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Liquid chromatographic–mass spectrometric determination of cyclooxygenase metabolites of arachidonic acid in cultured cells

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Abstract

A liquid chromatographic–electrospray ionization-mass spectrometric (LC-ESI-MS) technique was developed to simultaneously determine the cyclooxygenase metabolites of arachidonic acid (6-keto-$PGF_{1\alpha}$, PGD_2, PGE_2, $PGF_{2\alpha}$, and PGJ_2) produced by cultured cells. Samples were separated on a C_{18} column with water–acetonitrile mobile phase, ionized by electrospray, and detected in the positive mode. Selected ion monitoring (SIM) of m/z 353, 335, 335, 319, and 317 were used for quantifying 6-keto-$PGF_{1\alpha}$, PGD_2, PGE_2, $PGF_{2\alpha}$, and PGJ_2, respectively. Prostaglandins were detected at concentrations as low as 1 pg ($S/N = 3$) on the column. The method was used to determine the production of PGs from bovine coronary artery endothelial cells (ECs) and human prostate cancer cells (PC-3) with different degree of invasiveness. Bradykinin (10^{-6} M) stimulated a marked increase in the production of 6-keto-$PGF_{1\alpha}$, PGE_2, and $PGF_{2\alpha}$ and a small increase of PGD_2 by ECs. 6-Keto-$PGF_{1\alpha}$ was the major metabolite in these cells. The production of PGE_2 was threefold higher and PGD_2 was twofold higher in PC-3-S (invasive) cells than in PC-3-U (non-invasive) cells.

Keywords: Arachidonic acid; Cyclooxygenase

1. Introduction

Prostaglandin H synthase (known as cyclooxygenase, COX), lipoxygenase (LOX) and cytochrome P450 (CYP) metabolize arachidonic acid (AA) to many biologically active eicosanoids [1]. COX converts free AA to prostaglandin (PG) G_2 via its cyclooxygenase activity and reduces PGG_2 to PGH_2 via its peroxidase activity [2,3]. PGH_2 is then converted to biologically active prostanoids by cell-specific synthases and isomerases [4]. COX metabolites of AA, PGs and thromboxanes (TXs), have numerous physiological and pathological effects [5] such as the regulation of vascular tone, inflammation [6], pain [7,8], angiogenesis [9–12], and cancer [13–17]. Two COX isoenzymes, constitutive COX-1 and inducible COX-2, have similar subcellular localization in the endoplasmic reticulum and on the inner and outer membranes of the nuclear envelope [18,19]. A large number of studies demonstrate that COX-1 is constitutively expressed in most tissues and involved in the production of prostaglandins mediating cellular physiological functions [4,20,21]. On the other hand, COX-2 is induced in many cell types by mitogens, growth factors, cytokines, and tumor promoters. The COX-2 expression and prosta-

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glandins produced by COX-2 are associated with many diseases [6,21]. These two isozymes share more than 60% amino acid identity and have the same cyclooxygenase activity. However, small alterations in their structures are sufficient for development of specific COX-2 inhibitors as new non-steroidal anti-inflammatory drugs (NSAIDs) [22–26].

Prostaglandins exhibit many physiological effects and are closely associated with various pathological conditions. Thus, quantitative and qualitative analysis of prostaglandins may be a useful index of pharmacological, physiological, and pathological effects. Analytical methods for determination of these compounds need to have high sensitivity and selectivity because of the low concentrations and similarity of their chemical structures. Furthermore, the ability to determine these compounds simultaneously is very desirable because of the complexity of their enzymatic pathways. Radioimmunoassay has been successfully used to measure these prostanoids with high sensitivity but the method can measure only one compound at a time. High-performance liquid chromatography (HPLC) with fluorescence detection is a powerful technique for simultaneous determination of these compounds with high sensitivity. However, the method requires the derivatization of these compounds with fluorescent probes and the samples give high interference background. Gas chromatography–mass spectrometry has been one of the most useful techniques for determination of prostaglandins because of its sensitivity and selectivity. Again, the technique requires tedious and time-consuming sample derivatization and purification procedures. Recently, liquid chromatography–mass spectrometry (LC–MS) with either atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI) has evolved as a powerful tool in biochemical analysis [27–31] including PGE_2 [32], LOX metabolites of arachidonic acid [32–35], and non-enzymatic products of arachidonic acid, isoprostanes [36–38]. LC-ESI-MS-MS was used to determine PGE_2, 12-, 11-, and 5-HETE in human lung and rat leukemia cell lines [39].

