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On-line coupling of microdialysis sampling with liquid chromatography for the determination of peptide and non-peptide leukotrienes

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Abstract

An automated on-line sampling method was developed using microdialysis as the simultaneous sampling and sample pre-treatment technique. The extraction fraction values of microdialysis probes sampling different eicosanoids were investigated. The impact of cyclodextrins in the perfusion liquid used for sampling hydrophobic eicosanoids in biological systems was also studied. The total time for one analysis was 7.6 min allowing seven measurements per hour for monitoring kinetic changes in biological systems.

Keywords: Sample handling; Microdialysis; Leukotrienes; Eicosanoids

1. Introduction

In pharmaceutical drug research much effort is focused on trying to manipulate metabolic pathways by interrupting the progress of a disease through inhibition of a key target such as an enzyme or blocking a receptor binding site. Initially in the drug discovery process numerous drug candidates are synthesised and screened for affinity or activity with the isolated key biological target. Later in the process, different biological in vitro models e.g., cell cultures, isolated organs and whole animals, are used for the purpose of monitoring the physiological/biochemical state that one wants to influence and control. These models should help to understand the mechanism of action of the tested drug candidate, as well as to predict the action of the substance and thus reveal the positive and potential unwanted side effects, when in a later phase, it is tested in humans.

Arachidonic acid (AA) is one of the major constituents of cell membranes and is a precursor of the so called eicosanoids, i.e., leukotrienes, prostaglandins and thromboxanes. The eicosanoids exhibit important mediating roles in triggering various physiological and pathophysiological processes such as vascular resistance, thrombosis, wound healing, inflammation and allergic reactions [1,2]. The biosynthesis of eicosanoids is catalysed by primarily the 5-lipooxygenase and cyclooxygenase pathways, which thus are the key targets involved when wanting to control and influence these processes. Initially it is, however, necessary to study and follow the appearance and disappearance of the biomarkers themselves, i.e., the arachidonic acid metabolites, in biological model systems and later to test how these processes can be controlled and manipulated by

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potential drug candidates. The instability and rapid metabolism of these compounds is, however, critical and a crucial step during analysis is the sample handling from the time of collection, during storage and sample workup until as well as during the process of determination. These problems have to some extent been addressed and solved in previous works by the authors [3].

Microdialysis is a sampling tool that has previously been shown to be useful for off-line in vivo sampling of eicosanoids [4–12]. In the present work microdialysis sampling of eicosanoids was optimised by characterising different membrane types and sampling conditions and their impact on dialysis factor of various eicosanoid mediators. By combining microdialysis on-line with liquid chromatography (LC) we suggest the possibility for simple and relatively fast kinetic profiling of these biomarkers of limited size ($\leq$650 Da) in a complex biological cell model matrix, especially stimulated to study and understand the role and influence of these compounds in the inflammatory process. Microdialysis is an attractive tool for this purpose due to the discriminating nature of the microdialysis membrane towards large molecular mass interferences [13] and to other membrane interaction mechanisms [14,15]. In the present work, the resulting dialysates proved to be sufficiently clean to avoid the necessity of solid-phase extraction (SPE), prior to chromatographic analysis [3]. In addition microdialysis on-line with chromatography is relatively fast compared to traditional and previous techniques avoiding some of the stability problems mentioned above.

Four commercial microdialysis probes and one in-laboratory built tunable microdialysis probe were investigated using various commercial membranes. The influence of membrane properties, dialysis area, perfusion flow-rate and addition of cyclodextrins and EDTA on the extraction fraction of leukotriene B4 (LTB_4), leukotriene C4 (LTC_4), leukotriene D4 (LTD_4), leukotriene E4 (LTE_4) and prostaglandin B2 (PGB_2), were investigated.

2. Experimental

2.1. Chemicals

LTB_4, LTC_4, LTD_4, LTE_4 and PGB_2 supplied in ethanol were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). α-Cyclodextrin was obtained from Sigma (St. Louis, MO, USA). β-Cyclodextrin was purchased from Avebe (Veendam, Netherlands). γ-Cyclodextrin was obtained from Stadex (Malmö, Sweden). Acetonitrile, calcium dichloride, ethylenedinitrilotetraacetic acid disodium salt dihydrate (EDTA), magnesium dichloride, potassium chloride, 85% orthophosphoric acid 85%, sodium dihydrogenphosphate, sodium hydrogencarbonate, sodium chloride were all obtained from Merck (Darmstadt, Germany). Monocytic cells HL60 stimulated by A23187 were prepared in the laboratory.

