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Chemometrics-guided development of a cyclodextrin-modified micellar electrokinetic chromatography method with head-column field amplified sample stacking for the analysis of 5-lipoxygenase metabolites

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ABSTRACT

A new sensitive method using α-cyclodextrin-modified micellar electrokinetic chromatography has been developed to separate and quantify arachidonic acid metabolites of the lipoxygenase pathways in human polymorphonuclear leukocytes, i.e. leukotriene B_4, 6-*trans*-leukotriene B_4, 6-*trans*-12-*epi*-leukotriene B_4, 5(*S*)-hydroxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid, 12(*S*)-hydroxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid, and 15(*S*)-hydroxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid. The electrophoresis system was optimized with regard to the pH, boric acid, SDS and α-cyclodextrin concentration as well as separation voltage and temperature using a three level resolution IV fractional factorial design and a five level circumscribed central composite design. The resulting optimized conditions included 80 mM sodium borate buffer, pH 10.07, containing 16.6 mM sodium dodecyl sulfate, and 15 mM α-cyclodextrin, using a separation voltage of 12.5 kV at 23 °C. Sensitivity was enhanced employing head-column field amplified sample stacking which resulted in limits of quantification between 30 and 50 ng/mL and limits of detection between 10 and 17 ng/mL after solid phase extraction of the lipoxygenase products. The method was validated according to the recommendations of the International Conference on Harmonization and applied to the determination of the lipoxygenase metabolites in polymorphonuclear leukocytes upon stimulation with Ca^{2+}-ionophore A23187 and arachidonic acid. Robustness was confirmed using a three level resolution IV fractional factorial design. The novel method is suitable for the analysis of various arachidonic acid metabolites produced by cells and may be used for evaluation of lipoxygenase inhibitors.

1. Introduction

5-Lipoxygenase (5-LO) is the key enzyme in the biosynthesis of leukotrienes (LTs) [1]. The enzyme catalyzes the oxidation of arachidonic acid to the hydroperoxide 5(*S*)-hydroperoxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5(*S*)-HPETE), an intermediate that is further converted by 5-LO to the instable epoxide leukotriene A_4 (LTA_4). LTA_4 is either metabolized by LTA_4 hydrolase to leukotriene B_4 (LTB_4) or conjugated to glutathione by LTC_4 synthase to yield leukotriene C_4 (LTC_4). LTC_4 is subjected to elimination of glutamic acid by γ-glutamyltranspeptidase to LTD_4 and further to LTE_4 by a dipeptidase. Alternatively, LTA_4 can be hydrolyzed non-enzymatically to yield the diastereomers 6-trans leukotriene B_4 (6t-LTB_4) and 6-trans-12-epi leukotriene B_4 (6t,12e-LTB_4). A substantial amount of 5(*S*)-HPETE formed by the initial oxidation of arachidonic acid is released from 5-LO and subsequently converted by peroxidase(s) to 5(*S*)-hydroxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5(*S*)-HETE) [2]. In mammals, arachidonic acid is also a substrate for other lipoxygenases including platelet-type 12-lipoxygenase, 15-lipoxygenase-I and -II resulting in the formation of 12(*S*)-hydroxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (12(*S*)-HETE) and 15(*S*)-hydroxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (15(*S*)-HETE), respectively [3].

LTs are lipid mediators eliciting numerous inflammatory and immunological responses [4]. They have been implicated in the pathogenesis of human diseases including acute and chronic inflammatory diseases such as asthma, rheumatoid arthritis, psoriasis, nephritis, artherosclerosis and cancer [4,5]. This makes 5-LO and enzymes of the subsequent pathways attractive targets for pharmaceutical drug development. LTs are produced by pro-inflammatory cells such as polymorphonuclear leukocytes (PMNLs) or macrophages. These cells are relatively readily available and have been used in many studies on LT biosynthesis and in studies addressing the regulation of 5-LO.

Many methods have been published for the analysis of 5-LO products. Besides immunoassays [6], numerous chromatographic

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assays have been reported including HPLC with UV [7–10] or MS detection [11], UPLC-SRM/MS [12] or GC–MS [13,14]. With regard to electromigration methods, the capillary isotachophoretic separation of the cysteinyl-LTs C_4, D_4, and E_4, as well as prostaglandins and their metabolites has been published [15,16] and few research groups reported the separation of prostaglandins by capillary electrophoresis techniques [17–19]. Only one study investigated the separation of LTB_4 as well as the hydroxyeicosatetraenoic acid derivatives 5(*S*)-HETE, 12(*S*)-HETE and 15(*S*)-HETE using a MEKC method [20]. However, the method had a relatively high limit of quantitation in the range of 0.8–1.0 μg/mL and was not able to separate the diastereomers 6t-LTB_4 and LTB_4. It has been demonstrated that the diastereomers 6t-LTB_4 and 6t,12e-LTB_4 display differing effects on Ca^{2+} mobilization [21] so that a more selective assay is required to separate all LTB_4 epimers. Thus, the aim of this study was the development, validation and application of a sensitive capillary electrophoresis based method that allows the separation of all products of 5-LO including the diastereomers 6t-LTB_4 and LTB_4. Sample stacking was employed for increasing assay sensitivity.

2. Materials and methods

2.1. Chemicals

Prostaglandin B_1 (PGB_1), LTB_4, 6t-LTB_4, 6t,12e-LTB_4, 5(*S*)-HETE, 12(*S*)-HETE, and 15(*S*)-HETE were purchased from Biomol GmbH (Hamburg, Germany). α-CD, β-CD, γ-CD, carboxymethyl-α-CD (degree of substitution (DS) = 12), and carboxymethyl-β-CD (DS = 3) were obtained from Cyclolab Ltd. (Budapest, Hungary). Sulfated β-CD (DS = 7–11), sodium dodecylsulfate (SDS), Ca^{2+}-ionophore A23187 and arachidonic acid were from Sigma–Aldrich (Deisenhofen, Germany). 1 M sodium hydroxide was from Grüssing GmbH (Filsum, Germany). HPLC grade methanol was purchased from VWR International GmbH (Dresden, Germany). All other chemicals were obtained from commercial sources at the highest purity available and used without further purification. Water was purified with a Milli-Q Direct 8 system (Millipore, Schwalbach, Germany).

