Improved Quantification of Prostaglandins in Biological Samples by Optimizing Simultaneously the Relationship Eicosanoid/Internal-Standard and Using Liquid Chromatography Tandem Mass Spectrometry. Presented at "6th International Congress on Essential Fatty Acids and Eicosanoids" organised by the International Society for the Study of Fatty Acids & Lipids (ISSFAL), held in Cairns, Australia, July 2006

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Abstract

Although a wide variety of articles on quantification of eicosanoids by using internal standards are published every year, little has been done on how much internal standard should be added. This article demonstrates that the application of experimental design enables estimating the interaction eicosanoid/internal-standard and to select confidently an optimal amount of internal standard and a response factor (*RF*) for the analysis of eicosanoids in a high number of samples, where the amount of sample is limited and the unknown levels of eicosanoids are spanned in a wide range of concentrations. The results revealed that the interaction eicosanoid/internal-standard is an important factor that affects the validity of the *RF* and subsequently the accuracy of the analysis

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1. Introduction

Quantitative methods for the analysis of eicosanoids in biological samples are based on the construction of calibration curves. However, when various prostaglandins are being investigated simultaneously or when a large number of samples are analysed, it becomes difficult to make calibration curves for each prostaglandin. In such cases, the use of the internal standard approach in order to determine the relative response factors (*RF*) is advisable to achieve a rapid sample throughput with minimum cost, manpower, and instrument requirements.

The internal standard is a compound that mimics the eicosanoid of interest and is added to the sample before treatment. The internal standard should possess chemical, spectral, and chromatographic properties that are similar to those of the analyte, and should be resolved from the analyte of interest. The ideal internal standard is an isotopically labelled version of the analyte that will be coeluted with the analyte but will be resolvable by mass spectrometry or an alternative detection method [1]. Although the validity of the internal standard technique relies among others on the assumption of linearity of the detector response towards the analyte and the internal standard [2,3], a review of the current literature on eicosanoids quantification has revealed that details on the detector linearity towards the internal standard are generally omitted. In addition a key weakness in the majority of the literature on quantification is that it does not deal sufficiently with the contextual issue concerning the strategies behind the selection of a particular amount of internal standard especially in cases where the analytes can have a wide span of concentrations. How the concentration of internal

standard in eicosanoids' quantification has been selected in the reported works? In which extent the relationship analyte/internal-standard affects the quantitative determination of eicosanoids? A possible answer to the former question could be the application of trial and error methods or rules of thumb techniques such as targeting the internal standard to the lower 1/3 of the working standard curve [4-5] but to the latter question no answer has been given as yet. We have recently proposed a general method to determine a region where the *RF* does not change with changes in the analyte and internal standard concentrations [6] and have pointed out the potential applicability of this method in the analysis of biomedical samples. The goal of this article is to investigate how simultaneous changes in the concentrations of prostaglandin E2 (PGE_2) and its deuterated analogue PGE_2-d_4 affect the response factor and how the modelling of the relationship PGE_2/PGE_2-d_4 can assist the analyst in the selection of an optimal amount of internal standard in quantification experiments of a high number of samples where the amount of sample is limited and the unknown analyte may spanned in a wide range of concentrations.

2. Experimental

2.1. Reagents

PGE_2 and 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. Extraction procedure

The extraction procedure used in this work has been described elsewhere [7]. Briefly, a test tube containing PGE_2 and PGE_2-d_4 dissolved in acetonitrile was taken and evaporated to dryness under a stream of nitrogen at room temperature. An aliquot of 50 µl of blank human plasma was added in the test tube and vortex-mixed for 2 min. Successive aliquots of 100 µl of methanol:water (3:1) and acetonitrile were added,

vortex-mixed for 2 min, centrifuged at 3000 rpm for 10 min at room temperature and the supernatants collected, evaporated to dryness under a stream of nitrogen at room temperature, reconstituted in 30 μl of acetonitrile, transferred to an autosampler vial and submitted to LCMS/MS analysis.

