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Optimisation of an extraction method for the determination of prostaglandin E_2 in plasma using experimental design and liquid chromatography tandem mass spectrometry

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Abstract

A new extraction method has been developed for the extraction of prostaglandin E_2 (PGE_2) from human plasma of patients suffering chronic inflammatory disorders. The extraction solvents were optimised systematically and simultaneously by using a central composite design. The optimised method involves precipitation of the protein fraction, centrifugation, evaporation and dissolution of the supernatant in the mobile phase, screening to confirm the presence of the analyte, and quantification of the positive samples by liquid chromatography tandem ion-trap mass spectrometry. Tandem mass spectrometry in negative mode was performed by isolating and fragmenting the ion $[PGE_2\text{-H}]^-$ signal *m*/*z* 351. Identification and quantification was carried out by extracting the ion fragment chromatograms at 333, 315 and 271 *m*/*z*. The quantitative determination was linear for the low nanogram (1–50 ng/ml) and upper picogram (400–1000 pg/ml) range studied, using 15 and 0.5 ng/ml of internal standard, respectively. The lower limit of detection was 2.5 pg for an injection volume of 25 μl. The optimised extraction method showed high reproducibility (coefficients of variation < 4%) and recovery values, estimated from standard addition experiments, ranging from 96 to 98%.
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Keywords: Prostaglandin E_2; LCMS; Experimental design; Central composite design; Ion-trap mass spectrometry; Plasma extraction method

1. Introduction

Prostaglandins are members of the lipid class of biochemicals derived from arachidonic acid by means of the cyclooxygenase enzyme. These substances are known for their potency and varying physiological properties and pathological effects [1,2]. Prostaglandin E_2 (PGE_2), one of the most widely studied prostaglandins, has bronchodilator action [3], promotes renal natriuresis and diuresis [4,5], increases motility and secretions in the gastrointestinal tract [6] and protects the brain cells from stroke damage [7], but it is also associated with inflammation [8,9], pain [10,11], blood vessels constriction [12], blood clotting promotion [13], progression and metastases of a variety of animal and human tumours including breast, lung, and colon [14,15]. Due to their positive–negative health effect dichotomy prostaglandins have been of significant interest over the last several decades and attracted the attention of pharmaceutical manufacturers and nowadays there are many prostaglandins available commercially. Thus, qualitative and quantitative analysis of prostaglandins may be a useful index of pharmacological, physiological and pathological effects. Immunological assays (radioimmunoassay or enzyme immunoassay) are the most widely used methods for the estimation of prostaglandins due to their inherent sensitivity, inexpensiveness and simplicity. The main drawbacks of these assays are their lack of specificity for complex biological fluids, such as plasma and urine [16], trend to overestimate the levels of metabolites due to cross-reactivity, variability in the quantification of sequential samples and limitation to the detection of a single product at the time [17,18]. Single or coupled instrumental techniques, such as mass spectrometry (MS), liquid (LC) and gas (GC) chromatography, GCMS and LCMS are suitable and accepted alternatives to immunoassays in the analysis of prostaglandins [18]. Determination of prostaglandins in biological samples using single or coupled chromatography techniques has been carried out by means of a wide variety of analytical procedures, such as derivatisation, degradation, solid-phase or liquid extraction, thin layer chromatography and combination of all these procedures.

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Thus, following the rapid development of analytical techniques, there has been a growing trend in prostaglandin analysis in biological fluids towards faster extraction time, development of novel or simpler methods and improvement of the already existing methods in terms of simplicity, routine analysis, improved quantification and automation. Articles regarding the extraction and determination of prostaglandins in plasma using LCMS are not widespread; the current literature is focused on samples, such as cultured cell lines [17,19–21], renal tissue [22], liver microsomes [23], seminal fluids [24], gastric mucosa [25], etc. Recently, a simple and rapid extraction procedure based on the precipitation of the protein fraction of human plasma samples spiked with PGE_2 and subsequent LC ion-trap MS analysis of the supernatant has been proposed [26] but a glaring error, that is spiking the samples with PGE_2, standard and internal standards after the precipitation step has taken place, precludes any further conclusion on the accuracy and precision of this particular method. An extraction method which requires 1 h to derivatise the prostaglandins completely prior to LCMS quantification in a high concentration calibration range (30–3000 ng/ml) has been reported [27]. The aim of this study was to develop a rapid, simple and efficient method for the extraction of PGE_2 from human plasma samples and subsequent screening and quantification in the low nanogram range by using LC ion-trap MS/MS and experimental design. Screening methods based on LCMS/MS instrumentation are useful because they provide greater analytical efficiency and allow to discriminate endogenous levels (<0.012 ng/ml PGE_2, no detectable by LCMS) from pathological levels. This allows the analytical effort to focus on quantification of the positive samples.