2.2. Equipment

An overview of the analytical system is shown in Fig. 1. It consists of a high-pressure pump (LKB-Pharmacia 2150, Bromma, Sweden). A Rheodyne (Model 7000, Rheodyne, Cotati, CA, USA) injection valve with a 5-μl loop was used for the injection of standards. For the separation of leukotrienes and PGB_2, a 200×3.0 mm I.D. column packed with Nucleosil C_{18} material, 5 μm particle diameter, (Macherey–Nagel, Düren, Germany) was used. In the elution step the mobile phase was acetonitrile–phosphoric acid (45:55, v/v) at pH 2.6. The microdialysis equipment consisted of a CMA/100 syringe pump and an on-line injector CMA/160 equipped with a 5-μl loop, both from Carnegie Medicine Associates, Stockholm, Sweden. Five different microdialysis probes were used namely: CMA/20 PES, CMA/20 PA, CMA/20 PC, CMA/11 CU and an in situ tuneable microdialysis probe fitted with a SPS6005 membrane from Fresenius, Germany (see also Table 1). For the first series of experiments a UV–Vis detector (UV/VIS 119) from Gilson Medical Electronics, Villiers-le-Bel, France was used which in the final system was replaced with a photodiode array detection (PAD) system (HP1050, Hewlett-Packard, Palo Alto, CA, USA). The data were stored in a Hewlett-Packard Chemstation program (version 3.03).

2.3. Preparation of standards and samples

For the extraction fraction measurements, a 100

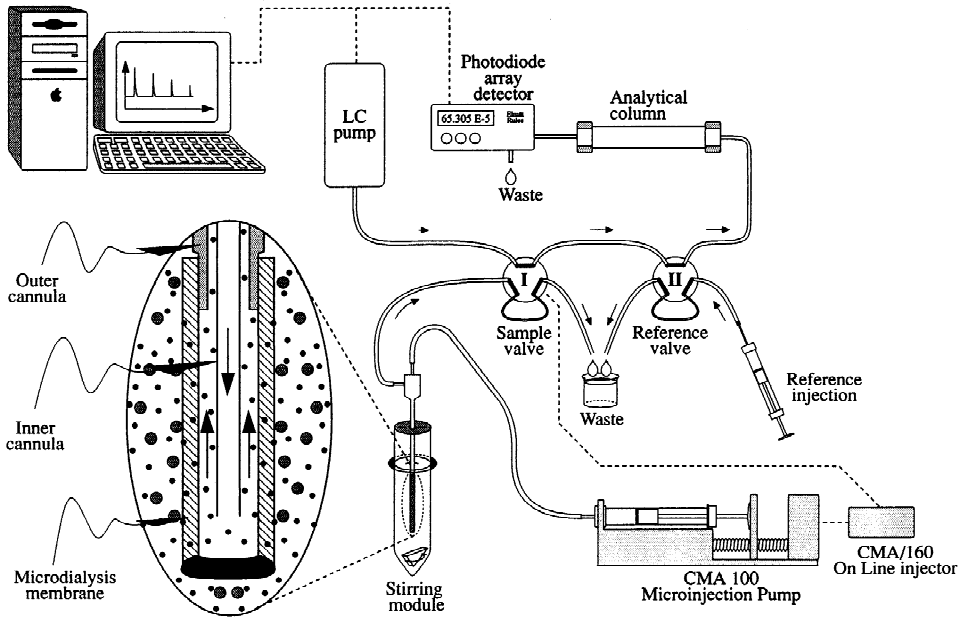

Fig. 1. Integrated on-line continuous sampling and chromatographic system.

mg/l standard solution of leukotrienes was prepared in a Ringer buffer containing 0.1% EDTA and 0.5% β-cyclodextrin. The perfusion liquid was identical to the standard solutions with the leukotrienes omitted. For the kinetic profiling experiments the monocytes were prepared as described earlier [3]. After evaporation of methanol the monocytes were suspended in Ringer buffer to a final concentration of 10^6 cells/ml. For the investigation of the impact of cyclodextrin on the extraction fraction and the sampling in the spiked monocytic cell system CMA/20 PC was used.

