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Development of a reliable analytical method to determine lipid peroxidation biomarkers in newborn plasma samples

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ABSTRACT

This paper describes a reliable analytical method based on ultra-performance liquid chromatography coupled to tandem mass spectrometry to determine F2-isoprostanes and other total byproducts (isoprostanes, isofurans, neuroprostanes and neurofurans) as lipid peroxidation biomarkers in newborn plasma samples. The proposed procedure is characterized by a simple sample treatment employing a reduced sample volume (100 μL). Also, it shows a high throughput and high selectivity to determine simultaneously different isoprostane isomers in a large number of samples. The reliability of the described method was demonstrated by analysis of spiked plasma samples, obtaining recoveries between 70% and 130% for most of the analytes. Taking into account the implementation of further clinical studies, it was demonstrated the proper sensitivity of the method by means of the analysis of few human newborn plasma samples.

In addition to this, newborn piglet plasma samples ($n=80$) were analyzed observing that the developed method was suitable to determine the analyte levels present in this kind of samples. Therefore, this analytical method could be applied in further clinical research about establishment of reliable lipid peroxidation biomarkers employing this experimental model.

1. Introduction

The isoprostanes (F2-isoP) are considered as the most reliable and sensitive markers of oxidative stress *in vivo* [1]. Other oxidation products were described by Roberts et al. [2], namely neuroprostanes (F4-NeuroPs). However, under hyperoxia conditions, isofurans (IsoFs) and neurofurans (NeuroFs) generation increases limiting the formation of F2-isoPs and F4-NeuroPs, respectively. All these compounds are stable products obtained from the arachidonic acid and docosahexaenoic acid peroxidation [2,3]. So, they are considered lipid peroxidation biomarkers.

Elevated levels of F2-isoPs, IsoFs, F4-NeuroPs and NeuroFs have been observed under different conditions related to increase of reactive oxygen species [4]. Therefore, the measurement of their concentrations in biological samples is an important tool to evaluate the role of oxidative stress in the pathogenesis of several human diseases [5,6]. In the perinatal field, the oxidative stress associated with physiopathological changes in several neonatal diseases (bronchopulmonary dysplasia, retinopathy of prematurity, hypoxic/ischemic encephalopathy) has been widely studied [7]. Among these pathologies and taking into account the high content of lipids in brain, the lipid peroxidation biomarkers could identify patients at risk of neuronal damage and subsequently encephalopathy development, as consequence of prolonged hypoxic–ischemic events [8,9]. So, it is very important to determine these biomarkers in newborn samples in order to initiate early therapeutic treatments [10].

Since in neonatology it is very important to develop a non-invasive diagnosis method, urine samples were the preferred kind of samples. In fact, a previous work described the F2-isoPs determination in neonate urine samples establishing reference ranges for extremely low gestational age neonates and term neonates, respectively [11]. In addition to this, the relative total contents of IsoPs, IsoFs, NeuroPs and NeuroFs was studied in urine samples from preterm infants and a comparison between infants who did not develop free radical associated conditions and patients who developed bronchopulmonary dysplasia was established [12]. The main drawback of urine analysis is the long-term after injury (several days) required to detect metabolic differences and

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diagnosis establishing [13]. As a consequence, treatment initiation would be delayed.

For this reason, other kinds of samples (blood, cerebrospinal fluid, and liquid amniotic) are studied in order to enhance an early diagnosis [14–16]. For these samples, the use of small volumes is a mandatory requirement in neonatology. Recently, we have developed a reliable analytical method to determine lipid peroxidation biomarkers in serum, and it was applied to the analysis of samples from severely depressed newborn infants [17].

In general, the clinical routine is more frequent to collect plasma than serum samples, it can be explained by some of the advantages of plasma over serum (greater yield from blood samples, no delayed clotting, less risk of hemolysis…) [18]. In addition to this, the coagulation cascade can affect the metabolites concentrations in serum samples, as well as potential interferences due to post centrifugal coagulation can be observed in serum but not in plasma samples [19]. Also, in previous works better reproducibility was obtained for plasma than for serum samples [20]. Nevertheless, few analytical methods applied to newborn plasma samples can be found in literature, and most of them just evaluate the utility of 8-iso-PGF2α as an ideal lipid peroxidation biomarker [21,22]. Moreover, these methods are characterized by complex sample treatment procedures and long analysis time [23]. So, the development of reliable analytical methods to determine several F2-IsoP isomers in newborn plasma samples would be required.

As regards plasma sample treatment, since F2-isoPs are mostly esterified, these samples required alkaline hydrolysis in order to break the protein-isoP adducts and determine total F2-isoPs (free+de novo released) [24]. Other approaches required were clean-up, preconcentration and derivatization steps [25–27]. The levels found in cord plasma samples were between 0.07 and 0.12 nmol L^{-1} depending on the delivery mode [26]. In addition to this, total F2-isoP levels found in term newborns were significantly lower than those found in preterm newborns. This fact could be explained by the lower levels of antioxidants in preterm newborns [10]. Specifically, Mestan et al. observed that elevated cord-blood 8-isoprostane concentration was associated with extremely preterm birth [14].

