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Research Article

Method Development by Design of Experiments for Quantification of Lipoxygenase Metabolites in Human Cancer Cells with UPLC-MS/MS

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Abstract

Lipoxygenase (LOX) products (5- and 12- HETE) have been implicated in carcinogenesis, contribution to invasion and cancer progression. Several LOX-inhibitors have been studied as potential cancer preventive agents, but the specificity of these inhibitors has been doubted. It is therefore important to have a highly selective method, as UPLC-MS/MS, for assessment of the mechanism of action for LOX inhibitors in the cellular context. Design of Experiments (DoE) is a chemo metrical approach which allows for optimization of a quantification method using a limited number of experiments and interaction between experimental factors can be observed. This paper presents optimization of an UPLC-MS/MS method for accurate quantification of the LOX products, 5- and 12- HETE, utilizing DoE. Significant interaction effects where seen between amount of organic solvent in the mobile phase at initial condition and gradient slope as well as between mobile phase flow rate and capillary voltage. A UPLC-MS/MS quantification method was developed and validated for 5- and 12- HETE. An intraday validation assessment showed that the quantitative determination was linear for 5- and 12- HETE in the range tested (1.00-100 ng/ml), and accuracy and precision met the acceptance criteria with a coefficient of variations lower than 15%. Auto-sampler stability was established for 12 hours at 4°C. The optimized method was specific for evaluation of LOX activity in cultured AsPC-1 pancreatic cancer cells. Without added Arachidonic Acid (AA) as substrate LOX products were not detected. After addition of AA, 5- and 12- HETE were quantified in both supernatants and cell lysates, demonstrating the usefulness for cell-based studies in the evaluation of LOX inhibitors.

Introduction

Lipids have a wide variety of functions in living cells, as building blocks, energy storage, as well as signaling and cell communication [1,2]. Lipid metabolism may be altered in cancer cells and some of these changes have been proposed to play a role in carcinogenesis and cancer progression [3]. Arachidonic Acid (AA), a 20-carbon polyunsaturated fatty acid, gives rise to many bioactive lipids collectively called eicosanoids. It cannot be synthesized de novo by animal cells and is obtained from dietary sources [4]. AA is metabolized through three key metabolic pathways, cyclooxygenases, producing prostaglandins, and Lipoxygenases (LOX), producing leukotrienes, as well as cytochrome P450 (CYP 450) [5].

AA is metabolized by 5-, 12- and 15-lipoxygenase to 5-, 12- and15-Hydroxyeicosatetraenoic Acid (HETE) respectively. 5-LOX metabolizes AA to the leukotriene LTA4 that is further metabolized to5-HETE or LTB4 by LTA4 hydrolase [6]. 5-HETE and LTB4 have been suggested to have a promoting effect on carcinogenesis and tumor progression. Evidence also suggests that LOX products from cancer cells are directly involved in carcinogenic processes, 5-HETE formation has been linked to carcinogenesis in several tissues whereas 12-HETE can contribute to invasion and metastasis. In contrast, 15-LOX products have been shown to promote cell differentiation, growth inhibition and apoptosis. The effect of 15-HETE is suggested to be mediated by antagonizing other LOX products, both LTB4 and 12-HETE [7]. Evidence of eicosanoids having a promoting effect on carcinogenesis and tumor progression is supported by clinical [6] as well as animal and cell-culture-based data [8].

Zileuton is the first and only selective 5-LOX inhibitor to get approval from the FDA for prophylaxis and chronic treatment of asthma [9]. Zileuton was recognized as a selective 5-LOX inhibitor and was shown not to inhibit 12-, 15- LOX and cyclooxygenase at concentrations up to 100μM [10]. Clinical use has been limited because of liver toxicity which is unrelated to the inhibition of 5-LOX. Zileuton has also been suggested to be beneficial in a number of diseases, ranging from inflammatory conditions to liver metastases. The effect of zileuton is linked to the inhibition of 5-LOX and the reduction in leukotriene formation; however, Rossi et al. showed that

Zileuton also inhibited prostaglandin production by interfering with the release of AA [9].

Several inhibitors of 5- and 12- LOX have been described and tested for anticancer activity [8]. Baicalein a bioactive flavonoid is recognized as a 12-LOX inhibitor and has in several studies been demonstrated to inhibit tumor cell growth and induce apoptosis [11-13]. It has been assumed that the effect is mediated by 12-LOX inhibition, but there is evidence that baicalein is not selective for 12-LOX and could be affecting different pathways [14]. We have described in-vitro anti-cancer activity of a lichen-derived compound, protolichesterinic acid with known 5- and 12-LOX inhibitor activity [15-17].

