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Ultra high performance liquid chromatography coupled to tandem mass spectrometry determination of lipid peroxidation biomarkers in newborn serum samples

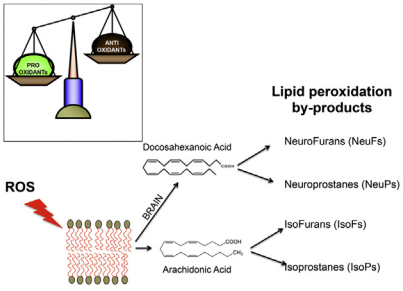
C. Cháfer-Pericás [a], L. Rahkonen [c], A. Sánchez-Illana [a], J. Kuligowski [a], I. Torres-Cuevas [a], M. Cernada [b], E. Cubells [b], A. Nuñez-Ramiro [b], S. Andersson [d], M. Vento [a, b], J. Escobar [a, *]

[a] Neonatal Research Group, Health Research Institute La Fe, Valencia, Spain
[b] Division of Neonatology, University & Polytechnic Hospital La Fe, Valencia, Spain
[c] Department of Obstetrics and Gynecology, University of Helsinki, Helsinki University Hospital, Helsinki, Finland
[d] Children's Hospital, University of Helsinki, Helsinki University Hospital, Helsinki, Finland

HIGHLIGHTS

- A validated liquid chromatographic method to determine peroxidation lipid biomarkers.
- Serum samples from severely depressed newborn infants (Apgar score 1 min <3; arterial cord pH <7.00).
- Small sample volume and simple sample treatment.
- High throughput of sample analysis and high selectivity for different isoprostanes isomers.

GRAPHICAL ABSTRACT



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ABSTRACT

Byproducts of arachidonic (AA) and docosahexaenoic acid (DHA) oxidation are highly relevant for the study of free radical associated conditions in the perinatal period. Plasma metabolites can provide the clinician with a snapshot of the oxidant status of patients before and after specific clinical interventions (e.g.: supplementation with oxygen). We describe a new andreliable ultra-performance liquid mass spectrometry method to determine F2-isoprostanes and other byproducts (isoprostanes, isofurans, neuroprostanes, neurofurans) in newborn serum samples. Cord blood samples were obtained from severely depressed newborn infants (Apgar score 1 min < 3; arterial cord pH < 7.00), and aliquoted for serum determination and stored at −80 °C. A UHPLC-MS/MS method was employed. It has a series of technical advantages: simple sample treatment; reduced sample volume (100 μL) which is essential for preterm neonates with low circulating blood volume, high throughput of sample analysis (96 samples in less than 24 h) and high selectivity for different isoprostanes isomers. Excellent sensitivity was achieved within limits of detection between 0.06 and 4.2 nmol L^{-1}, which renders this method suitable to monitoranalyte concentration in newborn samples. The method's precision was satisfactory; with coefficients of variation around 5–12% (intra-day) and 7–17% (inter-day). The reliability of the described method was assessed by analysis of spiked serum samples obtaining recoveries between 70% and 120%. The proposed method has rendered suitable for serum determination for newborn babies at risk of oxygen free radical associated conditions.

* Corresponding author. Neonatal Research Group Laboratory, Health Research Institute La Fe, Avenida Fernando Abril Martorell 106, E46026 Valencia, Spain. Tel.: +34 961 24 66 61; fax: +34 961 24 66 20.
E-mail address: justo.escobar@uv.es (J. Escobar).

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1. Introduction

Isoprostanes (isoPs) are stable products resulting of a non-enzymatic oxidation of polyunsaturated fatty acids (PUFAS). The isoprostanes obtained from the arachidonic acid (AA) peroxidation are called F2-isoPs. They are considered the most reliable markers of oxidative stress *in vivo* [1]. Actually, elevated levels of F2-isoPs have been detected in different conditions associated to an increase of reactive oxygen species (ROS) [2]. Therefore, measurement of their concentrations in biological samples is considered an important tool to evaluate the role of oxidative processes in the pathogenesis of human diseases and response to specific therapies [3].

Remarkably, oxidative stress is associated with the pathophysiology of the so-called free radical associated conditions of the neonatal period such as bronchopulmonary dysplasia (BPD), retinopathy of prematurity (ROP), hypoxic/ischemic encephalopathy (HIE) among others [4]. Fetal to neonatal transition causes the generation of a burst of ROS, and oxygen supplementation is the mainstay of the therapy for many of these conditions. In addition, neonates are endowed with an immature antioxidant defense system [5]. Therefore, the determination of reliable biomarkers of free radical associate conditions such as isoprostanes renders essential for diagnosis and therapy in the neonatal period [6].

In previous studies, the utility of 8-iso-$PGF_{2\alpha}$ as a suitable biomarker for determining oxidative reactions *in vivo* was been confirmed [7]. F2-IsoPs are commonly measured in urine, plasma and serum samples [8,9]. Since, isoprostanes in plasma and serum are generally esterified, these samples required alkaline hydrolysis in order to break the protein-isoPs adducts and determine total IsoPs (free + *de novo* released) [10]. Furthermore, it has been observed that hemolysis occurring in plasma samples could compromise the reliability of F2-isoPs determinations [11]. To avoid this inconvenient, several cleanup strategies such as solid phase extraction (SPE) combinations [12], liquid–liquid extraction (LLE) [13], and the use of affinity columns [14] were developed.