2.3. Experimental design

The behaviour of the *RF* when the concentrations of PGE_2 and PGE_2-d_4 were varied simultaneously was studied by using a uniform shell design developed by Doehlert [8]. A minimum number of seven experiments is suggested by this experimental design and distributed in the vertexes and centre of a hexagon as is depicted in Fig.1. According to this design, the two coded variables x_1 and x_2 are converted into the variables PGE_2 and PGE_2-d_4 respectively and the equally spaced coded levels -1, -0.5, 0, 0.5, 1 and -0.866, 0, 0.866 are converted into ng/ml values. The studied concentration ranges of PGE_2 and PGE_2-d_4 dissolved in blank plasma were 0.5-15 ng/ml and 5-15 ng/ml respectively and the injection volume was 25 μl. The *RF* at every experimental point was calculated by the expression:

$$RF = \frac{ng_{PGE_2}}{ng_{PGE_2d_4}} \times \frac{S_{PGE_2d_4}}{S_{PGE_2}} \qquad [1]$$

where the *ng* terms represent the amount in nanograms of analyte and internal standard injected in the chromatography system and the remaining terms represent the signal intensities of PGE_2 and PGE_2-d_4 in ion counts per second (icps).

2.4. Plasma samples quantification

Plasma samples were drawn from fasting patients suffering from inflammatory bowel disease (IBD) and under treatment with pharmacological medication supplemented with omega-3 polyunsaturated fatty acids (ω-3 PUFAs) from seal or whale oil. Indomethacin was added to the plasma samples to inhibit further synthesis of prostaglandins in *vitro*. The samples were kept at -80 ^{0}C prior to extraction and analysis by LCMS/MS.

2.5. Liquid chromatography ion-trap mass spectrometry (LCITMS)

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 × 4.6 mm, 5 μm (Agilent Technologies. Palo Alto, CA, USA) was kept in the column compartment at 40 ^{0}C. The solvent system operated in isocratic mode at 0.4 ml/min 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 ^{0}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 13000 *m*/z/sec (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.

2.6. Statistics

Data were expressed as mean values and standard deviations. A multiple regression analysis was performed and the statistical significance of the coefficients and the correlation was determined by the *F*-test at a 95 % confidence level. The regression analysis was done by Statgraphics Plus 5.1 software package.

3. Results and Discussion

3.1. Modelling of the relationship PGE_2/PGE_2-d_4

The analyte $[PGE_2\text{-}H]^-$ *m/z* 351 and the internal standard $[PGE_2\text{-}d_4\text{-}H]^-$ *m/z* 355 were isolated and the losses -H_2O-H, -2H_2O-H and -2H_2O-44-H monitored in both cases. A total of seven mixtures for PGE_2 and PGE_2-d_4 were prepared in triplicate according to the design described in Fig. 1 and measured randomly. The *RF*s were calculated

according to Eq. 1 and analysed and expressed as a function of the amount of PGE_2 and PGE_2-d_4 injected. A four terms first-order polynomial model was considered adequate to model the relationship PGE_2/PGE_2-d_4. The model is described by the equation:

$$\hat{y} = -0.04 + 0.34x_1 + 0.32x_2 - 0.96x_1x_2 \qquad [2]$$

The term $\hat{y}$ represents the estimated *RF* and the terms x_1, x_2 and x_1x_2 represent the nanograms of PGE_2, PGE_2-d_4 and their interaction respectively. The visualization of the *RF* behavior as a function of the amount of PGE_2 and PGE_2-d_4 (Fig. 2) was performed by using Eq. 2. The graphical display shows that in the whole PGE_2 analytical range studied (0.0125-0.375 ng), a constant response factor of 0.075 is obtained when the amount of PGE_2-d_4 is varied between 0.345-0.375 ng. Amounts of PGE_2-d_4 lower than 0.275 ng bring about a reduction in the dynamic analytical range. Fig.2 shows that when the amount of PGE_2-d_4 is fixed at 0.125 ng variations of the *RF* between 0.025-0.085 are observed between 0.013-0.375 ng of PGE_2. Interestingly, the previous mentioned fixed amount of internal standard (0.125 ng) was estimated by applying the rule of thumb of targeting the internal standard to the lower 1/3 of the working PGE_2 standard range displayed in Fig 2. Another important feature of the polynomial model proposed (Eq. 2) is the absolute magnitude of the interaction term (0.96) which causes the curvature observed in Fig. 2. This result indicates that the interaction PGE_2/PGE_2-d_4 is a key factor in the determination of an appropriate *RF* and consequently plays an important role in the accuracy of the determination. Unfortunately, it is common practice to overlook such an interaction term and accept without confirmation and regardless the analytical range the linearity of the detector toward both analyte and internal standard.

