Direct Injection of Redissolved Cell Culture Media into a Single-Column Liquid Chromatography Coupled to Mass Spectrometry for the Measurement of PGE_2

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Abstract: The traditional approach to assessing eicosanoids in cell cultures by liquid or gas chromatography coupled to mass spectrometry involves time-consuming procedures such as derivatisation, degradation, solid-phase extraction, liquid-liquid extraction, thin layer chromatography and sometimes, combination of all these procedures prior to the injection of the sample in the chromatography system. The performance of a method that allows the direct injection of redissolved cell culture media into a single-column liquid chromatography instrument coupled to mass spectrometry is evaluated. The external standard calibration curves were linear between 1-50 ng/ml for Williams' medium E and L-15 medium. The limit of detection and quantification were 0.5 and 1 ng/ml in both media respectively. The recovery values were 98.6% for Williams' medium E and 100.3% for L-15 medium. The internal standard method was not used for quantitative purposes due to the variability of the response factor. The proposed method has potential for broad implementation in monitoring biomarkers in cell cultures.

Keywords: Cell cultures, eicosanoids, prostaglandin E_2, LCMS, ion-trap mass spectrometry.

1. INTRODUCTION

Cell culture systems are suitable substitute methods for animal experiments. They have become increasingly important tools to investigate the production of eicosanoids, such as PGE_2 in various types of cell lines. The concentration levels of PGE_2 are an important criterion for the determination of pathophysiological events in invertebrates, vertebrates, and mammals [1]. Antibody-based assays such as enzyme-linked immunosorbent assay (ELISA) and radioimmunoassays are the most widely used methods for the estimation of PGE_2. However, the main drawbacks of these assays are: trend to overestimate the levels of metabolites due to cross-reactivity with related compounds resulting in reduced selectivity, lack of specificity for complex biological fluids, variability in the quantification of sequential samples and limitation to the detection of a single product at the time [2,3]. Gas chromatography and liquid chromatography coupled to mass spectrometry (GCMS and LCMS respectively) have proved to be reliable techniques capable of preventing the problems associated with antibody-based assays. A study aiming at comparing ELISA and GC/MS revealed that the estimated concentrations of 8-isoprostaglandin $F_{2\alpha}$ by ELISA were 30-fold greater than those determined by GCMS [2]. Nowadays, it is accepted that GCMS and LCMS outperform antibody-based techniques and they are commonly used in the determination of eicosanoids in a wide variety of samples, including cell cultures [3-6]. However, the determination of PGE_2 in cell culture systems by GCMS or LCMS demands some tedious and time-consuming preparation steps such as derivatisation, degradation, solid-phase extraction (SPE), liquid-liquid extraction (LLE), thin layer chromatography and sometimes, combination of all these procedures prior to the injection of the sample in the chromatography system. Thus, there has been a growing interest towards the development of simpler methods and improvement of the already existing sample protocols in terms of sensitivity, selectivity and simplicity of sample preparation. In some analytical areas, such as drug analysis, direct injection of a cell culture medium into the liquid chromatography system has been achieved by substituting the SPE and LLE steps for an automated on-line two-column-switching pre-concentration system [7]. Although, this proposed system is a reliable alternative to reduce sample preparation by enabling effective clean-up of cell cultures media, the required equipment is rather complex, involving a combination of several concentration and separation columns, pumps and switching valves under computer control. This significantly complicates the analysis and increases its cost. Direct injection of cell culture medium into a single column is generally overlooked in the analysis of eicosanoids due to the complexity of the culture medium which in turn can seriously affect the chromatography performance. The goal of this investigation is to propose a simple and efficient method that minimizes sample manipulation and allows the direct injection of redissolved cell culture samples into a single-column LCMS system. The investigation concentrates on the analysis of PGE_2 produced by mammalian and fish cells and determined by using a LC isocratic system coupled to a tandem MS in negative mode. The *m*/*z* 351 signal corresponding to the ion $[PGE_2\text{-}H]^-$ was isolated and fragmented and the identification and quantification carried out by extracting the ion fragment chromatograms at 333, 315 and 271 *m*/*z*.

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2. EXPERIMENTAL

2.1. Reagents

Prostaglandin E2 (PGE_2) and deuterated prostaglandin E2 (PGE_2-d_4) were 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 (Millipore, Milford, MA).