In order to assign a cancer preventive action to LOX inhibitors it is important to measure accurately a decrease in LOX products. Furthermore, it should be possible to reverse the effects by adding back the product in relevant concentration. Therefore, accurate measurements are necessary to assess the specificity and mechanism of action of LOX inhibitors in the cellular context.

The principle of the chemo metric method, statistical Design of Experiments (DoE) is to gather, obtain and interpret a high level of information from chemical data followed by application of a mathematical model, done with as few experiments as possible [18]. UPLC-MS/MS is composed of two systems working synergistically, ultra-performance liquid chromatography for separation of the analytes in the condense phase and mass spectrometer with an electro spray probe for ionization of analyte before introduction to the detector where the analytes are separated by mass to charge ratio (m/z). The system needs to be optimized for the analyte to be transferred from a condense phase to a gas phase where conditions are kept optimal in both UPLC and MS part of the system. The UPLC-MS/MS involves many experimental factors that are to be optimized simultaneously to obtain maximum sensitivity and limit the retention time while maintaining desired resolution [19]. The traditional way of achieving this is by Changing One Separate Factor at a Time (COST), One Variable at a Time (OVAT) or one factor at a time OFAT approaches [20-22]. None of these methods accounts for interactions between the experimental factors and the true optimum may not be achieved. Design of Experiments (DoE) is a tool to systematically induce controlled variation to evaluate variables effects and interactions utilizing statistical methods such as Partial Least Square (PLS) regression. It is used in analytical chemistry to get the most information about a system with a set of selected experiments that give a keen understanding of that system. DoE consist of mainly three steps, experimental screening, optimization and robustness testing. DoE is an excellent tool for optimization of an UPLC-MS/MS quantification method, allowing screening of significant experimental factors and optimization of the UPLC and the mass spectrometry simultaneously. Experimental screening evaluates the most influential experimental parameters and selects at what ranges the parameters should be inspected. Optimization is used to find a unique optimum and to evaluate the robustness of the method [22].

Advances in mass spectrometry in recent years have made gas chromatography and liquid chromatography coupled to a mass spectrometer the main analytical tool for evaluation of HETE´s. 5- and 12- HETE have been analyzed and quantified by several other methods [23], such as Enzyme-Linked Immune Sorbent Assays (ELISA), where questions remain on specificity due to possible cross-reactivity [24-26]. GC-MS has been recognized as the gold standard for eicosanoid analysis; however tedious derivation steps are needed for eicosanoid analysis with GC-MS [27]. The aim of this study was to develop a specific and accurate quantification method for measuring 5- and 12- LOX products in cultured cancer cells utilizing DoE.

Materials and Methods

Chemicals

5-HETE, 12-HETE, 5-HETE-d8 and 12-HETE-d8 where purchased from Cayman chemicals (Ann Arbor, MI USA). Acetonitrile, Chromasolv gradient grade 99.9% for HPLC, Hexane for HPLC, ethyl acetate, chromasolv 97%, 2-Propanol (IPA) for HPLC and Formic Acid (FA) for LC-MS was purchased from Sigma Aldrich (Germany) and de ionized water was produced with Milli-Q system (Millipore Billerica, MA, USA).

Culture and treatment of cells

AsPC-1 pancreatic cancer cell line was obtained from ATCC CRL-1682. This cell line was originally derived from a Caucasian 62-year-old human female, pancreas-derived adenocarcinoma from metastatic site, as cites. The cells were maintained in 75cm^2 culture flask (Nunc™, 156499) in 12ml RPMI medium (GIBCO™, 52400) supplemented with 10% fetal bovine serum (FBS, GIBCO™, 10270) and penicillin (100U/l) and streptomycin (100mg/l) (GIBCO™, 15140-148). The cells were incubated at 37°C in a 5% carbon dioxide atmosphere. Cells were passaged weekly by trypsination when they reached confluence.

AsPC-1 cells were seeded at 2.5x105 cells in 1 into 24-well culture plates (Nunc™, 152475) in RPMI medium without phenol red (to avoid interference with the mass spectrometry analysis)containing 10% FBS. After 24 hours medium was replaced by phenol-red-free RPMI medium without FBS and incubated at 37°C overnight to synchronize the cells. The experiment was started by replacing the medium with medium containing 10% FBS followed by incubation for 6 hours. Medium was then removed and each well washed with PBS. Medium without phenol red and FBS was added and incubation continued for 10 minutes. Then AA was added to the cells and incubated for further 5-30 minutes. Finally, the supernatant medium was collected and flash frozen in liquid nitrogen. The cells were collected as well using a cell scraper, counted and flash frozen at 2.5x105 cells in 1.0ml of PBS with liquid nitrogen. Each sample was prepared in duplicate.