2. Experimental

2.1. Reagents

Prostaglandin E_2 and deuterated prostaglandin E2 (PGE_2-d_4) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Acetonitrile and methanol were from Merck (Darmstadt, Germany). De-ionized water was purified in a Milli-Q system (Milli-Q system Millipore, Milford, MA).

2.2. Plasma samples

Plasma samples were drawn from fasting patients suffering from chronic inflammatory disorders and under treatment with pharmacological medication supplemented either with omega-3 polyunsaturated fatty acids (ω-3 PUFAs) from seal oil or ω-6 PUFAs from soy oil. Plasma samples were kept at −80 °C prior to analysis.

2.3. Extraction procedure optimisation design

Methanol, water and acetonitrile were the extraction solvents used in this work. Their selection was based on preliminary extraction experiments, using the Bligh and Dyer procedure [28] to extract PGE_2 from spiked plasma samples. The analyte was not detected in the lower phase (chloroform). After precipitating

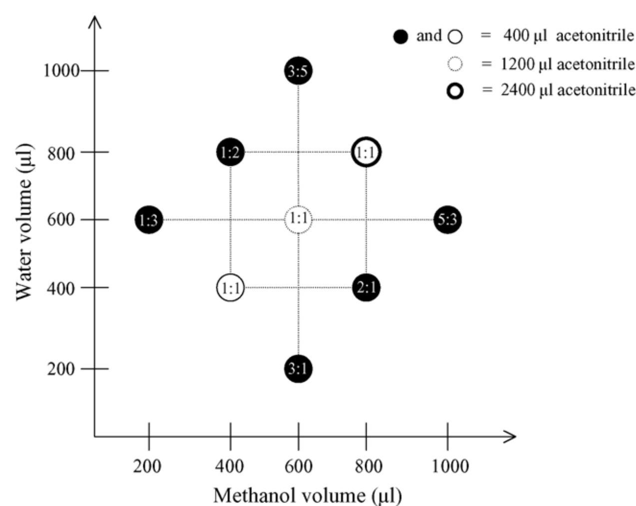

Fig. 1. Central composite design used in the optimisation of the extraction solvents.

the white cloud of protein micelles formed in the upper phase (methanol–water) with acetonitrile, the supernatant revealed the presence of PGE_2. A central composite experimental design [29] was used to optimise the proportions of these extraction solvents. The experiments were arranged according to Fig. 1 and the studied concentrations of PGE_2 standard dissolved in blank plasma were 30 and 0.5 ng/ml. The total number of experiments suggested by a central composite design is calculated according to the expression $2^x + 2x + 1$ where x represents the number of variables (methanol, water and acetonitrile). A total of 15 experiments are required ($2^3 + 2 \times 3 + 1$) in the present study, however it is important to note that the methanol and water proportions have been chosen purposely in order to bring about a region where the methanol:water (v/v) ratio remains constant (white circles in Fig. 1). In this way it is possible to include in this constant region a third factor, namely the influence of the amount of acetonitrile and optimise the 3 variables with 9 instead of the compulsory 15 experiments.