In this study, an LC–MS method with electrospray ionization was developed to simultaneously determine 6-keto-$PGF_{1\alpha}$, PGD_2, PGE_2, $PGF_{2\alpha}$, and PGJ_2. Its application was demonstrated in the determination of these prostaglandins in two unrelated biological systems, bovine coronary artery endothelial cells (ECs) and human prostate cancer (PC-3) cells with different degree of invasiveness. Prostaglandins in vascular cells such as endothelial cells are well-characterized [40–42]. In endothelial cells, prostacyclin (PGI_2), a vasodilator, is normally produced at very high concentration and detected in its stable form of 6-keto-$PGF_{1\alpha}$. COX-2 and prostaglandins (particularly PGE_2) have been recognized that they are involved in prostate cancer [43–52]. However, the mechanism and role of prostaglandins in prostate cancer are less well understood. PGI_2 was reported as a potential marker for prostate cancer [53,54]. PGI_2 and its analogs were found to inhibit the prostate cancer cell growth and metastasis [55–58]. PGD_2 can be converted to PGJ_2 and further converted to several products [59,60] that show growth inhibition of some cancer cells [61–63] including prostate cancer cells [64–66]. Among these prostaglandins, only PGE_2 has been measured in prostate cancer cells [67–71]. It has been shown to stimulate cancer cell proliferation [72–74]. In PC-3 cells, prostaglandins were simultaneously measured, and then indomethacin (non-specific COX inhibitor) and NS-398 (specific COX-2 inhibitor) were used to inhibit the COX enzyme and prostaglandin synthesis in order to identify the enzyme isoform and prostaglandin that correlate with the invasion of these cells.

2. Experimental

2.1. Materials

Arachidonic acid (AA), A23187 (calcium ionophore), indomethacin, and Western blot buffers were obtained from Sigma (St. Louis, MO, USA). NS-398 was obtained from Calbiochem-Novabiochem (La Jolla, CA, USA). Monoclonal antibodies against COX-1 and -2 raised in mouse were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 6-Keto-$PGF_{1\alpha}$, PGD_2, PGE_2, $PGF_{2\alpha}$, PGJ_2, [$^2H_4$]6-keto-$PGF_{1\alpha}$, [$^2H_4$]PGD_2, [$^2H_4$]PGE_2, and [$^2H_4$]$PGF_{2\alpha}$ were obtained from Cayman Chemical (Ann Arbor, MI, USA). Peroxidase-conjugated goat anti-mouse IgG, Fc fragment specific was obtained

from Jackson Immunoresearch Laboratories (West Grove, PA, USA). Ready Gels, Protein Assay Kit and nitrocellulose membranes were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Western Blot Chemiluminescence Reagent, ECL Detection Kit, was obtained from NEN Life Science Products (Boston, MA, USA). Bradykinin was obtained from Peninsula Laboratories (Belmont, CA, USA). C_{18} Bond Elut solid-phase extraction (SPE) columns (6 cc, 500 mg) were purchased from Varian (Harbor City, CA, USA). All other chemicals and solvents were of analytical or highest purity grades. Distilled and deionized water was used in all experiments.