Lipid extraction

Cell culture medium: Supernatants were collected from cultures. A 96-well plate Sirocco™ (Waters, Milford, MA, USA) protein precipitation plate was used for sample preparation of released lipids from medium. First, 0.60ml of methanol, containing 0.015µg/ml of internal standards; 5(S)-HETE-d8 and 12(S)-HETE-d8 were added to the Sirocco plate, after which 0.20ml of supernatant was added. The sample was collected by applying vacuum and analyzed by UPLC-MS/MS.

Cell lysates: Liquid-Liquid Extraction (LLE) was performed by transferring 1.0ml of cell lysates to a 14ml glass tube. 2ml of hexane: Ethyl acetate (1:1 v/v) containing 0.015µg/ml internal standards

for 12(S)-HETE-d8 and 5(S)-HETE-d8 was added to each sample. Samples were mixed and centrifuged for 10 min at 4100rpm and 10°C. The organic phase was collected into a new glass tube. The extraction process was repeated and the second organic phase collected into the same glass tube. Samples were evaporated to dryness at 35°C for 5 minutes and allowed to cool down at room temperature and subsequently dissolved in 0.10ml of ACN:H_2O (1:1 v/v) and analyzed by UPLC-MS/MS.

UPLC-MS/MS analysis

The quantification was performed with a Waters Acquity UPLC system, coupled to a Waters Quattro Premier XE triple quadruple mass spectrometer equipped with Electro Spray Ionization (ESI) probe. The mass spectrometer was optimized using Multiple Reactions Monitoring (MRM) in negative Electro Spray Ionization (ESI) mode to monitor Precursor ion > Product ion (m/z) at the quantification transitions m/z 318.99 > 114.64 for 5-HETE, m/z 327.18> 116.53 for 5(S)-HETE-d8, m/z 319.18>179.00 for 12-HETE and m/z 327.18 > 184.03 for 12(S)-HETE-d8. Data acquisition was carried out using Mass Lynx 4.1 software.

Method development with Design of Experiments

Design of experiments and all calculations were generated and carried out using the MODDE Pro 11 software (Sartorius Stedim Data Analytics AB, Umea, Sweden). D-optimal design was used for screening of experimental factors that had a significant effect on peak area, peak height and retention time of 5- and 12- HETE analyzed by UPLC-MS/MS. Table 1 shows the qualitative and quantitative factors chosen for the D-optimal design and at what range. All responses were centered and scaled to unit variance and log scale transformation was applied for both analytes (Table 1).

Following the D-optimal screening, experimental factors that significantly affected the responses for both HETE compounds, were optimized by Central Composite Face (CCF). The experimental factors chosen and their range are shown in Table 2. The performance of the optimized method was evaluated for linearity, limit of quantification, intraday accuracy and precision, recovery, matrix effect and 12-hours auto sampler stability at 4°C (Table 2).

Results

Method optimization by design of experiment

D-optimal Screening: D-optimal screening revealed that experimental factors influenced the results for peak area and peak height of both compounds, in the same manner. Therefore, it is possible to select between only evaluating peak area or peak height for both 5- and 12-HETE for optimization of sensitivity. This is clearly illustrated in the PLS loadings plot (see supplementary data figure S1).The plot also indicates that the retention time for both compounds is affected by chosen experimental variables in the same manner. Retention time was mostly influenced by gradient slope after which gradient curve was the second most influential factor, where curve a hade a negative effect, lowering the retention time, while curve type -B and -C had a positive effect, increasing the retention time of both compounds. Flow rate and organic phase start had almost equal negative effects on retention time. As expected an increase in gradient slope increased retention time, while an increase in flow rate and organic phase start decreased the retention time. Interactions between organic phase start and gradient slope also cause significant decrease in retention time for both compounds. Other factors had no effect on retention time as seen in the regression coefficient plot (see supplementary figure S2).The regression coefficient plot in figure 1 revealed that the type of organic mobile phase B had the greatest significant effect on peak area for both 5- and 12- HETE. Acetonitrile as mobile phase B had a significant negative effect and acetonitrile with 2-propanol added had a significant positive effect on peak area for both 5- and 12- HETE,showing that it was highly favourable for both compounds to add 2-propanol to the mobile phase-B. For 5-HETE, mobile phase flow rate had a significant negative effect and collision energy had significant positive effect on peak area and there was a significant interaction effect between mobile phase flow rate and capillary voltage. For 12-HETE flow rate and gradient slope had significant negative effect on peak area and significant interaction effects were detected. The curve of the gradient did not have a significant effect on the peak area for 5-HETE, but a significant positive effect was observed for linear gradient curve type (C) for peak area of 12-HETE and a negative significant effect was seen for gradient curve type (B). Collision energy had a non-significant effect on peak area of 12-HETE (Figure 1).

