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In vitro microdialysis recovery and delivery investigation of cytokines as prerequisite for potential biomarker profiling

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ABSTRACT

Cytokines as immunomodulatory proteins are secreted by immune and tissue cells mediating immune responses, e.g. inflammation. The use of microdialysis as a minimally invasive technique for sampling interstitial fluid might provide the basis for biomarker profiling for diseases and therapy monitoring. The objectives of this investigation were to develop reproducible methods and apply them to define applicability limits to quantify cytokines in microdialysate.

In vitro microdialysis recovery and delivery investigations were performed utilising a standardised system exploring analyte adsorption, pH effects, the infuence of cytokine concentration and temperature of the catheter surrounding medium. A Ringer's/human albumin solution was used as microperfusate and catheter surrounding medium; interleukin 6, 8 and 10 (IL-6, IL-8, IL-10) and tumour necrosis factor alpha (TNF-α) served as model cytokines. Microdialysate was sampled ($n = 3$) at flow rates of 0.3–5.0 μL/min using 3 linear probes. All samples were measured using a validated flow-cytometry method adapted to microdialysate.

Relative recoveries of the individual cytokines decreased exponentially with increasing flow rates and were not influenced by the catheter surrounding medium concentration but recovery of IL-6, IL-10 and TNF-α by the pH value. Relative recovery and relative delivery of IL-8 were of comparable extent and increased with higher temperatures. For the other cytokines, however, negative values occurred for relative delivery probably due to ultrafiltration.

Clinical application of microdialysis of cytokines is principally feasible if the many influencing factors are controlled. Since relative delivery determination is only reliable for IL-8, retrodialysis or similar calibration methods must be avoided. As future perspective, *in vivo* μD feasibility should next be demonstrated.

1. Introduction

Cytokines (M_r 8–80 kDa) as immunomodulatory proteins are secreted by innate and adaptive immune cells, e.g., macrophages, neutrophils, B- and T-cells, as well as by tissue cells (e.g. fibroblasts) mediating all types of immune responses such as inflammation, infection and allergic or autoimmune reactions. Therefore, monitoring of such mediator concentrations could provide important information about the state and progression of disease and the effect of drug therapy. However, in most cases cytokine concentrations in plasma do not represent concentrations in inflamed tissues. Microdialysis (μD) as a minimally invasive technique enables the sampling of analytes in the interstitial fluid (ISF) of tissues. Essentially, μD was designed to collect small molecules such as glucose or neurotransmitters from the extracellular fluid. Nonetheless, the most recent development of large-pore membranes (cut-off ⩾100 kDa) has enabled to sample molecules with higher molecular mass directly at the site of *in vivo* synthesis. Hence, it is appealing to apply μD as a technique to sample macromolecules in ISF, i.e., at the site of inflammation. The use of μD catheters might provide the basis for the development of a cytokine analysis procedure at the irritated tissue and, thus, as a biomarker profiling for state and progression of various diseases and monitoring of therapy effects.

μD catheters consist of a semipermeable membrane where an exchange of solute molecules in both directions occurs according to the concentration gradient. Due to the continuous flow of the microperfusate inhibiting equilibrium between ISF and microdialysate concentration, solute concentration at the end of the membrane or outlet tubing quantified in dialysate will only represent a fraction of the actual ISF concentration. Therefore, a catheter calibration has to be performed in order to determine the fraction relating the measured microdialysate concentration to the catheter

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surrounding concentration in *in vitro* settings or to ISF in *in vivo* settings (relative recovery, RR) (Plock and Kloft, 2005). The equality of analyte permeation behaviour between sampling (gain, i.e., during the collection of samples in an investigation) and delivery (loss, i.e., during the calibration procedure) provides the theoretical concept for most calibration techniques (Chaurasia et al., 2007). Applying the common method of retrodialysis the analyte is spiked to the microperfusate and the lost fraction of the 'delivered' analyte at the outlet is determined.

As first prerequisite for clinical studies the applicability and feasibility of cytokine μD as well as the dependence of recovery on μD conditions have to be explored *in vitro*. So far, results of published *in vitro* and *in vivo* μD investigations regarding RR and concentration levels of determined cytokines, respectively, revealed large variabilities probably due to the large heterogeneity of catheter material and microperfusate as well as the different flow rates (FR) used and other experimental conditions which all influence the extraction efficiency, i.e. relative recovery or relative delivery. Thus, the objectives of this investigation were to develop reproducible *in vitro* μD settings to sample cytokines and quantify them. As model cytokines for these μD investigations, the pro-inflammatory cytokines interleukin 6, interleukin 8 (IL-6, IL-8) and tumour necrosis factor-alpha (TNF-α) as well as the anti-inflammatory cytokine interleukin 10 (IL-10) were selected. The developed methods were applied to define the potential and limits of the applicability and feasibility of μD of cytokines. This would contribute to accurately implement the sampling of cytokines in future *in vivo* μD studies and assure the opportunity of prospective data comparison.