2.2. Methods

2.2.1. Culture of human prostate cancer cells

Human prostate cell carcinoma (PC-3) was obtained from the American Type Culture Collection, ATCC (Rockville, MD, USA). Cells were maintained in Eagle's minimal essential medium (RPMI) supplemented with 10% fetal bovine serum, L-glutamine (2 m*M*) and penicillin (50 U/ml). Cells were grown in 75-cm^2 polystyrene tissue culture flasks (Corning) at 37 °C in 5% CO_2. The PC-3-S (invasive) cell line was obtained from the unselected PC-3-U (non-invasive) cells in our laboratory by serial selection in Transwell® invasion assays. Briefly, the PC-3-U cells were transferred into the top well of a Transwell® culture apparatus and cultured for 48 to 72 h. The cells were allowed to invade through a membrane composed of 10% Matrigel on an 8.0-μm pore Transwell® filter. Cells that passed through the filter were monitored at 8–12-h intervals. When the cells had passed through the filter and began to grow independently, the upper chamber was removed. The selected invasive cells (PC-3-S) were then cultured and subjected to two additional rounds of selection by this invasion assay. The PC-3-S (invasive) cells typically exhibited about 8–15% invasion. These cells were frozen in the liquid nitrogen and used within 1 month.

2.2.2. Production of prostaglandins by human prostate cancer cells

When the cells grown in 75-cm^2 polystyrene tissue culture flasks reached 70–80% confluency, the cultured media were removed from the flasks and the cells were washed twice with HEPES buffer (pH 7.4, 10 m*M* HEPES, 155 m*M* NaCl, 5 m*M* KCl, 1.8 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, and 5.5 m*M* glucose). AA at the final concentration of 1×10^{-5} *M* in 5 ml HEPES was added and incubated at 37 °C for 10 min. After the incubation, A23187 was added to this solution at the final concentration of 1×10^{-5} *M* and incubated for 10 min. The cells were then scraped and the solution was transferred to 15-ml tubes. A 50-μl aliquot of each sample was saved for determination of protein concentration. The samples were transferred to reaction tubes, internal standards (deuterated PGs) at 5 ng each were added to the samples and mixed. The samples were extracted by solid-phase extraction.

2.2.3. Inhibition of prostaglandin production in prostate cancer (PC-3) cells

The PC-3-U cells and PC-3-S cells were incubated with either indomethacin (10^{-5} *M*) or NS-398 (10^{-5} *M*) at 37 °C for 15 min and washed three times with HEPES buffer prior to the incubation with AA (10^{-5} *M*). Then, the samples were prepared as described above.

2.2.4. Production of prostaglandins by bovine coronary artery endothelial cells

Bovine hearts were obtained from a local abattoir and used immediately. Endothelial cells (ECs) were isolated from bovine coronary arteries using a modification of the previously described technique [75]. In general, the cells were grown in 25-cm^2 flasks and used within the third passage.

When the cells reached about 80% confluency, the cultured media were removed from the flasks and the cells were washed three times with HEPES buffer. The cells were incubated with AA (10^{-5} *M*) in 2 ml HEPES buffer at 37 °C for 10 min. Then, the cells were washed once with 2 ml HEPES buffer and incubated with bradykinin (10^{-6} *M*) in 2 ml of HEPES buffer at 37 °C for 30 min. Cells incubated with 2 ml of HEPES buffer were used as the control. After the stimulation, A23187 at the final concentration of 10^{-5} *M* was added and incubated for 10 min. Then, the cells were scraped and transferred to 15-ml tubes. A 50-μl aliquot of each sample was saved for determination of protein concentration. The samples were transferred to reaction tubes, internal

standards (deuterated PGs) at 5 ng each were added to the samples, mixed and extracted by solid-phase extraction.

2.2.5. Determination of protein concentrations

The protein concentrations of the samples were determined using the Bio-Rad Protein Assay. The measured prostaglandins were normalized to the protein concentrations.