Figure 2 shows a 4D response contour plot for the peak area of 5- and 12- HETE as a function of capillary voltage and flow rate at both mobile phase B with acetonitrile and acetonitrile with 2-propanol at three different gradient slope times. The plot confirmed the results shown in figure 1, that using acetonitrile with 2-propanol as organic mobile phase results in increased peak area for both compounds. Furthermore, by decreasing the flow rate of the mobile phase and reducing the gradient slope, the sensitivity for both compounds was improved. Increased collision energy improved the sensitivity for 5-HETE, but the opposite was true for 12-HETE, decreasing the collision energy could improve the sensitivity for 12-HETE; however this was not a significant effect. Same results were observed for peak height (see supplementary figure S3). Therefore, the collision energy

Table 1: Experimental domain for the D-optimal design.

Experimental factors	Unit	Low	Center	High	Type of factors
Mobile phase B (OrgP)		ACN, ACN:IPA			Qualitative
Gradient Curve (Cur)[a]		A, B, C			Qualitative
Mobile phase flow rate (Flow)	ml/min	0.35	0.5	0.65	Quantitative
Initial amount of organic start (Org)	%	5.0	22.5	40.0	Quantitative
Gradient slope (Gra)	min	1.0	2.5	4.0	Quantitative
Capillary voltage (Cap)	kV	1.0	2.25	3.5	Quantitative
Collision energy (Col)	eV	10	15	20	Quantitative

[a]Gradient curve A is concave downwards; B is concave upwards, and C is linear. For center point, gradient curve C was chosen.

Table 2: Experimental settings for the CCF design.

Experimental factors	Unit	Low	Center	High
Mobile phase flow rate (Flow)	ml/min	0.35	0.50	0.65
Initial amount of organic solvent (Org)	%	5.0	22.5	40.0
Gradient slope (Gra)	min	1.0	2.5	4.0
Capillary voltage (Cap)	kV	1.00	2.25	3.50

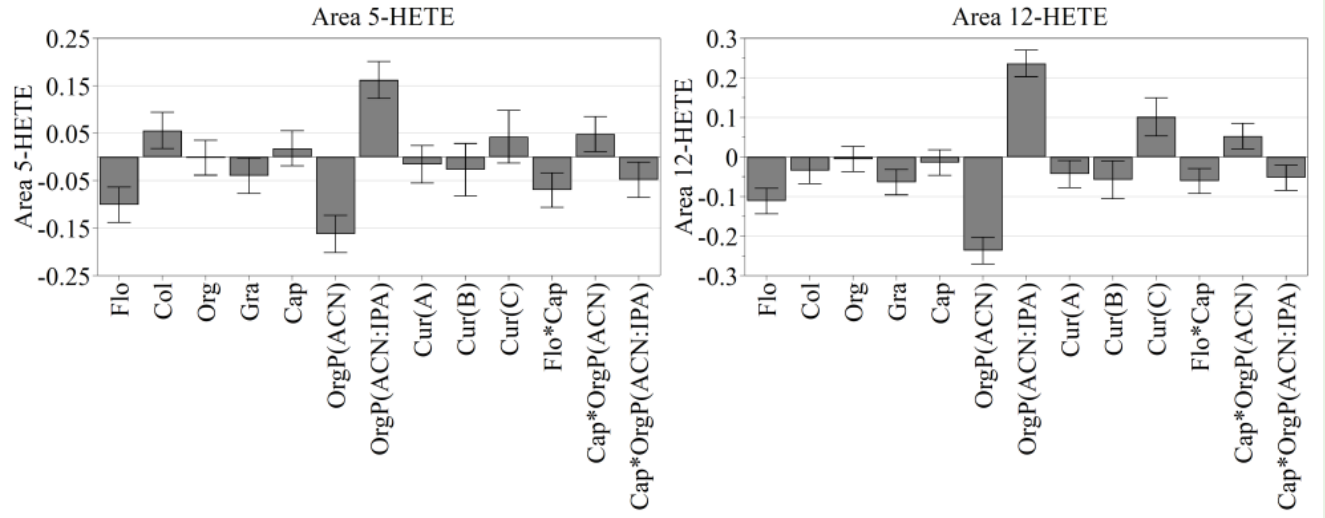

Figure 1: Regression coefficient plot for the peak area of 5-HETE (a) and 12-HETE (b). On the x-axis the experimental factors and factor interactions are shown and on y-axis the coefficients for peak area, scaled and centered. Error bars represent the 95% confidence interval. Flo, mobile phase flow rate (ml/min); Col: Collision energy (V); Org: Initial amount of organic start (%); Gra: Gradient slope (min); Cap: Capillary voltage (kV); OrgP: organic phase; ACN: Acetonitrile; ACN: IPA: Acetonitrile: 2-propanol, Cur: Gradient curve.

was not used as an experimental factor in the optimization and set separately for each compound (Figure 2).

