Micellar Phase-Transfer Catalysis: A Suitable Procedure to Derivatize Carboxylic Acids in Biological Matrices

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Summary: The potential of micellar-phase transfer catalysis (MPTC) is shown by derivatization of various carboxylic acids with 9-bromomethylacridine (Br MAC). These carboxylic acids were monitored in the pre-column derivatization mode by fluorescence detection in a completely automated system. The application of the methodology to analysis of the biological sample is shown.

Introduction

A novel micelle-mediated technique, termed micellar phase- transfer catalysis (MPTC) is known to be simple and rapid for the derivatization of various biologically active substances [1,2].

Since carboxylic acids are known as the important constituents of body, it becomes vital to have an assay to assess the levels of carboxylic acid under various pathological conditions.

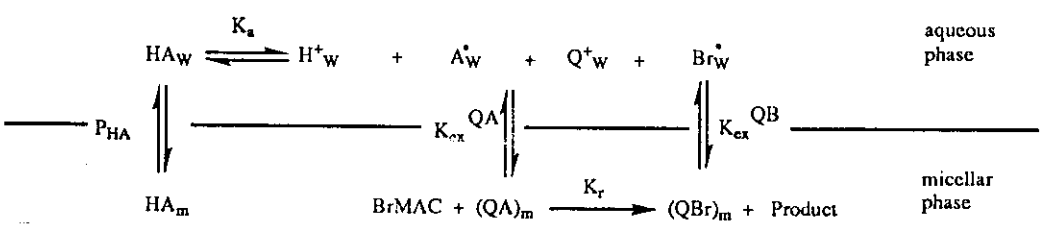
The most widely used methods are thin-layer chromatography (TLC) and column chromatography. The advantages of column chromatography when compared to TLC are higher yields, convenience of procedure, and smaller risk for auto-oxidation of carboxylic acids. Available gas-liquid chromatographic (GLC) and GLC-mass spectrometric (GLC-MS) methods require the synthesis of volatile derivatives in order to perform carboxylic acid analysis [3,4]. These procedures, besides being time consuming, may result in the loss of sample.

In this study, we report a newly developed, fully automated pre-column derivatization method for the analysis of free fatty acids. This method is based on derivatization of free fatty acids with 9-bromomethylacridine followed by separation on a reversed-phase column.

In this study, the potential of MPTC is demonstrated by showing that the technique is simple since no prior extraction with an organic solvent is required and the derivatization of lipophilic carboxylic acid is completed within 6 mins. The application of this method is shown on some representative biological samples.

Results and Discussion

A model has been developed for the mechanism of MPTC action, which is given below:



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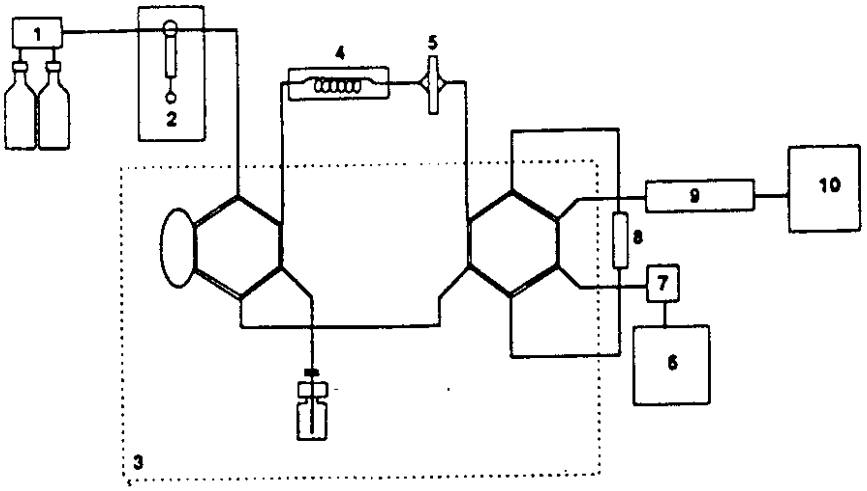

Fig. 1: Schematic of the automated MPT/RP-HPLC system for the determination of the BrMAC-derivatized free fatty acids. All the instrumental units used (1-10) are explained under experimental section.