3. Results and discussion

In a previous work we developed a two-step SPE method for sample pre-treatment before chromatographic analysis, initially using an off-line SPE step whereby the mediators were stabilised in their metabolisation [3]. Mediator stability was shown even during freezing of the SPE columns at −70°C for 10 months. In order to follow these biological patterns in real time, microdialysis was used in the present work as the sampling technique, resulting in simultaneous sampling and sample pretreatment,

Table 1
Characteristics of the membranes and probes used

Probe	Membrane	Cut-off (Da)	Membrane wall thickness (μm)	Inner diameter (mm)	Membrane surface area (mm^2)
CMA/20	Polycarbonate (PC)	20 000	26	0.5	17.3
CMA/20	Polysulfone (PES)	100 000	50	0.5	18.8
CMA/20	Polyamid (PA)	20 000	50	0.5	18.8
CMA/11	Cuprophane (CU)	6000	17	0.24	1.7
	Polysulfone (SPS 6005)	30 000	80	0.5	20.7

followed by an on-line chromatographic separation, see Fig. 1. A shorter sample handling time was required compared to traditional techniques, making it suitable for real time process monitoring.

3.1. Optimisation of microdialysis on-line with liquid chromatography

The five analytes studied vary in structure ranging from apolar to medium polar. Due to the hydrophobic nature of these fatty acids, hydrophobic interaction as a separation mechanism is a logical choice. The separation efficiency of the analytical column is evidently also critical for lipid mediator analysis in complex biological samples and was previously optimised [3]. LTC_4, LTD_4 and LTE_4 have peptide moieties in their structures and are known to be capable of chelation [16]. This is of special importance in trace analysis, since the samples are known to contain Ca^{2+} as well as other metal ions. In order to circumvent solute losses and irreproducible quantification due to metal chelation, EDTA was added to all samples [3]. Sampling in biological systems to monitor bioprocess dynamics by means of microdialysis requires some knowledge of the performance of the dialysis membranes used to obtain optimum extraction fractions. A variety of commercial microdialysis probes are available, varying in polymer structure and membrane cut-off. The present paper deals with the comparison of some commercial probes and an in-laboratory developed tunable probe [17] for the optimisation of the eicosanoid sampling process. The tunable probe was set to have an effective dialysis length of 10 mm resulting in approximately the same membrane area as the CMA types, i.e., 15.7 mm^2. Some characteristics of the probes are illustrated in Table 1. The inner diameters used were 0.5 mm except for the cuprophane probe (0.24 mm) and the membrane wall thickness varied between 17–80 μm. The extraction fraction will be governed by a number of factors such as the sample composition, and its impact on matrix–analyte membrane interactions. The true diffusion of the peptides and the non-peptide leukotriene over the membrane is controlled by the flux described by Fick's law, given in Eq. (1):

$$J = \frac{DA\mathrm{d}c}{\tau \mathrm{d}x} \quad (1)$$

where J is the flux in mol/s, D is the diffusion coefficient of the solute (m^2/s), A is the membrane area available for diffusion (m^2), dc/dx the concentration gradient across the membrane (mol/m^4), and τ the tortuosity of the membrane.

The microdialysis sampling was performed in a continuous flow microdialysis sampling (CFMS) mode as described by Torto et al. [18] and coupled on-line to LC, see Fig. 1. We measured the extraction fraction for the different probes at various flow-rates. The dialysate extraction fraction (E_d), described below by Eq. (2) [18,19], was determined for the probes at different flow-rates.

$$E_d = (C_{in}^{out} - C_{in})/(C_b - C_{in}) = C_{in}^{out}/C_b \quad (2)$$

C_{in}^{out} is the concentration of analyte in the outcoming dialysate, C_{in} ($=0$ in our experiments) is the concentration of the analyte in the incoming perfusion liquid and C_b is the concentration of analyte in the test tube. The extraction fractions were thus determined by injecting the standard from the test tube (C_{in}) and comparing it with the signal obtained received by injecting the outcoming dialysate (C_{in}^{out}) acquired at different flow-rates. At a minimum three injections were made at each flow-rate setting.