Central composite face Optimization: The method was optimized by Central Composite Face (CCF) design, where the following factors were studied: flow rate of the mobile phase, gradient slope and initial amount of organic solvent and capillary voltage. Affected response analyzed was peak area for both HETE compounds. Acetonitrile with 2-propanol was used as organic mobile phase, gradient curve was set curve A, which is a non-linear gradient curve, collision energy was set to 15V for 5-HETE and 10V collision energy for 12-HETE. The results from the optimization model are shown in figure 3. The response surface plot shows the effect of mobile phase flow rate and organic start on the peak area of 5-HETE, while gradient slope and capillary are held constant at values that give the highest peak area (same results were obtained for peak area of 12-HETE). By choosing an organic start of 22.5% and mobile phase flow rate of 0.4ml/min the highest peak area was obtained for both compounds (Figure 3).

Final method adopted for quantification of 5- and 12-HETE

The analytical column used was an ACQUITY UPLC BEH C18 (2.1mm x 100mm i.d.; 1.7µm) (Waters corp., Milford, MA, USA), that was maintained at 60°C. Mobile phase consisted of A: 0.1% formic acid B: 90:10, Acetonitrile: 2-Propanol, at a flow rate of 0.4ml/min. The gradient was non-linear; starting at 77.5% mobile phase A for 0.8 min. up to 95% of mobile phase B in 2.6 min and was held for 0.6 min before going back to the initial conditions. The total chromatographic run time was 6.5 min. Retention time of 5- and 12- HETE was 2.83 min and 2.76 min respectively. The sample manager temperature was maintained at 4°C. Capillary voltage was 3.5kV; cone voltage was set to 30 V for both 5- and 12- HETE. Collision voltage was set to 15V for 5-HETE and 10V for 12-HETE.

A series of test samples was injected to evaluate the developed method. Figure 4 compares the detection of 5- and 12- HETE before and after optimization with DoE. Following optimization of the

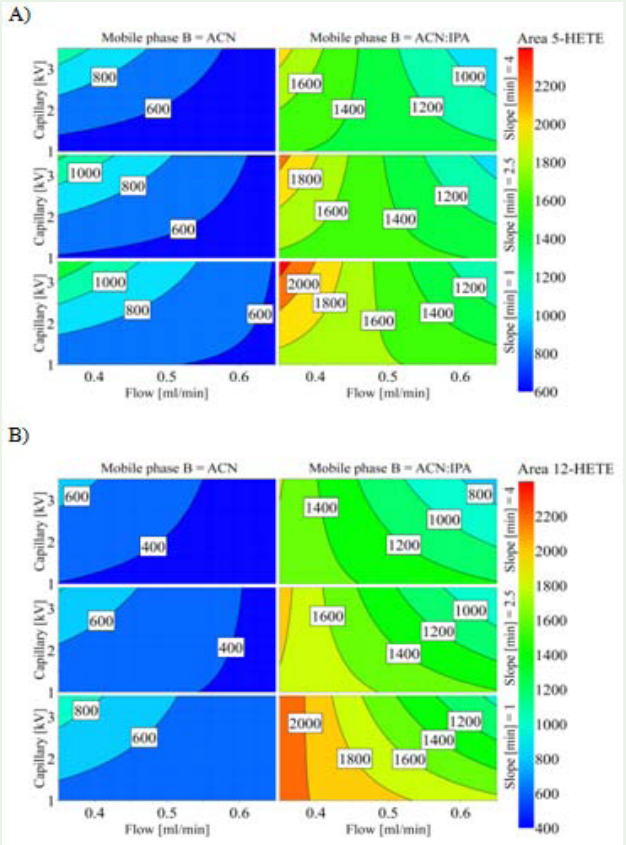

Figure 2: 4D response contour plot for the peak area of 5-HETE (a) and 12-HETE (b), showing capillary voltage, initial amount of organic start, type of organic mobile phase and type of slope. The color scale shows the peak area for the compounds, from lowest (blue) to highest (red) peak area. Flow: Mobile phase flow rate (ml/min); Capillary: Capillary voltage (kV); Slope: Gradient slope (min).