2.2. Cell Cultures

2.2.1. Hepatocyte Culture

Hepatocytes isolated from Wistar rats (200-250 g) were attached to dishes with 60 mm-diameter at a density of approximately 2×10^6 cells per dish and incubated in Williams' medium E (Sigma, St. Louis, MO) containing 10% v/v fetal bovine serum, and 1% v/v antibiotic antimycotic solution (Sigma, St. Louis, MO). After pre-incubation in an humified atmosphere (5% CO_2, 95% air) for 3 h at 37 °C, the medium was removed and the attached hepatocyte monolayer was washed gently with 3 ml of fresh Williams' medium E (WME) without fetal bovine serum to remove the unattached and non-viable cells. The incubation began with the addition of 4 ml of WME containing 1% antibiotic antimycotic solution and 200 μM of arachidonic acid bound to fatty acid-free bovine serum albumin (BSA) at a 4:1 molar ratio. Fatty acid peroxidation was prevented by adding 0.1% butylated hydroxytoluene and 20 μM α-tocopheryl succinate into the culture medium. The cells were incubated for 24 h without changing the medium.

2.2.2. Head Kidney Cultures

Head kidney cells isolated from Atlantic cod (800-1000 g) were seeded into a 12 well plate (Corning, New York, USA) with a density of 3×10^6 cells per well. A volume of 2 ml of L-15 medium (Sigma, St. Louis, MO) consisting of 1% glutaMax (Gibco-BRL, Cergy-Pontoise, France), 1% penicilline-streptomycine-amphotericine (BioWittaker, Petit Rechain, Belgium) and 10% fetal calf serum (BioWhittaker, Petit Rechain, Belgium) was added to each well. The cells were incubated at 9 ºC in a normal atmosphere incubator for 7 days without changing the medium.

2.3. Proposed Sample Preparation Protocol

After incubating hepatocytes and head kidney cells for 24 and 168 h respectively, 3 ml of the media (WME or L-15 medium) were centrifuged at 3000 g for 10 min at 4 ºC. An aliquot of 2 ml of supernatant is collected and freeze dried in a Hetosicc system (Heto, Birkerød, Denmark). An aliquot of 1 ml of acetonitrile was added to the dried sample, vortex-mixed for 1 min, centrifuged at 3000 g for 10 min and the supernatant collected. This step was repeated one more time. The collected supernatant was evaporated to dryness under nitrogen at room temperature and reconstituted in 30 μl of acetonitrile, vortex-mixed for 30 seconds and submitted to LCMS/MS.

2.4. Calibration

The prediction capability of internal standard and external standard calibration curves in the quantification of PGE_2 in the media was evaluated. The internal standard calibration curves were prepared as follows: two set of vials containing 50 μl PGE_2 (1, 15, 25, 35, 50 ng/ml) and a constant concentration of PGE_2-d_4 (5 ng/ml) in acetonitrile were prepared in triplicates and evaporated to dryness under a stream of nitrogen at room temperature. Aliquots of 50 μl of fresh culture medium (WME or L-15), with not detectable levels of PGE_2, were added into each set of vials and vortex-mixed for 2 min. The solutions were treated according to the proposed sample preparation protocol. The external calibration curve was constructed in a similar way but without adding PGE_2-d_4.

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. A Zorbax Eclipse-C_8 RP 150 × 4.6 mm, 5 μm column (Agilent, Palo Alto, USA) was used and kept in the column compartment at 40 °C. The solvent system operated in isocratic mode at 0.2 ml/min was acetonitrile with formic acid 0.1% (v/v) and UV detection at 254 nm. The total analysis time was 60 min. Nitrogen was used as nebulizing (50 psi) and drying gas (8 l/min) 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 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 351→333, 315, 271 *m/z* for PGE_2 and 355→337, 319, 275 *m/z* for PGE_2-d_4 and their intensities were recorded in ion counts per second (icps).

2.6. Statistics

Data are expressed as mean values and standard deviations. The statistical significance of the regression analysis was determined by the *F*-test at a 95% confidence level using Statgraphics Plus 5.1.