The optimisation strategy of the extraction procedure is as follows: Nine test tubes containing 200 μl of PGE_2 30 ng/ml in acetonitrile standard solution were taken and evaporated to dryness under a stream of nitrogen at room temperature. Aliquots of 200 μl of human plasma, drawn from a healthy patient with not detectable levels of PGE_2, were added into the test tubes and vortex-mixed for 2 min. Nine mixtures of methanol and water (I) were prepared according to the central composite design showed in Fig. 1. The data inside the circles in Fig. 1 represent the different methanol:water (v/v) ratios. Portions of 400 μl were taken from 6 of the I mixtures (indicated in Fig. 1 as black circles) and delivered into six of the nine test tubes containing 30 ng/ml PGE_2 dissolved in plasma and vortex-mixed for 30 s. Aliquots of 400 μl of acetonitrile (II) were added into these tubes followed by further 400 μl of the aforesaid six mixtures of I and vortex-mixed for 30 s after each addition. The addition sequence I → II → I and vortex-mixing for 30 s after each addition, is repeated on the remaining three test tubes containing the PGE_2 standard dissolved in plasma using 400 μl of I (white circles in Fig. 1 at constant methanol:water (v/v) ratios) and variable

amounts of II (400, 1200, 2400 μl). After addition of the solvents, the test tubes were centrifuged at 3000 rpm for 10 min at room temperature and the supernatants collected and evaporated to dryness under a stream of nitrogen at room temperature. The dried samples are reconstituted in 30 μl of acetonitrile, sonicated for 30 s, transferred to autosampler vials and submitted to LCMS/MS analysis.

The procedure described above was also applied to nine samples of blank plasma spiked with 0.5 ng/ml of PGE_2.

2.4. Screening of PGE_2 in plasma samples

Under optimal extraction conditions the procedure described above, without the spiking step, was applied on plasma samples ($n = 6$) from patients suffering chronic inflammatory disorders. The samples were submitted to LCMS/MS and the screening carried out by fragmenting the ion $[PGE_2\text{-H}]^-$ signal m/z 351 and identifying the characteristic product ions $[PGE_2\text{-}H_2O\text{-H}]^-$, $[PGE_2\text{-2}H_2O\text{-H}]^-$ and $[PGE_2\text{-2}H_2O\text{-44-H}]^-$ at 333, 315 and 271 m/z, respectively.

2.5. Calibration curves

Two calibration ranges 0–1 ng/ml (low range) and 1–50 ng/ml (high range) were studied. A plasma sample from a healthy patient was spiked with different PGE_2 and PGE_2-d_4 concentrations as is described above in order to construct the calibration curves. Six equally spaced PGE_2 levels were prepared in the low (0, 0.2, 0.4, 0.6, 0.8, 1 ng/ml) and high (1, 10, 20, 30, 40, 50 ng/ml) range using 0.5 and 15 ng/ml of PGE_2-d_4, respectively.

2.6. Plasma samples quantification

Positive samples from the screening step were spiked with PGE_2-d_4, treated according to the described extraction procedure and submitted to LCMS/MS quantification analysis.

2.7. Liquid chromatography ion-trap mass spectrometry

The LCITMS used in this study was an Agilent 1100 series LC/MSD trap, SL model with an electrospray interface (ESI), a quaternary pump, degasser, autosampler, thermostatted column compartment, variable-wavelength UV detector and 25 μl injection volume. The column used a Zorbax Eclipse-C_8 RP 150 mm × 4.6 mm, 5 μm (Agilent Technologies. Palo Alto, CA, USA) was kept in the column compartment at 40 °C. The solvent system operated in isocratic mode was acetonitrile with formic acid 0.1% (v/v) and UV detection at 254 nm. Nitrogen was used as nebulizing and drying gas at 350 °C. The ESI source was operated in negative ion mode and the ion optics responsible for getting the ions in the ion-trap, such as capillary exit, skimmer, lens and octapoles voltages were controlled by using the Smart View option with a resolution of 13,000 m/z/s (FWHM/m/z = 0.6–0.7). Complete system control, data acquisition and processing were done using the ChemStation for LC/MSD version 4.2 from Agilent. The transitions monitored were m/z 351 → 333, 315, 271 for PGE_2, m/z 355 → 337, 319, 275 for PGE_2-d_4.

2.8. Statistics

Data were expressed as mean values and relative standard deviations. The statistical analysis was done by Statgraphics Plus 5.1 software package.