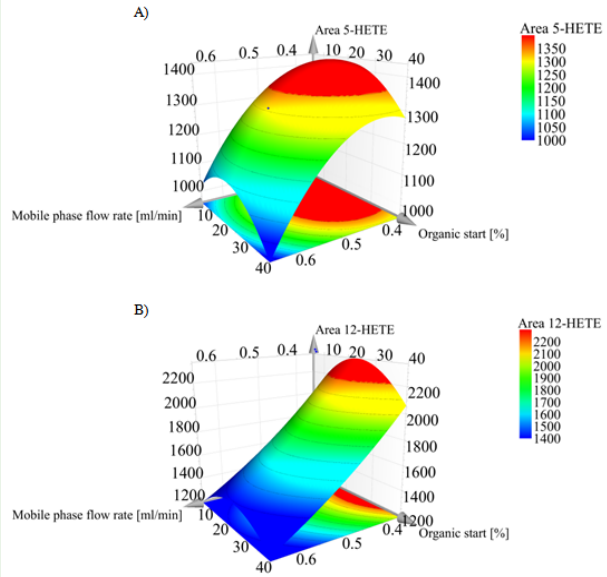

Figure 3: Response surface plot for 5- and 12-HETE.With initial amount of organic start (10 to 40%) plotted against mobile phase flow rate (0.35-0.65 ml/min). The vertical axis displays the area of each response.

method the sensitivity was increased by a factor of 5.4 and 2.6 for 5- and 12- HETE respectively (Figure 4).

Method performance

The performance of the optimized method was evaluated for concentration function, range, Limit of Quantification (LOQ), intraday accuracy and precision, recovery, matrix effect and 12 hours auto sampler stability at 4°C. The concentration function was investigated at concentration range from 3.00 to 100ng/ml for both HETE compounds. A Calibration curves were generated by plotting the corresponding peak area ratios for the analyte/IS versus corresponding concentrations in ng/ml using weighted (1/x) least squares quadratic regression. The LOQ were evaluated by replicate analysis (n=6) at the lowest concentration (3.00 ng/ml) for both compounds and the acceptance criteria with a coefficient of variations lower than 20% was met. The results are represented in Table 3. Intraday validation assessment was determined by replicate analysis (n=6) of the LOQ and for three Quality Controls (QC) at analyte concentration of 12.5, 20.0 and 60.0ng/ml for 5- and 12- HETE. The intraday accuracy and precision met the acceptance criteria with a coefficient of variations lower than 15%, as shown in Table 3. Degree of interference was assessed, and the conclusion was that no significant interfering peaks were detected. Stability of the analytes was established for 12-hours, suggesting that storage condition of the analytes at 4°C was appropriate (data not shown).Recovery and matrix effect were evaluated for 5- and 12- HETE for the sample preparation methods developed for the culture media and the culture cell lysate at one concentration, the QC-M which was 20.0ng/ml for both analytes. The peak area of QC-M (20.0ng/ml) for the analytes spiked in blank culture media and in neat solution was compared to the peak area of extracted QC-M. The results are summarized in Table 4. The results indicate that following protein precipitation of the cell culture media the signal of 5- and 12-HETE was enhanced (21% for 5-HETE and 15% for 12-HETE) and the recovery was between 41-50%. Following the liquid-liquid extraction of the cell lysates some matrix effect (-15% for 5- HETE and -24% for 12-HETE) was detected indicated by lowering of the signal intensity of both analytes. The recovery for 5-HETE was only 27%, while the recovery for 12-HETE was 74% (Table 3 and Table 4).

Table 3: Lower limit of quantification, intraday accuracy and precision.

Analyte	QC Level	Mean calculated concentration (ng/ml)	% CV[a]	% Bias[b]
5-HETE	LOQ (3.00ng/ml)	3.07	8.8	2.3
	QC-low (12.5ng/ml)	14.2	2.9	14
	QC-medium (20.0ng/ml)	21.9	7.4	9.7
	QC-high (60.0ng/ml)	56.6	8.3	-5.7
12-HETE	LOQ (3.00ng/ml)	3.27	8.0	8.9
	QC-low (12.5ng/ml)	14.2	8.8	13.3
	QC-medium (20.0ng/ml)	22.6	7.2	12.8
	QC-high (60.0ng/ml)	59.8	14.2	-0.3

[a]% RSD= (Standard deviation/mean)x100.

[b]% Bias= (Mean calculated concentration - Nominal concentration)/(Nominal concentration)x100. Each result is the average of 6 separate determinations.

QC: Quality Control; LOQ: Limit of Quantification; CV: Coefficient of Variation.