3. RESULTS AND DISCUSSION

3.1. Proposed Sample Preparation Method

After stimulating by arachidonic acid for 24 h, the medium of hepatocyte culture is dried and redissolved in acetonitrile as described above and injected into a single-column LCMS/MS system afterwards. The ion chromatogram of the released PGE_2 was recorded by isolating the signal 351 *m/z* and fragmenting it into the characteristic product ions $[PGE_2\text{-}H_2O\text{-}H]$, $[PGE_2\text{-}2H_2O\text{-}H]^-$ and $[PGE_2\text{-}2H_2O\text{-}44\text{-}H]^-$ at 333, 315 and 271 *m/z* respectively (Fig. **1a**). The results showed in Fig. (**1a**) were compared with those obtained by spiking a blank WME sample (containing only BSA) with a standard solution of PGE_2 (Fig. **1b**). The superimposition of the ion chromatograms of released PGE_2 from hepatocytes and spiked PGE_2 in blank medium, exhibits a perfect overlapping between the signals at 333, 315 and 271 *m/z*. Similar results were found when a sample of L-15 containing released PGE_2 from head kidney cells incubated for 7 days (results not shown) and a sample of hepatocytes plus medium incubated for 14 days (Fig. **1c**) were compared against spiked L-15 and WME respectively. These results give a

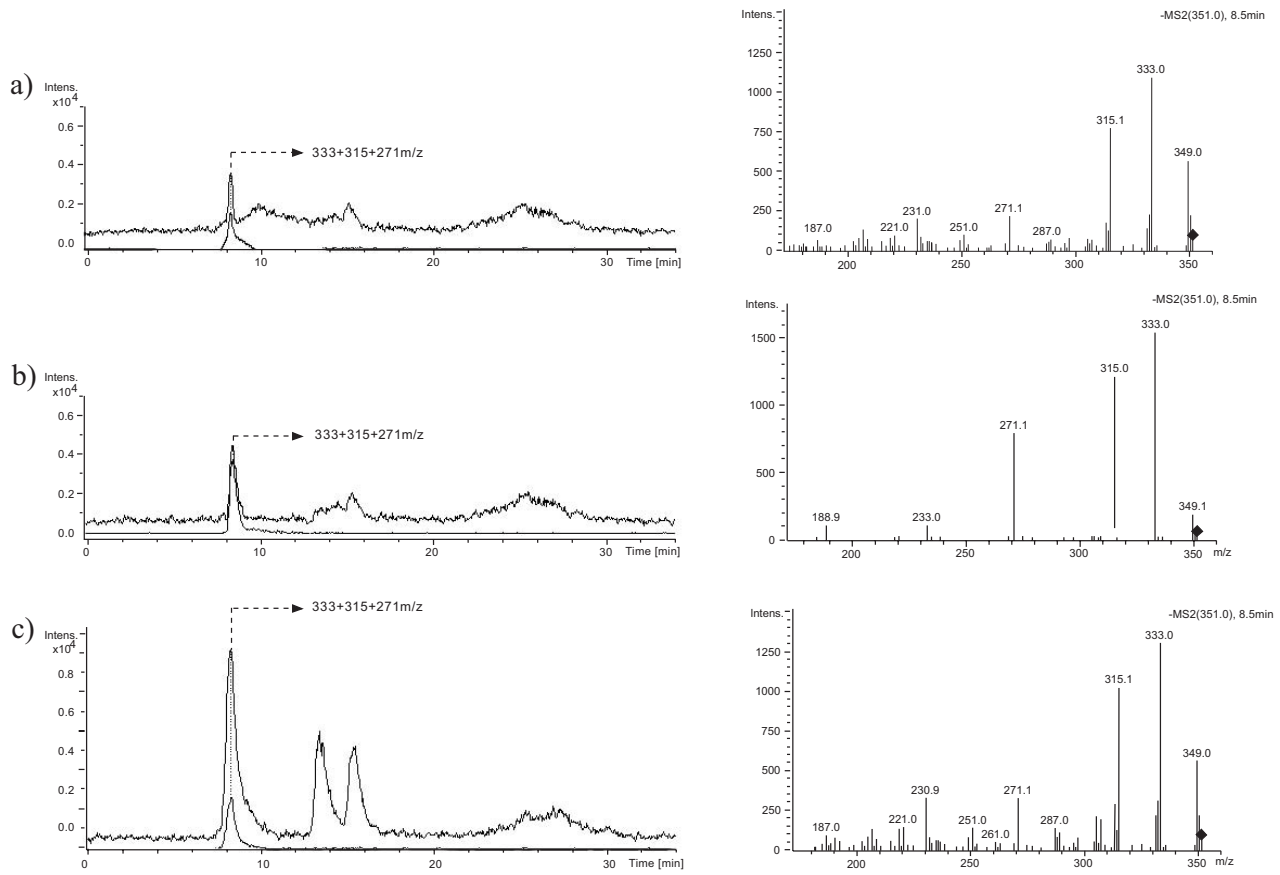

Fig. (1). Superimposed total ion current and extracted ion chromatograms (left) and corresponding ionization mass spectra (right) of (**a**) released PGE_2 in Williams' medium E by hepatocyte cells incubated for 24 h; (**b**) Williams' medium E spiked with 25 ng/ml of PGE_2; (**c**) hepatocyte cells plus Williams' medium E incubated for 14 days.

clear indication that the sample protocol described in this article is suitable for determining PGE_2 in cell culture systems either in mammalian or fish models.