3.2. Quantification of PGE_2 in plasma samples at an optimal level of PGE_2-d_4

Based on the above discussion and the observations derived from Fig. 2, it was decided that a *RF* of 0.075 and 0.360 ng of PGE_2-d_4 were optimal values to be used in the quantification of PGE_2 in plasma samples from IBD patients. 42 plasma samples (50 µl each) prepared in duplicate (42×2) were spiked with the internal standard, extracted as was described above and measured in random order. The *RF* was periodically checked in blank plasma samples spiked with 0.5, 7.0 and 15 ng/ml of PGE_2 and the fixed amount of 0.360 ng PGE_2-d_4, was estimated from Fig.2. In addition to this periodical checking, blank plasma samples were spiked with 0.5, 7.0 and 15 ng/ml of PGE_2 and the fixed amount of 0.125 ng of PGE_2-d_4, was estimated from 1/3 of the working range and also checked regularly. The results of these monitoring studies revealed no significant variations in the *RF*s at the highest and fixed amount of PGE_2-d_4 over the course of the analyses. In addition, there was no statistical difference between the average *RF* calculated regularly (0.077 ± 0.001) and the *RF* estimated from Fig. 2 (0.075). The *RF*s calculated at low level of PGE_2-d_4 were more variable than its high level counterpart. An average *RF* value of 0.047 ± 0.033 at low level of PGE_2-d_4 was estimated at the end of this study. The observed variations at low level of internal standard are direct consequence of the interaction PGE_2/PGE_2-d_4 which brings about a reduction in the dynamic analytical range. For instance, Fig. 2 shows that at 0.125 ng of PGE_2-d_4 seven *RF*s (0.025, 0.035, 0.045, 0.055, 0.065, 0.075 and 0.085) are obtained and the dynamic range 0.5-15 ng/ml (0.013-0.375 ng) of PGE_2 is split in seven analytical ranges accordingly. The results make clear that the interaction analyte/internal-standard is an important factor that affects the validity of the *RF* used in quantification experiments and consequently its determination in an appropriate analytical range is crucial for the accuracy of the analysis.

Assessing the within-preparation precision is a fundamental step in method validation, especially when a large number of samples is analysed and various analysts are engaged in the preparation process. To evaluate the within-preparation component, appropriate levels of PGE_2-d_4 and *RF* were estimated from the model and applied in the analysis of PGE_2 in 42 plasma samples from IBD patients. The samples were prepared in duplicates by two different analysts and submitted to LCITMS. The PGE_2 concentration in the 42 plasma samples ranged from undetectable to 3.130 ng/ml with a mean of 1.057 ng/ml and a median of 0.758 ng/ml. A scatter diagram of all the measurements was plotted in order to characterize the within-preparation precision. The scatter diagram displayed in Fig. 3, shows an excellent degree of correlation (r = 0.998) between every sample and its duplicate indicating a high degree of preparation precision over the course of the study.

4. Conclusions

The modelling of the relationship PGE_2/PGE_2-d_4 and the selection of an optimal amount of internal standard to be used in the quantification of PGE_2 in plasma from IBD patients have been achieved successfully by using experimental design and LCMS/MS.