2.2.6. Immunoblotting

Immunoblot analysis was performed as previously described [76] with slight modifications. Cell lysates (50 to 100 μg of protein per lane) were analyzed by 10% SDS–polyacrylamide gel electrophoresis (Ready Gels, Bio-Rad). After separation, the proteins were electrophoretically transferred to nitrocellulose membranes and non-specific binding was blocked by 20 m*M* Tris buffer, pH 7.8, containing 0.5 *M* NaCl, 0.1% sodium azide, and 5% bovine serum albumin for 2 h at room temperature with gentle rocking. After the solution was removed, the membranes were incubated with either mouse anti-COX-1 or mouse anti-COX-2 antibody at 1:500 dilution of the stock solution in blocking buffer for 2 h at room temperature. The solution was removed and the membranes were incubated with peroxidase-conjugated goat anti-mouse secondary antibody (1:20,000 dilution) for 1 h at room temperature. Immunoreactive bands were detected with a Western Blot Chemiluminescence Reagent Kit and Kodak BioMax MR film. Immunoreactive bands and band intensities were processed by Photoshop software.

2.2.7. Solid-phase extraction

The widely used method of C_{18}-column solid-phase extraction [77] was used for sample preparation with slight modifications. Briefly, ethanol (175 μl/ml sample) and acetic acid (20 μl/ml sample) were added to the samples, mixed, and centrifuged at 1500 rev./min for 3 min. The amounts of ethanol and acetic acid in the samples are important for extraction recovery of prostaglandins and other arachidonic acid metabolites [77,78]. These amounts of ethanol and acetic acid are optimal for most prostaglandins. The supernatant was applied to the C_{18} Bond Elut SPE columns that had been preconditioned with 5 ml of ethanol and then 15 ml of water. The samples were washed with 20 ml of water and the columns were allowed to run dry. Then, the samples were eluted from the columns with 5 ml of ethyl acetate. The ethyl acetate layer was removed from the water layer at the bottom of the reaction tubes. The water layer was then extracted twice with 1 ml of ethyl acetate. The ethyl acetate portions were combined for each sample and dried under the stream of nitrogen gas. Then, the sample was redissolved in 25 μl of acetonitrile, transferred to an insert in the sample vial and was ready for LC–MS analysis or frozen at −80 °C.

To determine the recovery of prostaglandins, the HEPES buffer incubation media of prostate cancer cells containing 1% bovine serum albumin (fatty acid free) were equally divided into 5-ml aliquots. Known amounts of prostaglandin standards (50 and 200 pg each) were added into two samples for each concentration. Three separate sets of recovery experiments were performed. The samples were extracted and analyzed for the recovery.

2.2.8. Liquid chromatographic–mass spectrometric measurements

Samples were analyzed by using liquid chromatography–electrospray ionization-mass spectrometry (LC–ESI-MS, Agilent 1100 LC/MSD, SL model). The samples were separated on a reverse phase C_{18} column (Kromasil, 250×2 mm, Phenomenex) using water–acetonitrile with 0.1% acetic acid as a mobile phase at the flow-rate of 0.3 ml/min. The mobile phase gradient started at 35% acetonitrile, linearly increased to 100% acetonitrile in 35 min, and held for 10 min. Drying gas flow of electrospray chamber was 12 l/min, drying gas temperature was 350 °C, nebulizer pressure was 35 p.s.i.g, vaporizer temperature was 325 °C, and fragmentor voltage was 120 V. The detection was made in the positive mode. For quantitative measurement, the *m*/*z* 353, 335, 335, 319, 317, 357, 339, 339, and 323 were used for measuring of 6-keto-$PGF_{1\alpha}$, PGD_2, PGE_2, $PGF_{2\alpha}$, PGJ_2, [$^2H_4$]6-keto-$PGF_{1\alpha}$, [$^2H_4$]PGD_2, [$^2H_4$]PGE_2, and [$^2H_4$]$PGF_{2\alpha}$, respectively. [$^2H_4$]PGD_2 was used as an internal standard for quantitation of both PGD_2 and PGJ_2. The standard curves were typically constructed over the range of 5 to 1000 pg per injection. The concentrations of these prostanoids in the sam-

ples were calculated by comparing their ratios of peak areas of compounds to the internal standards with the standard curves.