3.2. Comparison of different microdialysis membranes and probes

The extraction fraction values, depicted in Fig. 2, for the five leukotrienes are higher for the 100 kDa cut-off polysulfone probe compared to the polycarbonate and polyamide probes. The extraction fraction values for the 30 kDa cut-off polysulfone probe are, however, similar to the 100 kDa cut-off polysulfone probe in spite of the difference in pore size. It is thus likely that the chemical property of the membranes also contributes to the differences in the extraction fractions, rather than the size-exclusion effect of the membranes. The cuphrophane probe (CMA20/CU) gave lower extraction fraction (data not shown), which can be explained by the fact that the cuphrophane probe has a smaller surface area compared to the other probes (see Table 1). A low extraction fraction depends not only on membrane area but also to a great extent on the thickness of the cylindrical fluid path inside the membrane. A small

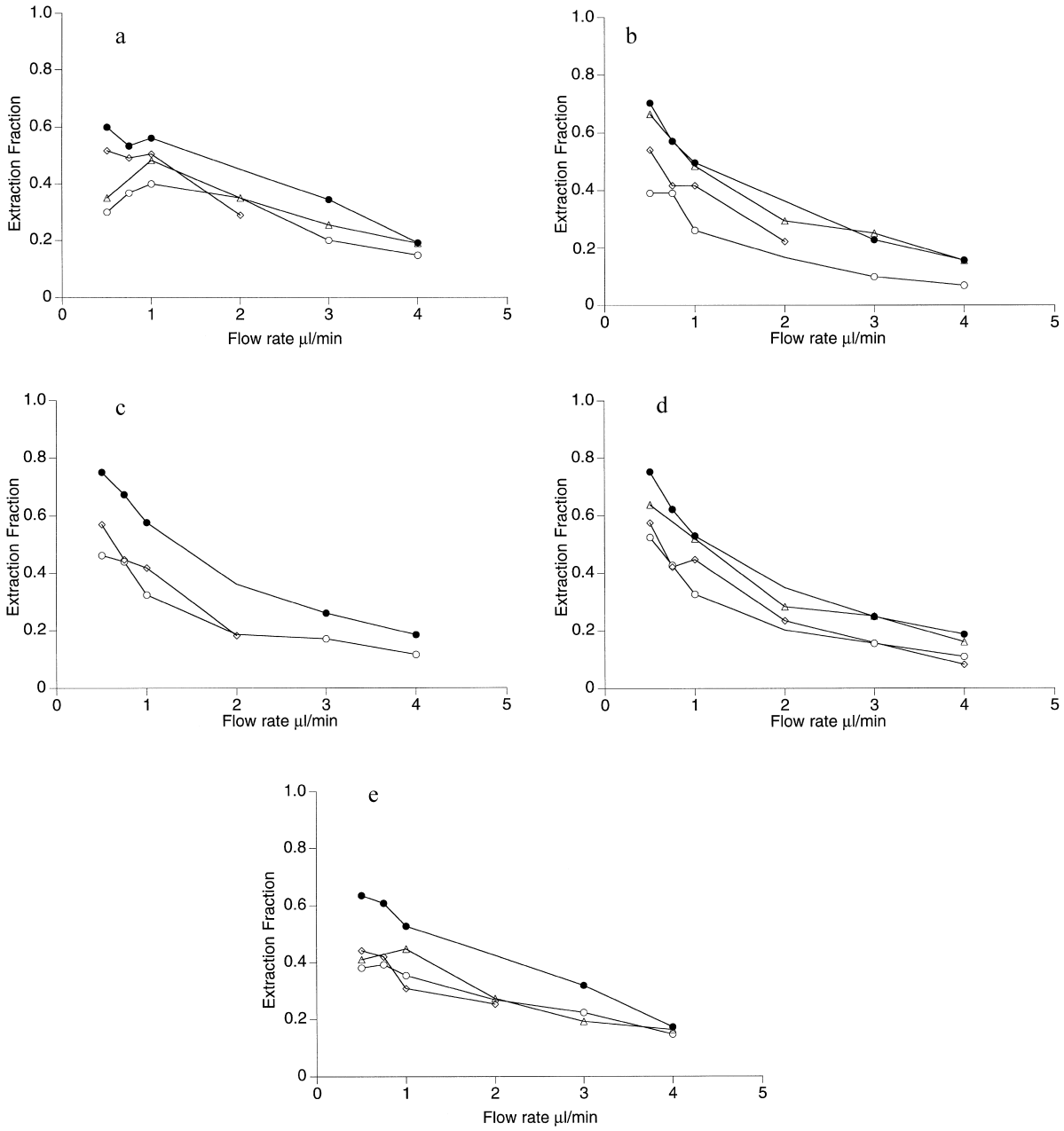

Fig. 2. The extraction fraction versus flow-rate of the perfusion liquid of five eicosanoides (a) LTB_4, (b) LTC_4, (c) LTD_4, (d) LTE_4 and (e) PGB_2, using four different probes: (○) polycarbonate, (△) polysulfone 30 kDa cut-off, (●) polysulfone 100 kDa cut-off, (◇) polyamide.

membrane inner diameter, such as that of the cuprophan probe (0.24 mm compared to 0.5 mm), most likely means a thin cylindrical fluid path and thus a high linear flow-rate that yields a lower extraction fraction. LTB_4 and PGB_2 show lower extraction fractions, i.e., 0.6 compared to 0.8 for the other leukotrienes, at a perfusion flow-rate of 0.5 µl/min using the M_r 100 000 polysulfone probe see Fig. 2.

The reason for these findings is not clear, however, might be explained by the possible adsorption to the polysulfone membrane since PGB_2 and LTB_4 are more apolar compared to the other leukotrienes. The polycarbonate membrane was chosen for further experiments in spite of the lower extraction fraction compared to the polysulfone membrane. The 100 kDa cut-off probe from CMA shows ultrafiltration effects at flow-rates over 2 μl/min. Together with the risk of sampling more unwanted components over the membrane this excluded the 100 kDa cut-off polysulfone probe from the further experiments. For the polysulfone 30 kDa cut off probe there was a co-eluting peak with the same retention time as LTD_4, and thus there are no extraction fraction values for the 30 kDa cut off probe, see Fig. 2c. The problem with a co-eluting peak has never been seen before with that type of membrane in this laboratory. The unidentified peak could arise from the glue which sticks the membrane on the probe. The CMA 20/PA probe displayed similar extraction fraction to that of the CMA 20/PC probe and no major difference in performance could be observed. The CMA/PC 20 probe was chosen for further experiments.