Production of 5- and 12-HETE by ASPC1 cell line

The optimized method was tested by studying5- and 12- HETE production by the pancreatic cell line As PC-1. None of these LOX products were observed without AA addition. With added AA, products of both 5- and 12- LOX could be detected in medium, with 5-HETE being more abundant than 12-HETE.Two concentrations of added AA were evaluated, 30µM and100µM of AA showing a clear dose relationship for production of HETE´s. Incubation time had no significant effect on production, showing similar results for both time points. Comparable results were obtained from LOX products released into medium and cell lysates, but concentrations were 10 times lower in the lysates as seen in (Figure 5), implying rapid release of the products from the cells.

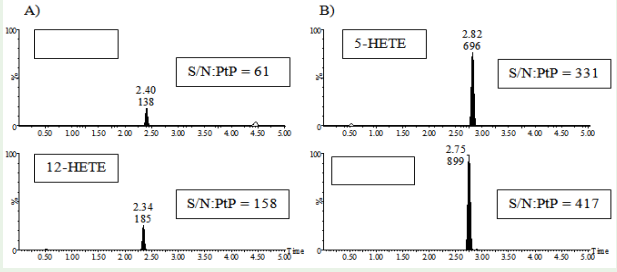

Figure 4: MRM chromatogram of 5- and 12- HETE before (A) and after (B) chemo metric optimization. Y axis shows relative ion abundance (%) at the same scale. X axis shows time in minutes.

Citation: Eiriksson FF, Thorsteinsdottir UA, Halldorsson H, Ögmundsdottir HM and Thorsteinsdottir M. Method Development by Design of Experiments for Quantification of Lipoxygenase Metabolites in Human Cancer Cells with UPLC-MS/MS. SM Anal Bioanal Technique. 2018; 3(2): 1017.

Table 4: Recovery and matrix effect.

Sample preparation	Analyte					
	5-HETE			12-HETE		
Protein precipitation	Extracted QC-M	Post-spike QC-M	Neat spiked QC-M	Extracted QC-M	Post-spike QC-M	Neat spiked QC-M
Mean peak area	312	754	621	612	1238	1075
Recovery	41%			50%		
Matrix effect	21%			15%		
Liquid-liquid extraction	Extracted QC-M	Post-spike QC-M	Neat spiked QC-M	Extracted QC-M	Post-spike QC-M	Neat spiked QC-M
Mean peak area	1971	7086	8309	4667	6259	8270
Recovery	27%			74%		
Matrix effect	-15%			-24%		

Each result is the average of 3 different determinations.

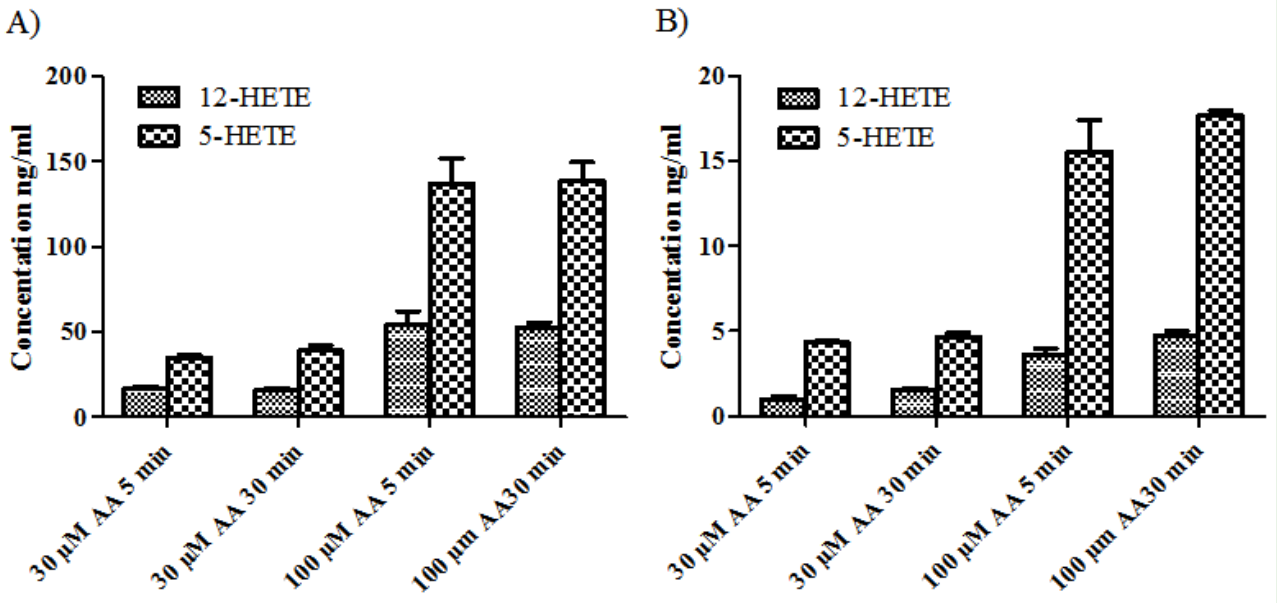

Figure 5: Production of 5- and 12-HETE in AsPC-1 following addition of arachidonic acid (AA). A) Concentration of LOX products released into medium. B) Concentration of LOX products in cell lysates. Note different scales in A and B.