2.2. Capillary electrophoresis

Capillary electrophoresis was performed on a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Krefeld, Germany), equipped with a diode array detector and a sample tray temperature control system, using 50 μm inner diameter, 363 μM outer diameter fused-silica capillaries (BGB Analytik, Schloßböckelheim, Germany) with an effective length of 40 cm and a total length of 50.2 cm. New capillaries were rinsed with 0.1 M sodium hydroxide for 30 min, water for 15 min, and background electrolyte (BGE) for 15 min. Each day, the capillaries were rinsed with 0.1 M sodium hydroxide for 30 min, water for 5 min, and BGE for 5 min. At the end of each day, the capillaries were rinsed with 0.1 M phosphoric acid for 5 min, 0.1 M sodium hydroxide for 5 min, and water for 5 min, and stored in water. Between the analyses, the capillary was dipped in vials containing water for 10 s and rinsed for 6 min with 0.4 M sodium hydroxide, and 6 min with BGE. The optimized conditions consisted of a BGE composed of 80 mM sodium borate at pH 10.07, containing 16.6 mM SDS and 15 mM α-CD, an applied voltage of 12.5 kV and a separation temperature of 23 °C. UV detection was carried out at the cathodic end at 235 nm for the hydroxyeicosatetraenoic acid derivatives and at 280 nm for PGB_1 (used as internal standard) and the LTs, respectively. All solutions were filtered through 0.2 μm polyester membrane filters and sonicated for 3 min.

Sample solutions of the analytes were prepared in methanol. The total sample volume was 80 μL. The sample tray was cooled at 10 °C to prevent solvent evaporation. For the stacking procedure a plug of 90% methanol was injected for 60 s at 3.4 kPa into the BGE filled capillary followed by electrokinetic injection of the sample at −5 kV (reversed polarity) until 95% of the maximum current of −9.8 μA was reached. Subsequently, a voltage of 12.5 kV was applied for analyte separation.

2.3. Incubation of polymorphonuclear leukocytes

Human PMNL cells were isolated from fresh blood of adult, healthy female volunteers at the University Hospital, Jena, Germany, as described elsewhere [22]. The incubation of PMNL was performed using 5×10^6 cells as well as 2.5 μM Ca^{2+}-ionophore A23187 or 2.5 μM Ca^{2+}-ionophore A23187 and 20 μM arachidonic acid in presence of 1 mM $CaCl_2$ according to [23]. Briefly, cells were suspended in 1 mL phosphate-buffered saline (PBS), pH 7.4, containing glucose (1 g/L). Stimulation was achieved by addition of 5 μL of a solution of 0.5 mmol/L Ca^{2+}-ionophore A23187 in methanol or 5 μL of a solution containing 0.5 mmol/L Ca^{2+}-ionophore A23187 and 4 mmol/L arachidonic acid in methanol as well as 2.5 μL 0.4 mol/L $CaCl_2$ in water. The samples were vortexed and incubated at 37 °C for 10 min. The reaction was stopped by placing the samples on ice and by addition of 1 mL methanol, 0.5 mL PBS, 30 μL 1 M HCl and 10 μL internal standard solution containing 20 μg/mL PGB_1 in methanol. The samples were centrifuged for 10 min at $800 \times g$ and the supernatants were applied to endcapped C_{18} solid-phase extraction columns from UCT (Bristol, England) which were preconditioned with 1 mL methanol and 1 mL water. The columns were washed with 1 mL water and 1 mL 25% methanol (v/v). The analytes were eluted with 500 μL methanol and lyophilized. The residue was dissolved in 300 μL methanol.

2.4. Chemometric design

Modde 9.1 (Umetrics AB, Umeå, Sweden) was used for chemometric design and statistical analysis. For each model, the used factors $x_{1,\ldots,n}$ are fitted using a polynomial function:

$$y = b_i + b_1x_1 + b_2x_2 + b_{12}x_1x_2 + b_{11}x_1^2 + \cdots + \varepsilon$$

For each response y, the intercept b_i, the main coefficients $b_{1,\ldots,n}$, the interaction coefficients $b_{12,\ldots,(n-1)n}$, the quadratic coefficients $b_{11,\ldots,nn}$, and the residual ε are calculated. The goodness of fit (R^2 = (total sum of squares − sum of squares for residuals)/total sum of squares) and the goodness of prediction (Q^2 = 1 − (prediction residual sum of squares/total sum of squares)) were subsequently optimized, while non-significant combined terms and combined terms with deteriorating influence were removed from the equation. R^2 and Q^2 close to 1 describe an excellent model, $Q^2 > 0.5$ a good model and $Q^2 > 0.1$ indicates a significant model [24].

3. Results and discussion

3.1. Initial separation conditions

The structures of the LTs and hydroxyeicosatetraenoic acid derivatives are shown in Fig. 1. Prostaglandin B_1 (PGB_1) was selected as internal standard [8]. Due to the lipophilicity of the compounds and their comparable charge densities because of the similar structures, a MEKC system consisting of a borate buffer, pH 8.3, containing 12.5 mM SDS was initially used, which is comparable to a previously published method [20]. The three hydroxyeicosatetraenoic acid derivatives as well as the diastereomeric compounds 6t-LTB_4 and LTB_4 could be separated using an