3.2. Internal Standard Calibration Studies

Calibration graphs were constructed in two different blank matrices: WME and L-15 medium. Both media were treated according to the proposed sample preparation protocol. The internal standard calibration curve was prepared by spiking cell medium (WME or L-15) with different concentrations of PGE_2 (1, 15, 25, 35, 50 ng/ml) and keeping constant the concentration of the internal standard (5 ng/ml of PGE_2-d_4). The preparation error was checked by preparing every calibration point in triplicates. The calibration graphs were constructed using least-squares regression of PGE_2/PGE2-d_4 peak area ratios (designated as $S_{PGE_2}/S_{PGE_2\text{-}d_4}$) against PGE_2 concentrations (designated as $[PGE_2]$). The analytical characteristic of the PGE_2 curves were $S_{PGE_2}/S_{PGE_2\text{-}d_4} = 0.41 \times [PGE2] - 0.87$ and $S_{PGE_2}/S_{PGE_2\text{-}d_4} = 0.52 \times [PGE2] - 0.92$ with correlation coefficients of 0.995 and 0.996 for WME and L-15 medium respectively. Comparison of the theoretical and back-calculated PGE_2 concentrations by using the peak area ratios and the analytical characteristic of the regression curves revealed recovery values that ranged between 270-83% and 225-84% for WME and L-15 medium respectively when the concentration of PGE_2 was varied between 1-15 ng/ml in both media (Fig. **2**). These variable recovery values indicate very low accuracies at these levels of concentration in both media. Relatively constant recovery percentages averaging 99.6 ± 5.0 and 100.6 ± 7.1% were observed between 25-50 ng/ml of PGE_2 for WME and L-15 medium respectively (Fig. **2**). The preparation error was not significant at the 95% confidence level.

3.3. Response Factor Accuracy and Recovery

In order to explain the low accuracies found between 1-15 ng/ml of PGE_2 in the above experiments, the internal standard response factor was calculated at every experimental point by multiplying the PGE_2/PGE2-d_4 concentration ratio by the PGE2-d_4/PGE_2 area ratio. The regression of the calculated response factors against the concentrations of

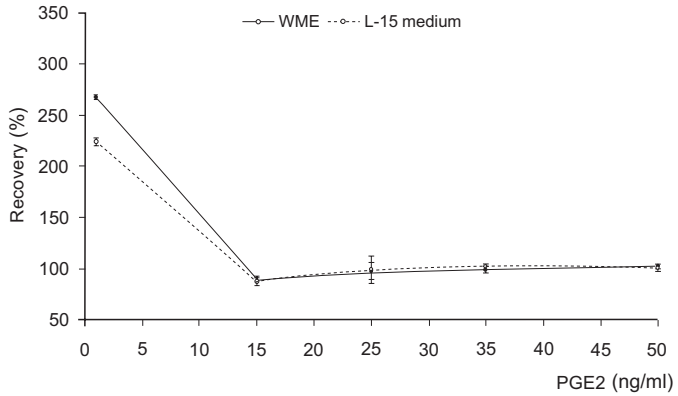

Fig. (2). Recovery of PGE_2 in Williams' medium E and L-15 medium using the internal standard technique.

