) Gradient HPLC of the plasma free fatty acids derivatized with BrMAC using the fully automated system; (B) plasma processed without BrMAC reagent; (C) processed reagent blank. For HPLC conditions, see Experimental. Peaks: 1 = $C_{18:3}$; 2 = $C_{20:4}$; 3 = $C_{16:1}$; 4 = $C_{14:0}$; 5 = $C_{18:2}$; 6 = $C_{18:1}$; 7 = $C_{16:0}$; 8 = $C_{17:0}$ (I.S); 9 = $C_{18:0}$.

CONCLUSIONS

We have demonstrated that plasma samples can be determined fully automatically by RP-HPLC, after derivatization with the fluorophore BrMAC in an on-line MPTC unit. The complications of protein precipitation during derivatization and chromatography can be solved by using an in-line filter, mounted in front of the injection valve of the precolumn, and a column-switching unit, respectively. More than 100 samples can be processed using a single precolumn. The recovery and relative standard deviation of the determination of free fatty acids in plasma are 92 ± 4% and less than 3%, respectively, and the limit of determination is about 300 n*M*.

ACKNOWLEDGEMENTS

We are grateful to Meyvis (Bergen op Zoom, The Netherlands) for putting the Gilson 232/401 autosampler at our disposal.

REFERENCES

1 H. Miwa, M. Yamamoto, T. Nishida, K. Nunoi and M. Kikuchi, *J. Chromatogr.*, 416 (1987) 245.
2 C. de Ruiter, R. R. Otten, U. A. Th. Brinkman and R. W. Frei, *J. Chromatogr.*, 436 (1988) 429.
3 E. V. Dehmlow and S. S. Dehmlow, *Phase-Transfer Catalysis*, Verlag Chemie, Weinheim, 1980.
4 C. M. Starks and C. Liotta, *Phase-Transfer Catalysis, principles and Techniques*, Academic Press, New York, 1978.
5 F. A. L. van der Horst, M. H. Post and J. J. M. Holthuis, *J. Chromatogr.*, 456 (1988) 201.
6 F. A. L. van der Horst, J. M. Reijn, M. H. Post, A. Bult, J. J. M. Holthuis and U. A. Th. Brinkman, *J. Chromatogr.*, (1990) in press.
7 F. A. L. van der Horst, G. G. Eikelboom and J. J. M. Holthuis, *J. Chromatogr.*, 456 (1988) 191.
8 F. A. L. van der Horst, M. H. Post, J. J. M. Holthuis and U. A. Th. Brinkman, *Chromatographia*, 28 (1989) 267.

9 F. A. L. van der Horst and J. J. M. Holthuis, *J. Chromatogr.*, 426 (1988) 267.
10 K. Akasaka, T. Suzuki, H. Ohrui, J. Meguro, Y. Shindo and H. Takahashi, *Anal. Lett.*, 20 (1987) 1581
11 V. Rogiers, *Clin. Chim. Acta*, 78 (1977) 233.
12 M. Hatsumi, S. Kimata and K. Hirosawa, *J. Chromatogr.*, 380 (1986) 247.
13 J. D. Baty, S. Pazouki and J. Dolphin, *J. Chromatogr.*, 395 (1987) 403.