However, as the commercially available microdialysis probes lack the possibility to adjust the effective dialysis length and thus the extraction fraction, the lack of this feature favours the use of the in-laboratory-made tunable probe in the future. The possibility to tune the extraction fraction with the same probe could be of great importance when coupling microdialysis to bio-, or immunoassay techniques due to the limited concentration dynamic range.

3.3. Cyclodextrin impact on the extraction fraction

The stability aspect of eicosanoids is a key factor in qualitative and quantitative determinations. The stability of the leukotrienes varies not only in biological cell systems but also in aqueous media, depending on buffer composition and pH. Adsorption effects are pronounced if appropriate additives are not added to the cell media. The cyclodextrins were added to the analytes and the perfusion liquid to suppress adsorption. Without the addition of cyclodextrin the extraction fraction values for the solutes were found to be considerably lower (data not shown). No biological restrictions were observed when α-, β- and γ-cyclodextrin were added to the perfusion liquid and the standard solutions i.e., the cells used in these experiments were compatible with the concentration of cyclodextrins added. In Fig. 3 the extraction fraction for the different eicosanoids using a CMA/20 PC probe and different cyclodextrin additives are illustrated. As seen, the γ-cyclodextrin causes the lower adsorption thus yielding a higher signal for all of the analytes. The difference between γ- and α-cyclodextrin was not as pronounced for LTB_4 and PGB_2. The extraction fraction at different flow-rates for LTB_4 and PGB_2, seen in Fig. 3, shows that the β-cyclodextrin yields lower extraction fraction curves than α- and γ-cyclodextrin, respectively. However, it is of vital importance that small fluctuations in the flow-rate do not alter the extraction fraction significantly during sampling. The typical relative standard deviation values for quantification of the five mediators during on-line sampling and chromatography was found to vary from 4.5% to 13.4%. The analysis showed that the stability of the mediators under study was good, without using any specific sample handling procedures.