spiked PGE_2 in both media (Fig. **3**) reveals that the response factor does not remain constant throughout the analytical range considered. The dashed line in Fig. (**3**) illustrates the ideal behaviour of the response factor as a function of the analytical concentration. It is evident from Fig. (**3**) that the response factor (designated as RF) decreases logarithmically in both media as the concentration of PGE_2 is increased, according to the equations $RF = -0.10 \times Ln[PGE_2] + 0.87$ and $RF = -0.11 \times Ln[PGE_2] + 0.81$ with regression coefficients of 0.973 and 0.987 for WME and L-15 medium respectively. The observed variability of the RF as a function of the PGE_2 concentration between 1-15 ng/ml as well as its constancy between 25-50 ng/ml (Fig. **3**) explain the different recovery values (and consequently the variability in accuracy) found at different PGE_2 concentration ranges in the previous experiments. Recent findings on the variability of the response factor as a function of the concentration [8, 9] suggest that the degree of ionization of the internal standard in the electro-spray ion source and the interaction PGE_2/PGE2-d_4 could be significant factors that affect the accuracy of the determination. The addition of PGE_2-d_4 before submitting the sample to any treatment procedure can help to compensate for recovery losses, provided that the degree of ionization of the fixed amount of PGE_2-d_4 is not affected by the amount of PGE_2 in the entire analytical range investigated, in that way the response factor remains constant and the results are meaningful. In a recent comparative study between the internal standard and the traditional calibration method by using LCMS/MS [9], it is reported that the degree of ionization in the electro-spray ion source is strongly dependent on the amount of molecules resulting in a non-linearity in the concentration/response ratio. It must be said, that several quantitative studies [10-12] have demonstrated the variability of the RF as a direct result of varying the analyte and internal standard concentrations indicating clearly that among the possible variables affecting the RF accuracy, the interaction analyte/internal-standard could play an important role which is generally omitted in quantification studies.

3.4. Direct Quantification of Cell Culture Media

Based on the previous results, calibration curves were constructed by spiking separately fresh WME and L-15 medium with different concentrations of PGE_2 (1-50 ng/ml) without adding internal standard. Three replicates were prepared at each level of concentration. The calibration curves showed good linearity over the investigated concentration range with a correlation coefficient of 0.999 in both media and recovery values that ranged between 98.6 ± 3.1% and 100.3 ± 1.7% for WME and L-15 medium respectively. The preparation error checked in both media was not significant at the 95% confidence level. The limits of detection and quantification defined as the analytical concentration giving a signal equal to three and six times the standard deviation of the blank medium signal and calculated from the calibration curves were 0.5 ng/ml and 1 ng/ml respectively in both media. The released PGE_2 into WME and L-15 medium by hepatocytes and head kidney cells, with and without stimulation ranged from 5.5 to 8.3 ng/ml and 3.4 to 30.2 ng/ml respectively.

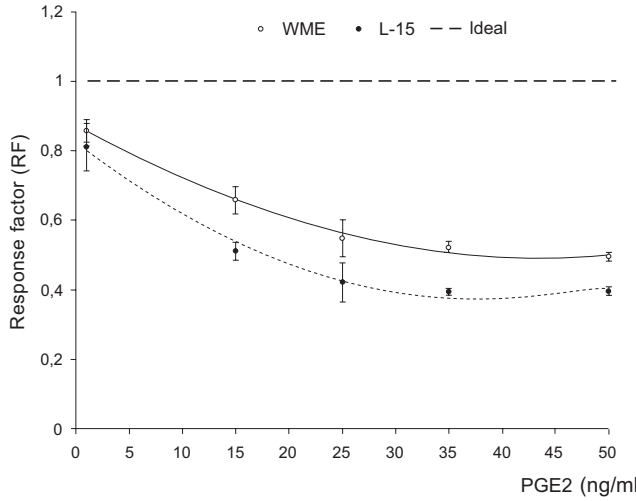

Fig. (3). Variability of the response factor as a function of the PGE_2 concentration at a fixed level of PGE_2-d_4 (5 ng/ml) in Williams' medium E (solid line) and L-15 medium (dotted line). The ideal behaviour is represented by a dashed line. Every point represents the average of 3 measurements.

4. CONCLUSIONS

Although the results obtained by using the internal standard method are less satisfactory than those obtained with the external standard method, it must be said that a comparison between both methodologies in the context of the present investigation is not appropriate considering that the internal standard method is affected by factors such as the interaction PGE_2/internal-standard which could not be estimated by the classical methodology used in the present work. By performing a multivariate study could be possible to determine systematically and simultaneously a PGE_2 and PGE_2-d_4 concentration region where the response factor remains constant.

To our knowledge this is the first reported method that allows the direct injection of redissolved culture medium or culture medium containing cells into a single-column LCMS system. The proposed method is an effective, simple and rapid strategy for the analysis of PGE_2 in cell cultures that minimizes sample manipulation and potential contamination, reduces the requirements for handling potentially infectious biomaterial and improves reproducibility and most impor-

tantly, the proposed method has potential for broad implementation in monitoring biomarkers in cell cultures.

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