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Development and validation of an extraction method for the determination of pro-inflammatory eicosanoids in human plasma using liquid chromatography–tandem mass spectrometry

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ABSTRACT

A simple method coupled to liquid chromatography–tandem mass spectrometry (LC–MS/MS) was developed and validated for the extraction of prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) from human plasma. The extraction protocol consisted of adding formic acid (10 μL) and acetonitrile (140 μL) to human plasma (50 μL) and further injection of the supernatant (25 μL) into the LC–MS/MS. The method was selective for PGE_2 and LTB_4 and the regression models, based on deuterated internal standards (30 ng mL^{-1} PGE_2-d_4 and 40 ng mL^{-1} LTB_4-d_4), were linear over the concentration range 1.0–50.0 ng mL^{-1} with limits of detection ($3 \times \sigma_{blank}$) and quantification ($6 \times \sigma_{blank}$) of 0.5 ng mL^{-1} and 1 ng mL^{-1} for both eicosanoids. The recovery ranges were 95.1–104.7% for PGE_2 and 86.4–103.2% for LTB_4. The developed method was successfully implemented on plasma samples from patients before and after exposure to certain anti-inflammatory treatment.

1. Introduction

It has been acknowledged that the number of patients suffering from chronic inflammatory disorder is increasing worldwide. A survey conducted in 16 European countries confirms that around 20% of adult Europeans suffer from moderate to severe intensity chronic pain which is seriously affecting the quality of their social and working lives [1]. High prevalence of chronic pain (53.7%) has been reported in Sweden, indicating considerable socioeconomic costs [2].

Arachidonic acid metabolites, such as prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) are generated through the cyclooxygenase and lipoxygenase pathways, respectively. The plasma levels of these eicosanoids in healthy and pathologic states have been extensively studied. Normal levels in plasma have been estimated to range between 0.8–846.0 pg mL^{-1} for PGE_2 [3–6] and 33.9–60.9 pg mL^{-1} for LTB_4 [7–9]. They have been recognized to play a major role in the pathogenicity of inflammatory processes and cancer and also in the activation of physiological responses such as fever, redness, swelling, pain, etc. The degree of inflammation and pharmacological response might be determined by the ratios of PGE_2 or LTB_4. For example, lower levels of PGE_2 in blood plasma and in gingival crevicular fluid after treatment were signs of improvement of periodontal diseases [4]. The inhibition of LTB_4 synthesis in a rat model by using bestatin, was associated with the reduction of oesophageal adenocarcinoma incidence [10]. Increased concentrations of PGE_2 and LTB_4 have been found in various types of cancer, including colon, lung, breast, head and neck, pancreatic and prostate cancer, and their presence is often associated with a poor prognosis [11–17]. Elevated plasma PGE_2 and LTB_4 have been reported in mice given a tobacco-specific carcinogen agent in drinking water [18]. Therefore, the development of analytical methods for the detection and quantification of PGE_2 and LTB_4 is of great interest as they play vital roles in a range of inflammatory pathologies and also it might help to monitor appropriate pharmacological therapies. In particular, health centres are in urgent need of simple and fast sample treatment methods and analytical techniques suitable for routine analysis in clinical investigations of eicosanoid biomarkers. In addition, dietary studies on the effect of omega-3 and omega-6 rich foods and oils, and also clinical intervention studies on the effect of specific fish oils on inflammatory conditions require a rapid and reliable

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extraction method for determining eicosanoids in plasma samples. Several analytical methods have been reported for the analysis of eicosanoids with different detection and quantification principles and among them enzyme- and radio-immunoassays are the most popular methods due to their sensitivity and inexpensiveness. The main disadvantages of these immunological assays are their lack of specificity for complex biological fluids (e.g. plasma and urine), metabolite overestimation due to cross-reactivity and limitation to a single metabolite at the time [19–21]. To overcome the drawbacks associated with immunological assays, chromatography techniques such as gas and liquid chromatography, coupled to mass spectrometry (GC–MS and LC–MS) have been proposed as reliable alternatives [21,22]. The literature on extraction and quantification of eicosanoids by LC–MS is generally focused on cell cultures [19,23–27], tissue [28], liver microsomes [29], seminal fluids [30], gastric mucosa [31], etc. In contrast, articles on plasma samples are less often reported [22], despite the fact that the levels of eicosanoids in this type of sample may be a useful index of pharmacological, physiological and pathological effects. In recent years, excellent articles on the quantification of single and multiple eicosanoids in plasma by LC–MS and extracted by means of solid phase extraction (SPE) [32–37], protein precipitation, solvent extraction [22,33,38–40] and derivatization [41] have been reported. It must be said that the attractiveness of a particular preparation protocol for plasma is generally assessed by the required sample volume and processing speed, while the instrumental technique by the quantitative figures of merits (e.g. limits of detection and quantification). In this respect, SPE and derivatization protocols require large volumes of sample, are time consuming and economically disadvantageous for routine analyses. Precipitation methods are not suitable for the detection of eicosanoids at low concentrations [42]. Solvent extraction efficiency is highly dependent on the polarity of the selected solvents (e.g. organic solvents are poor at extracting hydrophilic compounds such as tetranor-PGEM, tetranor-PGFM, and leukotrienes).