The conjugated base A is extracted from the aqueous phase by a quaternary ammonium ion Q^+ into the aprotic micellar core as an ion-pair complex AQ. In the non-ionic micelle, A^- reacts with the fluorescence reagent BrMAC [5]. K_{ex} QA and K_{ex} OB are the extraction constants of A^- and the counterion Br^- respectively. The partition coefficent P_{HA} determines the partitioning of the non-dissociated acid HA to the micelle. K_a is the acidity constant of the acid in water and K_r is the reaction constant in the micelle.

This reaction demonstrates that high reaction rates can be obtained if (a) the acid is present only as the A^- species i.e. the derivatization has to be carried out at netural pH which is about 2 units above the pK_a of common carboxylic acids in micellar solution and (b) K_{ex} is large i.e. a lipophilic ion-pair agent should be used.

Fig. 2 shows the separation of methylacridine derivatives of several carboxylic acids which are obtained after derivatization for 6 min with BrMAC at pH 7.0 and 60°C. The procedure of derivatization and chromatographic separation parameters are reproducible and the signal (fluorescence) is related to the concentration of each carboxylic acid giving a linear detector response of each fatty acid in the concentration range of 1-100 µmol/l. The linearity remained valid in the µmol range of each carboxylic acid giving a correlation of variation of (r^2) equal to unity [5].

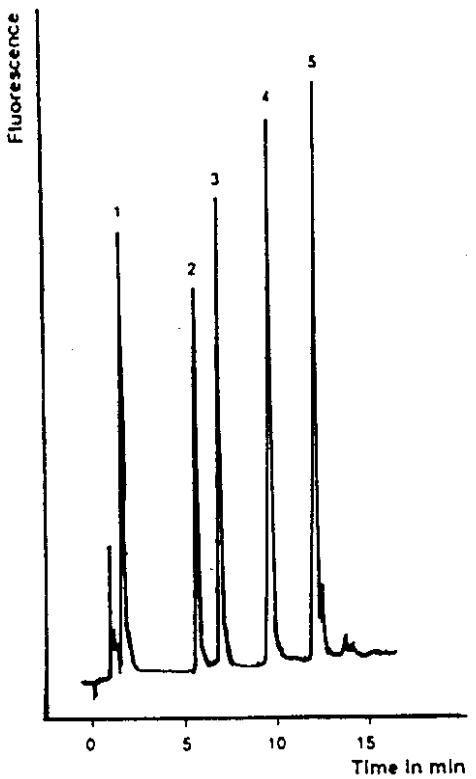

Fig. 2: RP-HPLC Chromatogram of the BrMAC derivatized carboxylic acids using fluorescence detection. Peaks 1) BrMAC 2) Salicyl-MAC 3) Ibuprofen-MAC 4) propylpentanoyl-MAC 5) Choyl-MAC. The separation is achieved using a linear gradient from methanol-water (20:80) to 100% methanol in 12 min.

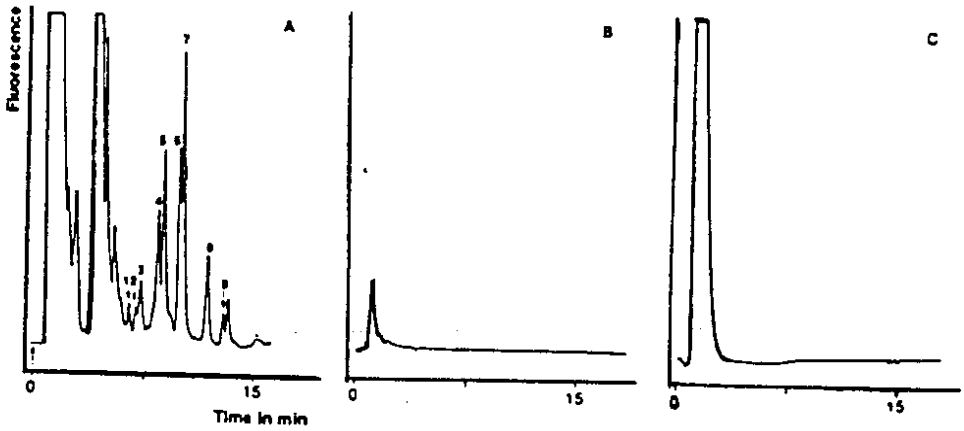

Fig. 3: RP-HPLC separation of the methylacridine derivatives of the plasma free fatty acids (A) plasma sample completely processed (B) plasma sample without BrMAC label and (C) blank reagent. Peaks 1=$C_{18:3}$, 2=$C_{20:4}$, 3=$C_{16:1}$, 4=$C_{14:0}$, 5=$C_{18:2}$, 6=$C_{18:1}$ 7=$C_{16:0}$, 8=$C_{17:0}$ (internal standard) and 9=$C_{18:0}$. Experimental conditions were similar as given in Fig. 2.