The design used in the present study can estimate simultaneously the effect of PGE_2, PGE_2-d_4 and their interaction with a minimum of seven experiments, making it more desirable than the conventional trial and error approaches or techniques aimed at the lower 1/3 of the working standard curve.

The modeling of the eicosanoid/internal-standard relationship emerges as a powerful tool for the improvement of eicosanoids quantification. In addition, such a tool allows comparing different response factors in conjunction with their optimal eicosanoid and internal standard working ranges in an easy and comprehensive way.

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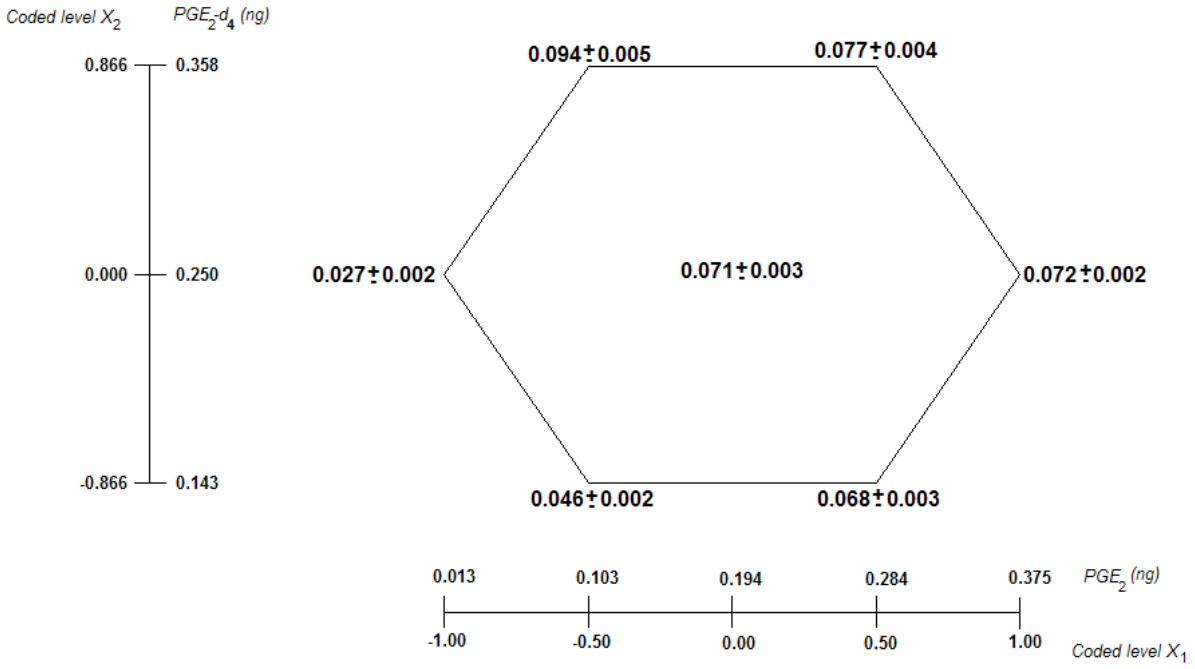

Fig. 1. Uniform shell design used to estimate the response factors represented at the vertexes and centre of the hexagon and expressed as mean and standard deviation values (n = 3). PGE_2 and PGE_2-d_4 concentrations in ng/ml are obtained by dividing the analytical amounts (ng) by 25×10^{-3} ml.