3.4. Sampling in a spiked monocyte cell system

The complete system, shown in Fig. 1, was applied to increasing levels of LTB_4, LTC_4, LTD_4, LTE_4 and PGB_2 added to ionophor (A23187) stimulated human monocyte cell line supernatant by standard addition to study the possibility of kinetic profiling for these compounds under real conditions. In these human cell lines the parent compound arachidonic acid metabolizes to LTA_4 and PGB_2, following one pathway. LTA_4 can then subsequently be transformed to LTB_4 in another pathway by LTA_4-hydrolase or form LTC_4 by the action of LTC_4-synthetase by a glutathione incorporation. LTC_4 can further be transformed to LTD_4 and LTE_4, which are considered to be rather stable end metabolites. We have, however, previously found that LTE_4 under certain circumstances is transformed to *N*-acetyl LTE_4 and LTE_4- sulfone [20]. LTC_4, LTD_4 and LTE_4, the non-peptide leukotriene LTB_4 and the prostaglandin PGB_2 were sampled and analysed in a 7.6 min cycle time as seen in Fig. 4. In Fig. 4a, the

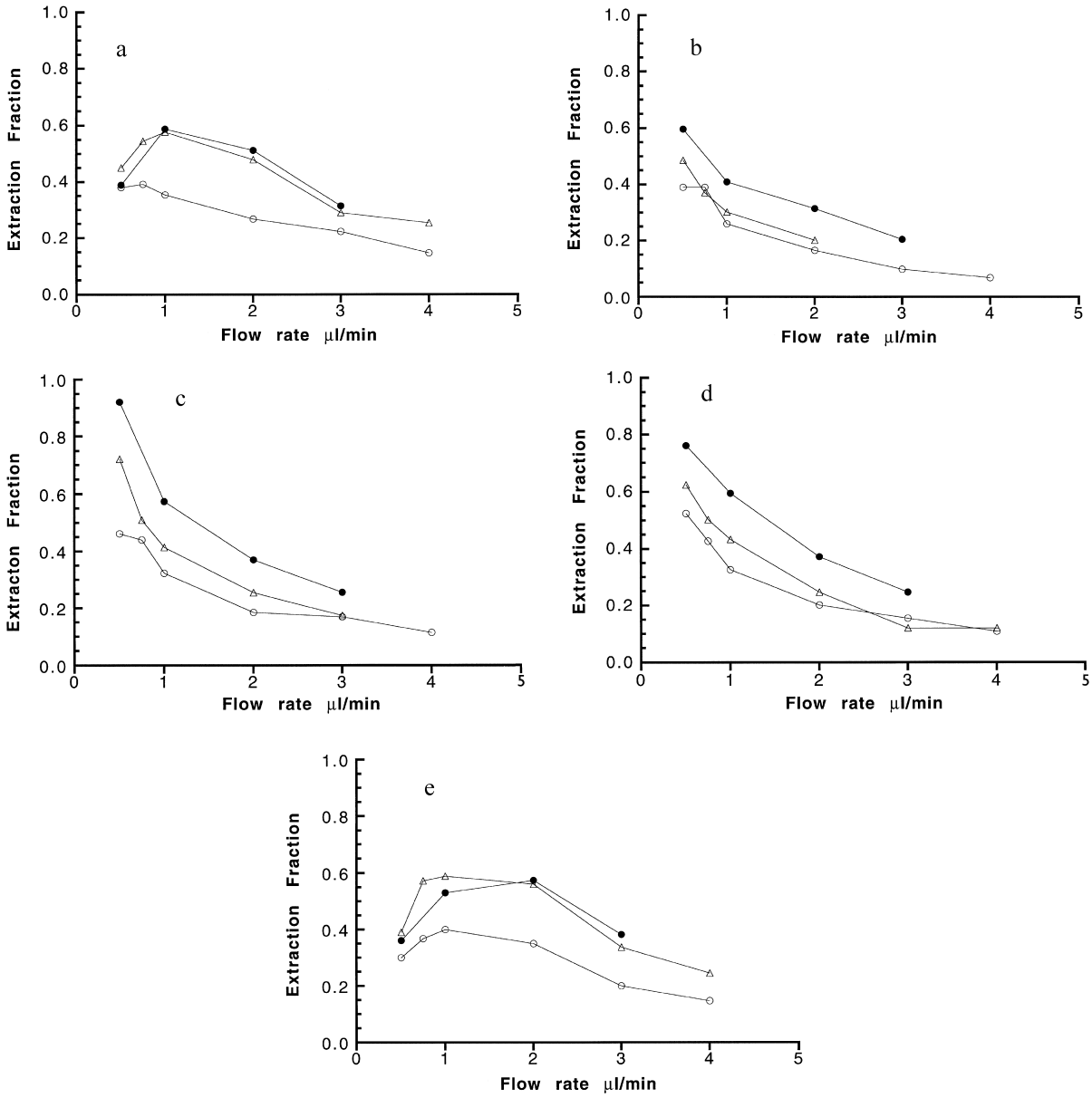

Fig. 3. A comparison of extraction fractions versus flow-rate for five eicosanoides (a) LTB_4, (b) LTC_4, (c) LTD_4, (d) LTE_4 and (e) PGB_2, using a 20 kDa cut-off polycarbonate probe with different cyclodextrin additives: (△) α-cyclodextrin, (○) β-cyclodextrin, (●) γ-cyclodextrin.

background of the blank (unspiked monocyte solution) is shown, displays no major cell component interfering during the analysis. The chromatogram also illustrates the selectivity of the microdialysis probe chosen (CMA 20/PC) as indicated by the absence of interfering peaks.

In a series of separations Fig. 4b–d, the total eicosanoid level was increased from 0.8 to 6 nmol. The absolute detection limits for these mediators in this system are seen in Fig. 4b, while raising to the final level do not give any qualitative or quantitative problems. The time for a chromatographic run was 7.6 min as shown in Fig. 4 which indicates that a perfusion flow-rate of 1.32 µl/min is suitable for

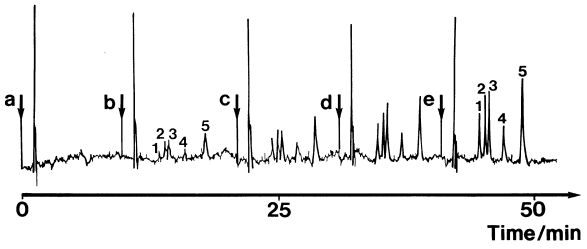