The aim of this work is to develop a reliable and sensitive method for the determination of a large number of F2-isoPs and other total peroxidation byproducts in newborn plasma samples, which are the most employed in early diagnosis studies. For this purpose, we will use small sample volume, simple sample treatment and UPLC–MS/MS. To our knowledge this is the first validated liquid chromatographic method to determine simultaneously several F2-isoPs in small volume of plasma samples. It will be applied first to an experimental model to establish potential biomarkers of neonatal encephalopathy, and then to evaluate newborn infants with encephalopathy in the frame of a multicenter clinical trial.

2. Materials and methods

2.1. Plasma collection and storage

Plasma samples from newborn piglets ($n=80$) were obtained at the Department of Pediatric Research and Institute of Surgical Research, Oslo University Hospital Rikshospitalet. National Animal Research Authority (NARA) approved the experimental protocol and the animals were cared for and handled in accordance with the European Guidelines for Use of Experimental Animals by certified FELASA fellows (Federation of European Laboratory Animals Science Association). The peripheral blood samples were collected in heparine containing cryo-tubes. Finally, the samples were centrifuged at 2000*g* for 10 min at room temperature, and the supernatant (plasma) was separated and aliquoted. Thereafter, samples were stored at −80 °C until analysis.

Plasma samples from newborn infants ($n=4$) were obtained at the Department of Neonatology, University and Polytechnic Hospital La Fe. The cord blood samples were collected in heparinized syringes. They are processed within 30 min from the extraction. First, they are centrifuged at 1500*g* for 10 min at room temperature. The supernatant (plasma) is separated and centrifuged at 2500*g* for 15 min in order to remove most of the platelets contained in the plasma samples. Then, the plasma samples are aliquoted and stored at −80 °C until analysis.

2.2. Reagents

Isoprostane standards of 8-iso-15(R)-PGF2α, 1a1b-dihomo-PGF2α, 2,3-dinor-iPF2α-III, 8-iso-15-keto-PGE2, 8-iso-15-keto-PGF2α, 8-iso-PGE2, 5-iPF2α-VI, 8-iso-PGF2α, the prostaglandins PGE2 and PGF2α, as well as the deuterated internal standard (IS) PGF2α-D4 were purchased from Cayman Chemical Company (Ann Arbor, Michigan, USA). Potassium hydroxide was from Sigma Aldrich Química SA (Madrid, Spain). Methanol (LC-MS grade) and n-heptane (analytical grade) were obtained from J.T. Baker (Avantor Performance Materials B.V., The Netherlands). Formic acid (98%) and ethyl acetate (analytical grade) were from Panreac (Barcelona, Spain).

2.3. Solutions

Individual stock solutions of 8-iso-15-keto-PGE2, 8-iso-15-keto-PGF2α, PGE2, 8-iso-PGE2, 8-iso-PGF2α, PGF2α and PGF2α-D4 standards at a concentration of 1 mmol L^{-1} were prepared in H_2O (pH 3, 0.1% v/v HCOOH):CH_3OH (85:15 v/v) and kept at −20 °C. Stock solutions of 8-iso-15(R)-PGF2α, 1a1b-dihomo-PGF2α, 2,3-dinor-iPF2α-III and 5-iPF2α–VI standards were purchased in methyl acetate or ethanol, at concentrations of 2800, 26,000, 300 and 300 μmol L^{-1}, respectively. A 5 μmol L^{-1} working solution of the set of target analytes was prepared in H_2O (0.1% v/v HCOOH):CH_3OH (85:15 v/v) by mixing appropriate volumes of the above mentioned individual solutions, and kept at −20 °C. The calibration curves were prepared daily by serial dilution in H_2O (0.1% v/v HCOOH):CH_3OH (85:15 v/v) at concentrations ranging from 5 μmol L^{-1} to 2 nmol L^{-1} of each analyte. Potassium hydroxide solution was prepared at 15% (w/v).

2.4. Materials

SPE-96 well plates (Discovery® DSC-18, 100 mg) were used for sample solid-phase extraction (SPE) (Sigma-Aldrich, St. Louis, MO, USA). Ultrasonic bath (Bandelin Sonorex Digitec, Berlin, Germany) was used to improve the hydrolysis efficiency. Vortex mixer was from VelpScientifica (Usmate, Italy). Centrifuge Biocen22R was from OrtoAlresa (Madrid, Spain). Thermomixer HLC from Ditabis (Pforzheim, Germany). Speed vacuum concentrator (mi Vac) was from Genevac LTD (Ipswich, United Kingdom). 96-well sample plates (Acquity UPLC 700 μL) from Waters (Barcelona, Spain).

2.5. UPLC/MS/MS chromatographic system

The chromatographic system used consisted of a Waters Acquity UPLC-Xevo TQDsystem (Milford, MA, USA). The conditions employed were optimized in a previous work [17]. Briefly, negative electrospray ionization (ESI^-), capillary voltage 3.5 kV, source temperature 120 °C, desolvation temperature 300 °C, nitrogen cone and desolvation gas flows were 25 and 680 L h^{-1}, respectively. Dwell time was set to 5 ms. Separation conditions were