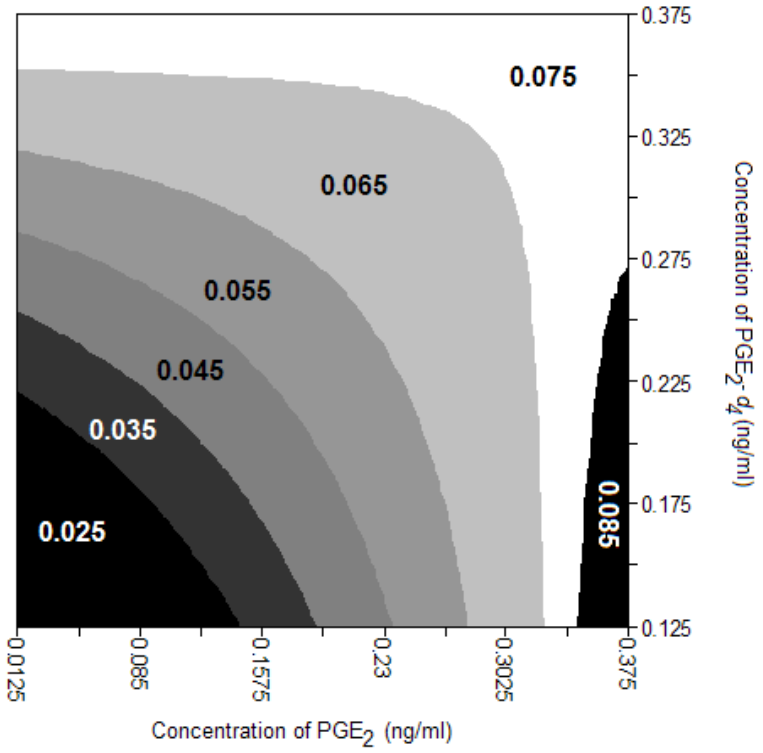

Fig. 2. Response factor contour plot as a function of the amount of PGE_2 and PGE_2-d_4.

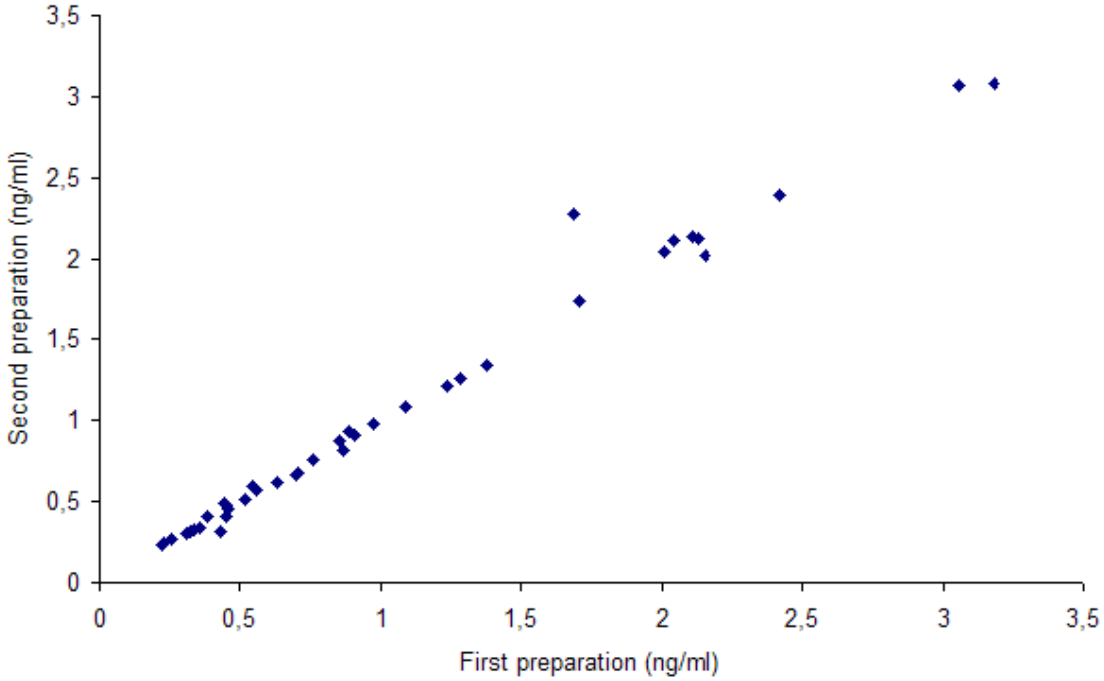

Fig. 3. Scatter diagram for the 42 plasma samples from inflammatory bowel disease patients prepared in duplicates.