3. Results and discussion

3.1. Liquid chromatographic–mass spectrometric characteristics

Prostaglandins in this study have similar molecular structures with some differences in their functional groups as shown in Fig. 1. These functional groups provide distinct chromatographic and mass spectral characteristics of the compounds. Fig. 2 shows the positive ion mass spectra of 6-keto-$PGF_{1\alpha}$, PGD_2, PGE_2, $PGF_{2\alpha}$, PGJ_2 standards. Under the conditions used, all prostaglandins exhibited the ions that correspond to the loss of x molecules of water, $[M-xH_2O+H]^+$. 6-Keto-$PGF_{1\alpha}$ has m/z 353 ($[M-H_2O+H]^+$) as the most abundant ion and two minor m/z 335 and 317, which correspond to the subsequent loss of more water molecules. PGD_2 and PGE_2 have two hydroxyl groups, and they exhibited the loss of one and two water molecules. $PGF_{2\alpha}$ contains three hydroxyl groups, and it exhibited three major ions with m/z 337, 319, and 301, which correspond to the loss of one, two, and three water molecules, respectively. PGJ_2 has only one hydroxyl group and it has only one major ion of m/z 317 ($[M-H_2O+H]^+$). Most prostaglandins have the highest abundance of m/z resulting from the loss of one water molecule except $PGF_{2\alpha}$. $PGF_{2\alpha}$ has the highest abundance at m/z 319 ($[M-2H_2O+H]^+$). Thus, the abundance of this m/z was used for quantitation measurement of this prostanoid.