3. Results and discussion

3.1. Solvents optimisation

The analyte $[PGE_2\text{-H}]^-$ m/z 351 was isolated and fragmented into the ions $[PGE_2\text{-}H_2O\text{-H}]^-$, $[PGE_2\text{-2}H_2O\text{-H}]^-$ and $[PGE_2\text{-2}H_2O\text{-44-H}]^-$. The summation of the intensities of the characteristic fragments (333, 315 and 271 m/z) in ion counts per second (icps) was recorded in order to select the optimal extraction solvent condition. The extraction procedure and instrumental precision were monitored by replicating the former three times at every experimental point in Fig. 1 and the latter by measuring repeatedly ($n = 3$) some of the individual extractions. Table 1 shows the experimental results obtained after performing the extractions of 30 and 0.5 ng/ml PGE_2 dissolved in plasma at different methanol:water (v/v) ratios and different volumes of acetonitrile. It can be seen from this table that the highest magnitudes of the analytical signal, at low and high PGE_2 concentration, were obtained at a methanol:water (v/v) ratios of 3:1 and 2:1 at high and low concentration of PGE_2. A multiple range test that allows the statistical comparison of the recorded signals did not reveal significant differences between the signals at 30 ng/ml PGE_2 when the proportion of methanol is lower than the proportion of water (ratios 1:3, 1:2 and 3:5). At these ratios there was no detection of the analytical signal at the low level of prostaglandin studied in this work. There were not significant changes in the signal, at the two analytical concentration levels, when the ratio methanol:water was kept constant and the volume of acetonitrile was varied. In addition, the signals at 30 ng/ml of PGE_2 recorded at the extraction point 5:3 ratio of methanol:water and 400 μl of acetonitrile did not differ from those found at constant ratio of methanol:water. The results in Table 1 indicate clearly, that the efficiency of the extraction procedure in terms of signal magnitude and analytical concentration is increased when the proportion of methanol is higher than the proportion of water, hence a methanol:water (v/v) ratio of 3:1 and the minimum volume of acetonitrile used in this work, namely 400 μl can be considered as optimal condition for the extraction of PGE_2 from plasma samples. The instrumental precision, at both levels of concentration, reported as coefficient of variation was $<3.17\%$ and considered acceptable for the analysis of PGE_2 from plasma samples.

3.2. Plasma samples screening

Using the optimised extraction condition described above and plasma samples ($n = 6$) from patients suffering chronic inflammatory disorders, four positive identifications were made during the LCMS/MS screening step. A representative ion chromatogram of one of the positive-identified samples at 351 m/z is shown in Fig. 2A. Species with identical molecular weights to