Fig. 4. Injections of a monocyte solution spiked with increasing concentrations of a mediator mixture: (a) blank, (b) 100 ng/ml, (c) 200 ng/ml (d) 300 ng/ml and (e) 400 ng/ml. Peaks: 1=LTC_4, 2=LTD_4, 3=LTE_4 and 4=PGB_2 and 5=LTB_4.

continuous injections allowing a double loop filling (totally 10 μl). This perfusion flow-rate provides an extraction fraction of around 0.5. The concentration of the leukotrienes in the cell system which should be examined will determine the final perfusion flow-rate and the time for one analysis with a minimum time of 7.6 min.

Diode array detection allowed us to make spectrum identification by library comparisons. Many of the resulting metabolites have similar retention times and this qualitative identity could be made more probable by making comparisons with diode array spectra from our own library.

4. Conclusions

These experiments illustrate the possibility to use this integrated sampling–chromatographic system for studying the kinetic profile of the appearance and disappearance of the arachidonic acid metabolites in a cell line system especially stimulated to study the role of these mediators in the inflammatory process. This approach excludes the time consuming step of sample pretreatment and is possible due to the fact that the microdialysis membrane prevents the monocytes from entering the perfusion liquid which normally would cause column clogging. The possibility for continuous leukotriene monitoring could be of great importance in the study of inflammatory diseases since these compound markers rapidly metabolises. Further, using γ-cyclodextrin as a perfusion enhancer will result in an overall increase in signal response of around 20% compared to β-cyclodextrin. In order to measure in vivo models improvements of the sensitivity needs to be made, this can be achieved for this integrated analytical system by using, e.g., post-column derivatisation with a fluorescent label or by an immunodetection principle as was previously published [16,20].

Current work in our group focus on further characterisation of peptide structured biological markers with the use of the in-laboratory-designed tunable microdialysis probe [21].

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