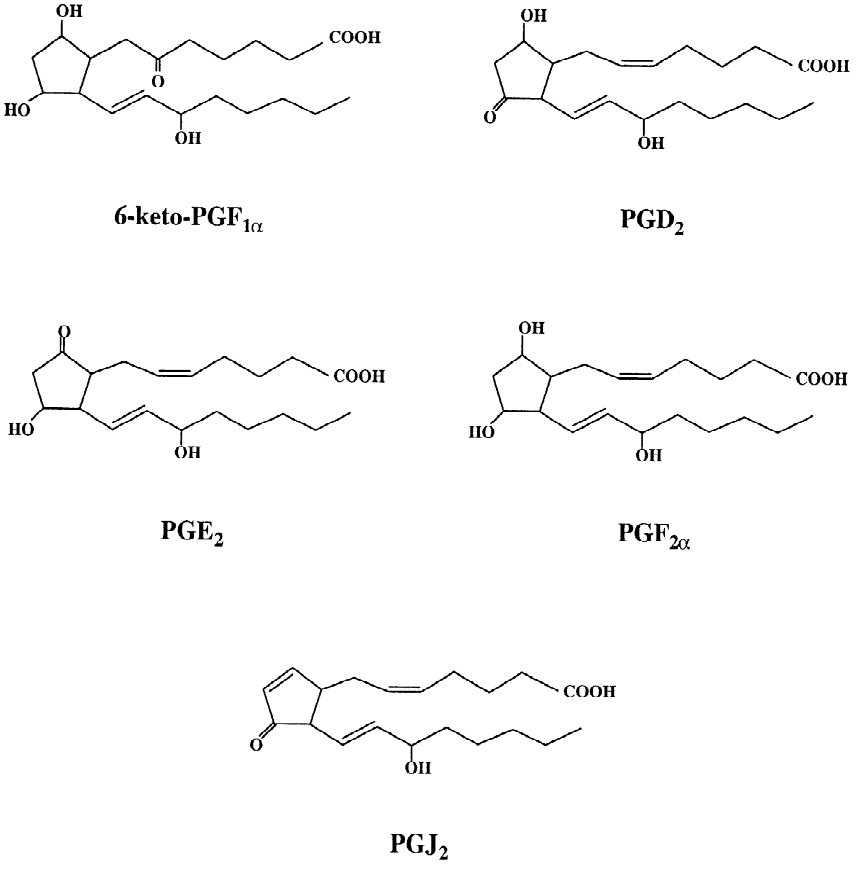

Fig. 1. Molecular structures of 6-keto-$PGF_{1\alpha}$, PGD_2, PGE_2, $PGF_{2\alpha}$, and PGJ_2.