Sensitive and selective analytical methods have been developed to determine F2-isoPs in biological samples. The majority of the methods that have been described are based on gas chromatography (GC) coupled to mass spectrometry (MS) [15]. However, GC-MS methods have some disadvantages such as tedious and time-consuming derivatization to achieve volatile compounds, as well as low capacity to identify different F2-isoPs' isomers. Most of them require SPE, LLE as well as thin layer chromatography and immune-affinity chromatography for the purification of the samples and to achieve highly specific determination of isoprostanes [11,16]. Moreover, in previous GC-MS/MS developed methods, large sample volumes were needed [17].

Immunoassays using the ELISA methodology have also been employed. The main drawback of commercially available ELISA kits is the poor selectivity, with cross-reactivity between 5 and 20% [18]. Each assay kit can measure just one isomer of F2-isoprostanes, and the results obtained are not comparable to chromatographic results [19].

In contrast, the employment of liquid chromatography (LC) coupled to mass spectrometry (LC-MS/MS) provides high sensitivity and specificity, and it allows the simultaneous measurement of different isomers of F2-isoPs. However, it has yet received limited attention, and few LC methods have been applied to human biological samples [20]. These methods are characterized by employing LLE sample treatment approach, high retention times, and long chromatograms [10,13].

We have recently described an LC-MS/MS method to determine isoprostanes in newborn urine samples, and have established reference ranges for lipid peroxidation byproducts in this biofluid [21].

Previously validated methods are characterized by employing large sample volumes, single analyte determination and long chromatograms [22]. Regarding methods to determine F2-isoP in serum samples, no validated LC based method was found in literature. Only in one previous work free isoprostanes were determined in children's serum samples in order to establish correlation with oxidative damage and cognition deficits [23].

The concentrations found in literature for total esterified human plasma F2-isoPs were between 0.11 and 24 nmol L^{-1} [23,24]. Particularly, the levels of 8-iso-$PGF_{2\alpha}$were between 0.11 and 0.5 nmol L^{-1} for healthy subjects [22]. In newborns, 15-F(2t)-isoprostane cord plasma levels were between 0.07 and 0.12 nmol L^{-1} depending on the delivery mode [25], total F2-isoPs were significantly higher for term (0.73 nmol L^{-1}) and especially preterm (1.02 nmol L^{-1}) newborns comparing with adults (0.52 nmol L^{-1}). Total F2-isoP significantly decreased with advancing gestational age, it could be due to the higher levels of pro-oxidants and/or the lower levels of antioxidants in newborns [26]. Specifically, Mestan et al. observed that elevated cord-blood 8-isoprostane concentration was associated with extremely preterm birth. This high concentration was also associated with decreasing birth weight, clinical chorioamnionitis, fetal inflammatory response of the placenta, and signs of perinatal depression [27]. The limit of detection obtained in this work for 8-isoprostane by using enzymatic immunoassay was 0.01 nmol L^{-1}. In addition to this, a previous work did not find correlation between plasma isoprostanes in mothers and newborns [28]. All these results suggest that some form of lipid peroxidation is active in the fetus.

The aim of this work was to develop a reliable and sensitive method for the determination of a large number of F2-isoPs and other total peroxidation byproducts in newborn serum samples. For this purpose, we used small sample volumes, simple sample treatment and UHPLC-MS/MS. To our knowledge this is the first LC validated method developed to determine F2-isoPs in newborn serum samples.

2. Materials and methods

2.1. Serum collection and storage

Serum samples were obtained from whole cord blood of infants born in the Helsinki University Central Hospital (Finland). All infants were born at gestational age between 37 and 42 weeks and had Apgar scores at 1 min < 3. The study was approved by the Ethics Committee of the University Central Hospital of Helsinki, and informed consent was obtained from the parents. The blood samples were collected in a dry cryotube. The tube was held in vertical position and at room temperature to improve the coagulation during 30 min after the extraction. Then, the sample was centrifuged at 2000 g for 10 min at room temperature, and the supernatant (serum) was separated and aliquoted. Thereafter, samples were stored at −80 °C until analysis.

2.2. Reagents

Isoprostanes' standards of 8-iso-15(R)-$PGF_{2\alpha}$, 1a,1b-dihomo-$PGF_{2\alpha}$, 2,3-dinor-i$PF_{2\alpha}$-III, 8-iso-15-keto-PGE_2, 8-iso-15-keto-$PGF_{2\alpha}$, 8-iso-PGE_2, 5-i$PF_{2\alpha}$-VI, 8-iso-$PGF_{2\alpha}$, the prostaglandins PGE_2 and $PGF_{2\alpha}$, as well as the deuterated internal standard (IS) $PGF_{2\alpha}$-D_4 were purchased from Cayman Chemical Company (Ann Arbor, Michigan, USA). Other total peroxidation byproducts (isoprostanes, isofurans, neuroprostanes and neurofurans) were not commercially available. In a previous work we generated them from the oxidation of arachidonic acid and docosahexanoic acid in order to charac-