Rapid and cost-effective methods for extracting multiple eicosanoids from human plasma and subsequent quantification by LC–MS/MS represent important tasks for both research and routine clinical analysis. The present study aims at developing a streamlined method for extracting the pro-inflammatory eicosanoids PGE_2 and LTB_4 from human plasma and subsequent detection using LC–MS/MS. The development of such methods will assist the analysis of eicosanoids in research and routine studies and could also have the potential to become national or international benchmark references.

2. Experimental

2.1. Reagents

Prostaglandin E_2 (PGE_2, 99%), deuterated prostaglandin E_2 (PGE_2-d_4, 99%), leukotriene B_4 (LTB_4, 97%), deuterated leukotriene B_4 (LTB_4-d_4, 99%) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Acetonitrile (liquid chromatographic grade, 99.8%), formic acid (98%) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Methanol (liquid chromatographic grade, 99.9%) was from Merck (Darmstadt, Germany) and isopropanol (100%) obtained from Kemetyl Norge (Vestby, Norway). A Millipore Milli-Q water system (Millipore, Milford, USA) was used.

2.2. Plasma samples

A blank plasma sample was drawn from a fasting and healthy female. Ten plasma samples were drawn from fasting patients suffering from different inflammatory conditions, and thirty plasma samples were from a nutritional intervention study involving salmon fish and vitamin D (kindly donated by Dr. Ingvild E. Graff). Indomethacin was added to all the samples to inhibit the in vitro synthesis of eicosanoids. The samples were kept at −80 °C prior to extraction and analysis by LC–MS/MS.

The samples were collected after approval by the local ethics committee and written informed consent.

2.3. Selection of the optimal extraction solvents

The selection of the optimal extraction system consisted of sequentially adding pure solvents (formic acid, acetonitrile, methanol and water) to 100 μL of blank plasma spiked with equal concentrations of PGE_2 and LTB_4 (100 ng mL^{-1} of each). The proportions of the solvents were estimated using an augmented lattice design (Fig. 1) and the total volume was always 400 μL. Every solvent addition was followed by vortex-mixing (1 min) and after completing the addition of the three solvents the system was centrifuged (10 min) and the supernatant collected. The monitored responses were clarity of the supernatant, precipitation in the collected supernatant after adding subsequent aliquots of acetonitrile (4×100 μL) and the extracted ion chromatogram intensities for PGE_2 and LTB_4. Injection of the supernatant into the LC–MS system was performed only after confirming lack of precipitation with acetonitrile. The results of the solvent optimization are presented in Section 3.1.

2.4. Selection of the optimal concentrations of internal standards

As will be shown in Section 3.1, the preferred extraction system consisted of consecutive addition of formic acid (10 μL) and acetonitrile (140 μL spiked with the internal standards) to 50 μL of blank plasma (spiked with the analytes). The appropriate concentrations of analytes and internal standards and the calculation of their associated response factors were found using a Doehlert design strategy described elsewhere [27,38,43], employing five levels for the analyte concentrations and three for the internal standard concentrations.

The general protocol for selecting appropriate amounts of internal standards was as follows: aliquots of a stock blank plasma sample spiked with equal concentrations of PGE_2 and LTB_4 (500 ng mL^{-1}) were taken and diluted with blank plasma to produce five different analytical levels (1.0, 13.5, 25.0, 37.5 and 50.0 ng mL^{-1}). Seven extractions (in triplicate), arranged as indicated in Table 1 (I → VII), were performed by using 50 μL of the described five plasma solutions (1.0, 13.5, 25.0, 37.5 and 50.0 ng mL^{-1}), 10 μL of formic acid and 140 μL of acetonitrile containing equal amounts of PGE_2-d_4 and LTB_4-d_4 at three different concentration levels (3.4, 25.0 and 47.5 ng mL^{-1}). The analytical response factors at the seven experimental extractions points (Table 1) were calculated and modelled as described elsewhere [27,38,43].

2.5. Analytical validation

The developed extraction method in conjunction with the optimal amounts of internal standards was submitted to a validation protocol. The evaluated performance parameters were selectivity, range, recovery, precision, limit of detection (LOD), limit of quantification (LOQ) and dilution integrity.

The selectivity was assessed by using spiked (between 1.0 and 50.0 ng mL^{-1}) and unspiked blank plasma samples. The PGE_2, LTB_4, PGE_2-d_4 and LTB_4-d_4 extracted ion chromatograms from both, spiked and unspiked samples were compared to establish whether or not the developed extraction