Discussions

In this study we have successfully utilized chemo metrics to develop a method for accurate quantification of products of 5- and 12- lipoxygenases in cancer cell cultures. Chemo metrics allowed us to develop the method very efficiently and with confidence of finding the true optimum. Experimental screening could be run overnight without supervision and results were complete on the following day for modeling and interpretation of the model. We developed methods for appropriate culture conditions, extraction of lipids from various matrices and analysis by mass spectrometry. Metabolites of LOX, both released to the medium and intra-cellular, analyzed in cell lysates, were quantified by the method.

D-optimal design, allows for screening of both qualitative and quantitative factors in the same model and significant factors can be identified with few experiments. All the data were gathered overnight and the model could be interpreted the day after. To facilitate the interpretation of the model we use the coefficient plot that demonstrates how different factors and factor interactions affect the response, this allows for simplification of the model before optimization is performed. D-optimal screening helped to eliminate qualitative factors before optimization. Three experimental factors were selected from the data gathered in D-optimal design before moving on to the CCF optimization. Mobile phase containing 2-propanol was selected as it was advantageous for both compounds increasing sensitivity and lowering retention time. Selection of the gradient curve was based on a compromise between sensitivity and separation of both compounds. By selecting a non-linear gradient curve, we lowered the retention time and increased the separation between 5- and 12- HETE. Collision energy was set individually for each compound since we got the highest sensitivity at different values. D-optimal screening permits investigation of factors that affect readout as well as indicating the need for range modification. Chemo metrics is an excellent tool to interpret data for decision making in method development when used as a comprehensive approach to take account of several experimental factors simultaneously rather

than with the standard COST (Change One Separate Factor at a Time) method. By using DoE to facilitate the method development a large amount of information is acquired in few experiments and interactions between experimental factors are detected.

To increase robustness and uptime of the mass spectrometer it was necessary to eliminate phenol red from the medium as this caused precipitation on the cone of the mass spectrometer. As Fetal Bovine Serum (FBS) contains lipids including both 5- and 12- HETE, FBS-containing medium was removed and replaced by serum-free medium before addition of AA to the AsPC-1 cells. HETE production by AsPC-1 in standard medium was below the limit of detection, but after AA addition the products of LOX were detected in medium and cell lysates.

Immunoassays and GC-MS have generally been used for the quantification of eicosanoids. The specificity of ELISA for HETE quantification has been questioned and it has been suggested that ELISA needs to be validated with the GC-MS gold standard method [27]. Commercially available 5-HETE ELISA kits specify in their manual that there is no significant cross-reactivity or interference between human 5-HETE and analogues, although they note that they are limited by current skills and knowledge and it is not possible for the manufacturer to complete the cross-reactivity detection for all analogues [28]. ELISA for 12-HETE has also been validated for cross-reactivity with a panel of related eicosanoids [29].

GC-MS methods require tedious derivatization steps before separation on a column and mass spectrometric detection [24]. Eicosanoids are also thermally unstable and can therefore not be easily analyzed by this technique. Rapid advances in UPLC-MS/MS technology have made this technique highly feasible for quantification of eicosanoids with excellent selectivity and specificity without requirement for derivatization.

In terms of costs and availability of equipment the cost per sample is higher for ELISA than mass spectrometry which offers the possibility to quantify several compounds within one sample simultaneously. However, the equipment cost is lower for ELISA.

We have already applied this method successfully and shown that AsPC-1 cancer cells treated with Protolichesterinic Acid (PA) showed reduced production of 5- and 12-HETE. The inhibiting effect of PA on 5- and 12-LOX activity was, however, only observed in cells at the highest concentrations and above those inducing anti-proliferative effects in the cell line [30].

Conclusions

In conclusion, a chemo metric approach was successfully implemented to optimize an analytical method by liquid chromatography coupled to mass spectrometry. The method was developed for accurately assessing LOX activity in pancreatic cancer cells with AA added as substrate. The method is suitable for screening compounds for evaluating LOX inhibitory activity and will also be useful for further studies of LOX activity in cancer cell lines and cancerous tissue.

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