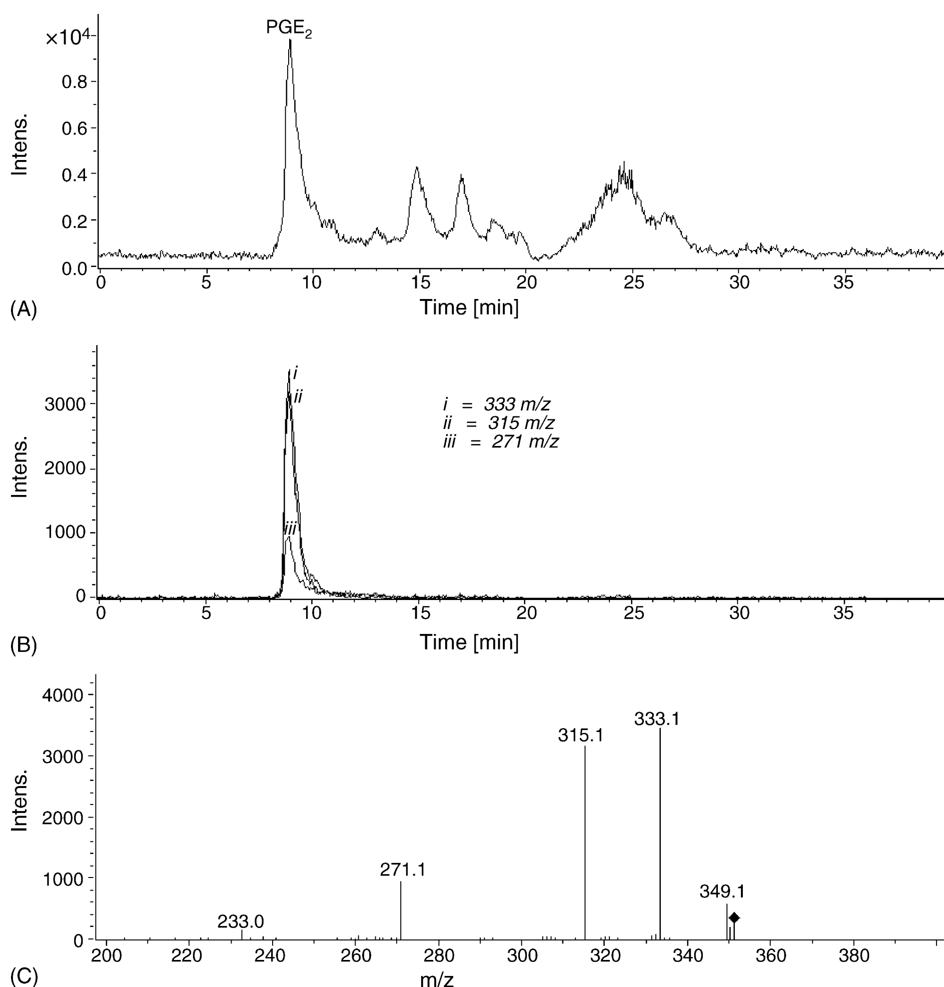

Fig. 2. Representative ion chromatogram (A), superimposed characteristic ion fragments chromatograms at 333, 315 and 271 *m/z* (B) and MS/MS product ion spectrum of the PGE_2 anion (C) of pathological levels of PGE_2 extracted from plasma using the optimised protocol outlined in this figure.

$[PGE_2\text{-H}]^-$ are observed in this figure hence a positive identification is accomplished by comparing the elution time of PGE_2 standard dissolved in blank plasma (8.4 min) and those showed in Fig. 2A, by performing coelution experiments to confirm the analyte identity and by extracting the characteristic ion fragment chromatograms from Fig. 2A. The superimposed ion chromatograms at 333, 315 and 271 *m/z* extracted from Fig. 2A and showed in Fig. 2B revealed that the species eluting at 8.4 min correspond to $[PGE_2\text{-H}]^-$ effectively and that the presence of structural isomers of PGE_2, such as PGD_2, 8-iso PGE_2, 8-iso-15-keto PGE_2, which could yield identical product ion spectra with PGE_2, can be discarded. Further experiments aiming at eliminating suspicion of simultaneous elution of PGE_2 and its isomers under the conditions used in this work were conducted by using the extraction and chromatographic protocol reported by Yang et al. [19] who separated successively PGE_2 from its isomers. The chromatographic results (not shown) resemble those in Fig. 2A but with different retention times (9.7 min for PGE_2). The MS/MS spectrum at the PGE_2 ion chromatogram peak maximum (Fig. 2C) revealed that under the experimental conditions used in this work the abundance of the ions 333 and 315 *m/z*, resulting from the loss of one and two molecules of water, did not show any differences between them. Thus, the summation of these ions was used for quantitative measurements of PGE_2.

3.3. Extracted standard calibration curves

The lower range studied containing PGE_2 (0–1 ng/ml) and PGE_2-d_4 (0.5 ng/ml) dissolved in plasma, revealed that the lower limit of quantification, defined as the lowest concentration analysed with acceptable accuracy and precision, corresponding to ten times the standard deviation of the blank plasma and for an injection volume of 25 μl was 0.4 ng/ml. The calibration graphs for plasma spiked with PGE_2 and internal standard were linear over the concentration ranges 0.4–1 ng/ml ($r^2 = 0.988$) and 1–50 ng/ml ($r^2 = 0.988$). The analytical characteristics of these curves determined by unweighted least-squares regression were $\hat{y} = -0.147 + 2.879 \times [PGE_2]$ and $\hat{y} = 0.0012 + 0.081 \times [PGE_2]$ for the low and high range, respectively. The term $\hat{y}$ in both equations represents the estimated signal as PGE_2 to PGE_2-d_4 ratio calculated by using the summation of the characteristic fragment peaks of PGE_2 (333 and 315 *m/z*) and PGE_2-d_4 (337 and 319 *m/z* which were also similar in abundance). The detection limit corresponding to three times the