One-line derivativatization

The manual sample handling is limited to a 10-fold dilution of the plasma sample with the micellar solution to reduce protein aggregation during derivatization and the addition of an internal standard. The 2 μm screen ensures that small particles such as aggregated proteins do not enter the chromatographic system while the pre-column is used for sample clean-up and pre-concentration. Using this system, more than 200 samples were processed without any problem as regards back-pressure build-up.

A typical chromatogram of the carboxylic acid derivatives of the plasma sample is shown in Fig. 3. The identification of each in the sample was achieved according to their retention times obtained during analysis of the standard mixture.

The recovery measured by addition of carboxylic acid standards to a plasma sample from 10-100 μmol/l was 92% for all carboxylic acids. The detection limit for each carboxylic acid at signal to noise ratio of 3 is 1 μmol/1 [5].

In conclusion, MPTC is a novel technique with good potential for the esterification derivatization of carboxylic acid with common fluorescence labels in aqueous media. Virtually no manual sample handling is involved as no prior extraction of the analyte into a suitable organic solvent is required. Furthermore, carboxylic acids can be rapidly derivatized their methylacridine derivatives within a mere 6 min at 60°C and pH 7.0 and the procedure is a suitable alternative to gas-liquid chromatography methods.

The work is under progress to apply this technique to the patients with various clinical disorders.

Experimental

Chemicals

The nonionic surfactant Arkopal N-130 (a polyoxyethylene- nonylphenyl ether) was purchased from Hoechst, Amsterdam. The Netherland, the ion-pair agent tetrakis-decyl ammonium bromide (TDeAbr) from Fluka Chemical Company, Buchs, Switzerland, while the fluorescence reagent 9-bromomethylacridine (BrMAC) was kindly provided by Professor Erland Johansson from Clinical Research Centre, University of Uppsala, Sweden.

The micellar derivatization mixture was prepared by adding selected amounts of Arkopal N-130 and TDeARb to a 50 mM phosphate buffer (pH=7), followed by sonification of 10 min. The BrMAC reagent was dissolved in acetone at a concentration of 28 mg/ml. All other chemicals were of highest purity available and were obtained from various commercial sources.

HPLC system

A schematic assembly of the automated MPTC/RP-HPLC system is shown in Fig. 1. The entire system consisted of 1) a low pressure solvent selector, 2) a low-pressure syringe pump (Model 402), 3) an autosampler (Model 232), 4) a reaction Coil kept at 60°C, 5) a 2-µm Filtration Unit, 6) a high-pressure solvent delivery system - all obtained from Gilson Chromatographic System, Paris, France. The system also consisted of 7) an additional injection Valve (obtained from Rheodyne, Cotati, USA, 8) a pre-column containing C_{18} (100 x 2 mm i.d.), 9) an analytical column 3 µm Spherisorb C_{18} (100 x 3 mm i.d.) both supplied by Chropack, Stockhlom, Sweden and 10) a detection unit i.e. fluorescence detector S 531 supplied by Shimadzu, Tokyo, Japan.

Derivatization procedure

The following procedure was performed automatically. The micellar agent is added to the sample to yield a final concentration of 25 mM Arkopal N-130 and 6 mM TDeABr. Next, the sample is placed in a Gilson 232 autosampler and processed fully automated as schematically presented in Fig. 1. Firstly, the BrMAC reagent solution is added to the sample. Then 50 µl of the sample is transferred to a reaction coil which is thermostated at 60°C. After 6 min, the sample is transferred through a stainless steel 2 µm screen to a pre-column. Finally, the pre-column is switched on-line to the RP-HPLC system, and the analytes are separated on the analytical column with a linear elution gradient from water : methanol (80:20) to 100% methanol in 12 min, and subsequently monitored with the fluorescence detection set at E_{ex} =365 and E_{em} = 420 nm.

Analysis of a plasma sample

Blood sample was obtained from a healthy male volunteer (35 years old) and was collected into lithium heparin test tube maintained at 4°C. The plasma was promptly separated by centrifugation at 1500 x g for 5 min at room temperature and then stored at -70°C until the time of analysis. Prior to derivatization of plasma sample with BrMAC, the sample was deproteinized with 10% Sulphosalicylic acid and the pH of the supernatant was adjusted to 7.0 with 0.1 M NaOH.

References

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