2. Material and methods

2.1. Bioanalytical quantification method of cytokines

For quantification of cytokines in small sample volumes a mixture of Ringer's solution (RS) and human albumin solution (HA) (RS/HA, 9/1 v/v, HA concentration 0.5% m/v) was used as alternative matrix for μdialysate due to its very limited availability (sample volumes not sufficient for assay development plus assay validation). All matrix solutions were spiked with the 4 model cytokines IL-6, IL-8, IL-10 and TNF-α, and measured using BD™ Cytometric Bead Array (CBA) and BD FACSArray™ Bioanalyzer. The commercially available BD™ CBA assay for cytokines from cell culture supernatant samples was adapted for the quantification of cytokines from the considered matrix, i.e., microdialysate sampled with 100-kDa cut-off μD catheters. Since samples obtained via μD are less in volume, the reduction of required sample volume (from 50 to 25 μl) was investigated. The upper limit of quantification was intended to be increased via an additional dilution step during sample preparation. Validation was performed complying with the European Medicines Agency Guideline on Bioanalytical Method Validation (EMA, 2011) while applying 12 calibration solutions and 6 quality control (QC) solutions. Concentrations of calibration and QC solutions were 4, 6, 10, 20, 50, 100, 500, 1000, 4000, 6000, 9000, 10,000 pg/ml and 5, 15, 200, 1000, 7000, 9500 pg/ml, respectively.

2.1.1. Effect of pH value on quantification of cytokines

As a prerequisite for the investigation of effect of pH value on RR and analysing the robustness of the bioanalytical method, pH effect on the quantification of cytokines was determined. For this purpose, 3 matrix solutions were spiked with different amounts of all 4 model cytokines to obtain a final concentration of $<10^1$, $<10^2$ and $<10^4$ pg/ml of each cytokine. Each individual solution was split into 3 aliquots. The first and the third aliquot were adjusted with HCl or NaOH solution to pH 5 and pH 8, respectively, whereas the second aliquot remained untreated with a pH value of 7 (i.e. pH value of RS/HA solution). Subsequently, 3 replicates of each of the 9 solutions were analysed and relative deviance of concentration of the acidic or basic pH samples from concentration of the sample with pH 7 was calculated (concentration recovery).

2.2. In vitro microdialysis system

All μD experiments were performed with an *in vitro* μD system shown in Fig. 1 previously developed to standardise conditions (e.g. heights, distances, lengths, temperature, etc.) for *in vitro* μD investigations (Simmel and Kloft, 2010). Three linear μD catheters (CMA 66/66 linear μD catheters, 30 mm membrane length, 100 kDa cut-off; M Dialysis AB, Johanneshov, Sweden) were fixed within a vial containing the catheter surrounding medium. This medium vial was tempered at the intended temperature and medium was stirred at 600–700 rpm. The inlet tubing of the '66 linear μD catheters' was connected to a syringe (BD Luer-Lok™ 1 ml syringe, made of polycarbonate) placed in the μD pump. Microperfusate and catheter surrounding medium consisted of a mixture of RS and HA (9/1 v/v, HA concentration 0.5% m/v). Before each experiment, catheters were rinsed with microperfusate and equilibrated in tempered surrounding medium for at least 60 min.

2.3. Adsorption of cytokines to the catheter components

Polyurethane tubings, 0.38 mm in diameter and 400 mm in length, were perfused with microperfusate containing high ($C_{IL\text{-}6}$ = 600 pg/mL, $C_{IL\text{-}8}$ = 500 pg/mL, $C_{IL\text{-}10}$ = 300 pg/mL, C_{TNF} = 800 pg/mL) and low ($C_{IL\text{-}6}$ = 100 pg/mL, $C_{IL\text{-}8}$ = 90 pg/mL, $C_{IL\text{-}10}$ = 40 pg/mL, C_{TNF} = 80 pg/mL) concentrations of the model cytokines. Following an initial perfusion at a FR of 2.0 μl/min for 4 intervals of 15 min (30 μL per sample), FR was switched to 0.5 μl/min and 3 aliquots were sampled in 60 min intervals (30 μL per sample).

In the second part of adsorption testing, 3 catheter surrounding medium vials were filled with 12 ml of medium containing 1000–4000 pg/ml of cytokines. Three CMA 66 catheter membranes and 100 mm tubings were placed in the solutions and equilibrated for 10 min at 37 °C. Afterwards, aliquots of the surrounding solutions were sampled. The set of adsorption samples was analysed and MFI (median fluorescence intensities) signals were compared with the ones of the microperfusate or medium samples, respectively.

2.4. Relationship between relative recovery of cytokines and flow rate at 25 °C and 37 °C

In vitro μD RR was determined employing FR of 0.3, 0.5, 1.0, 2.0 and 5.0 μl/min and catheter surrounding medium spiked with 10^3–10^4 pg/ml for each separately spiked cytokine. Recovery investigations were performed over 3 subsequent sampling intervals per FR at surrounding medium temperatures of 25 °C and 37 °C, respectively leading to microdialysate volumes of 30–60 μl. *In vitro* μD RR was calculated from medium and microdialysate concentrations:

$$\mathrm{RR}(\textit{in vitro}),\% = \frac{C_{\mu\mathrm{dialysate}} - C_{\mu\mathrm{perfusate}}}{C_{\mathrm{medium}} - C_{\mu\mathrm{perfusate}}} * 100 \quad (1)$$

As $C_{\mu\mathrm{perfusate}}$ = 0, RR simplifies to:

$$\mathrm{RR}(\textit{in vitro}),\% = \frac{C_{\mu\mathrm{dialysate}}}{C_{\mathrm{medium}}} * 100 \quad (2)$$

$C_{\mu\mathrm{dialysate}}$, $C_{\mu\mathrm{perfusate}}$ and C_{medium} represented the microdialysate, microperfusate or catheter surrounding medium concentration of the respective cytokine, respectively.