Table 1
Summary of the central composite design and precision results at different PGE_2 concentrations

Extraction solvents added sequence (I → II → I)			Extraction procedure ($n = 3$)				Instrumental precision ($n = 3$)			
I	II	I + II + I	30 ng/ml		0.5 ng/ml		30 ng/ml		0.5 ng/ml	
CH_3OH:H_2O (μl/ratio)	Acetonitrile (μl)	Total volume (μl)	Average response (icps × 10^5)	Coefficient of variation (%)	Average response (icps × 10^3)	Coefficient of variation (%)	Average response (icps × 10^5)	Coefficient of variation (%)	Average response (icps × 10^3)	Coefficient of variation (%)
400/1:3	400	1200	0.63^{α}	6.76	nd	–	0.61	1.16	–	–
400/1:1	400	1200	2.22^{β}	3.62	2.64^{ε}	4.35				
400/1:2	400	1200	0.77^{α}	14.41	nd	–				
400/3:1	400	1200	2.67^{γ}	3.42	4.12^{ζ}	3.94	2.66	1.93	3.94	3.17
400/1:1	1200	2000	2.23^{β}	3.38	2.50^{ε}	4.22	2.15	0.92	2.61	2.12
400/3:5	400	1200	0.74^{α}	12.83	nd	–	0.71	1.86	–	–
400/2:1	400	1200	2.57^{γ}	3.01	3.85^{ζ}	5.61				
400/1:1	2400	3200	2.17^{β}	3.27	2.64^{ε}	3.92				
400/5:3	400	1200	2.23^{β}	3.52	3.52	4.72	2.20	1.86	3.35	2.56

icps = ion count per seconds; Greek superscripts denote homogeneous signal group averages; nd: not detected.

Table 2
Determination of PGE_2 in plasma from patients suffering chronic inflammatory disorders using extracted standard and standard addition calibration curves

Sample	Extracted standard curve ($n = 3$)		Standard addition curve ($n = 3$)	
	Mean (ng/ml)	Coefficient of variation (%)	Mean (ng/ml)	Coefficient of variation (%)
S1	8.060	7.695	8.037	2.212
S2	30.556	2.045	29.580	2.004
S3	dl (∼0.1)	–	–	–
S4	27.824	0.807	28.078	0.161

dl: detection limit.

standard deviation of the blank plasma and for an injection volume of 25 μl was 2.5 pg of PGE_2 on the column.

3.4. Plasma samples quantification

The quantification of the four positive screened samples was carried out by means of extracted standard calibration curves and by standard addition curves. The results (Table 2) showed good agreement between both calibration methods. The recovery, estimated according to the 2002 IUPAC recommendations [30] and by using standard addition curves ranged from 96 to 98%.

4. Conclusions

The extraction procedure for the analysis of PGE_2 in plasma described in this work and based on the precipitation of the proteic fraction and further LC ion-trap high order MS detection of the supernatant has been shown to be a useful approach for a rapid, simple and efficient screening and quantification of the analytical prostaglandin in a wide range of concentrations.

The small amount of plasma required coupled with the low solvents consumption, fast extraction time and rapidity with which the sample is processed make the present approach highly suitable for routine analysis and clinical investigations of PGE_2 in patients suffering from chronic inflammatory disorders.

The experimental design has been a valuable tool for the systematic variation and simultaneous optimisation of the extraction solvents used in this work. It is important to highlight the substantial reduction of time and resources when the central composite design is manipulated. The optimisation of the three extraction solvents has resulted in a 40% reduction of the total number of experiments.

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