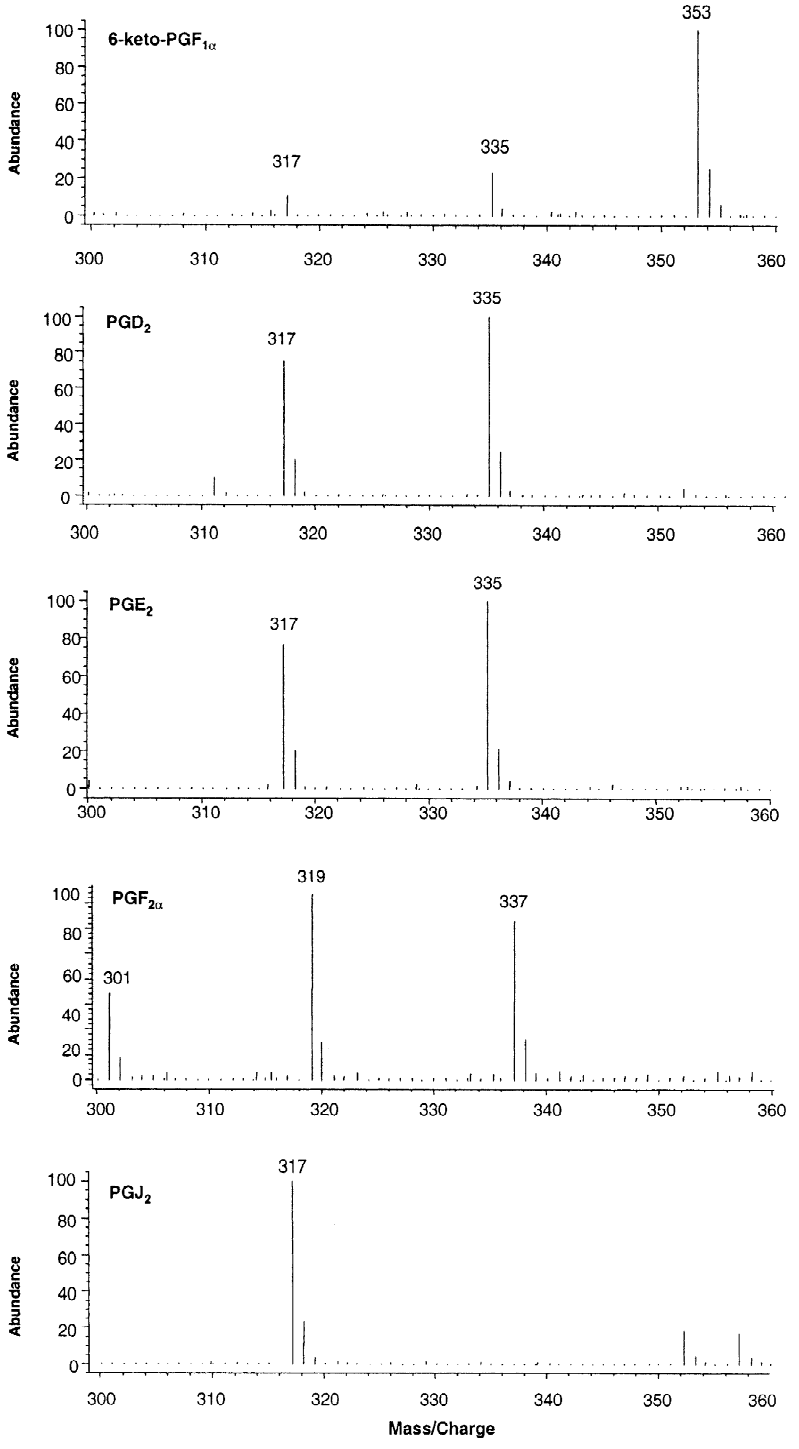

Fig. 2. The positive ion-electrospray ionization-mass spectra of 6-keto-$PGF_{1\alpha}$, PGD_2, PGE_2, $PGF_{2\alpha}$, and PGJ_2 (see the conditions in the text). These spectra exhibit the loss of water molecules from the compounds. The number of detected major ions from each prostaglandin corresponds to the number of hydroxyl groups present on the molecules. The m/z with the highest abundance was used for quantitation of each prostaglandin.

The differences in functional groups and their positions are sufficient for a good chromatographic separation by a reverse phase C_{18} HPLC column using water–acetonitrile mobile phase. The selected ions of 6-keto-$PGF_{1\alpha}$ and [$^2H_4$]6-keto-$PGF_{1\alpha}$ (m/z 353 and 357) were detected from 0 to 7.75 min; $PGF_{2\alpha}$ and [$^2H_4$]$PGF_{2\alpha}$ (m/z 319 and 323) were detected from 7.75 to 9.80 min; PGD_2, PGE_2, [$^2H_4$]PGD_2 and [$^2H_4$]PGE_2 (m/z 335 and 339) were detected from 9.80 to 14.50 min; and PGJ_2 (m/z 317) was detected from 14.50 min to 20 min. Fig. 3A shows the selected ion chromatograms of 6-keto-$PGF_{1\alpha}$, PGD_2, PGE_2, $PGF_{2\alpha}$, PGJ_2 standards at 500 pg each on the column. Their retention times were 4.39, 11.16, 10.08, 8.75, and 15.56 min, respectively. The internal standards, deuterated prostaglandins, eluted at slightly shorter retention times than the non-deuterated prostaglandins. The retention times for [$^2H_4$]6-keto-$PGF_{1\alpha}$, [$^2H_4$]PGD_2, [$^2H_4$]PGE_2, and [$^2H_4$]$PGF_{2\alpha}$ were 4.38, 11.10, 10.04, and 8.71 min, respectively. Examples of selected ion chromatograms of samples from invasive human prostate cancer cells (PC-3-S) and bovine coronary artery endothelial cells stimulated with bradykinin (10^{-6} M) are shown in Fig. 3B and C, respectively.

3.2. Standard curves and sensitivities

Table 1 shows the statistical analysis parameters of standard curves from six separate runs of 6-keto-$PGF_{1\alpha}$, PGD_2, PGE_2, $PGF_{2\alpha}$ and PGJ_2. The ratios of peak areas of m/z 353 to 357, 335 to 339, 335 to 339, 319 to 323, and 317 to 339 were plotted as a function of the amount of 6-keto-$PGF_{1\alpha}$, PGD_2, PGE_2, $PGF_{2\alpha}$, and PGJ_2, respectively. The curves are linear over the range of 5 to 1000 pg on the column. The detection limits (signal-to-noise ratio = 3) of these prostaglandins are 1.0 pg. This detection limit of PGE_2 is similar to or better than the previous reports of 1.3 pg [39] or 20 pg on the column [32]. The use of a positive mode of detection may contribute to the higher detection limit of PGE_2 of the latter [32]. All curves have slight positive intercepts due to the small contribution of the non-deuterated prostanoids present in the deuterated internal standards and they were corrected in calculation.

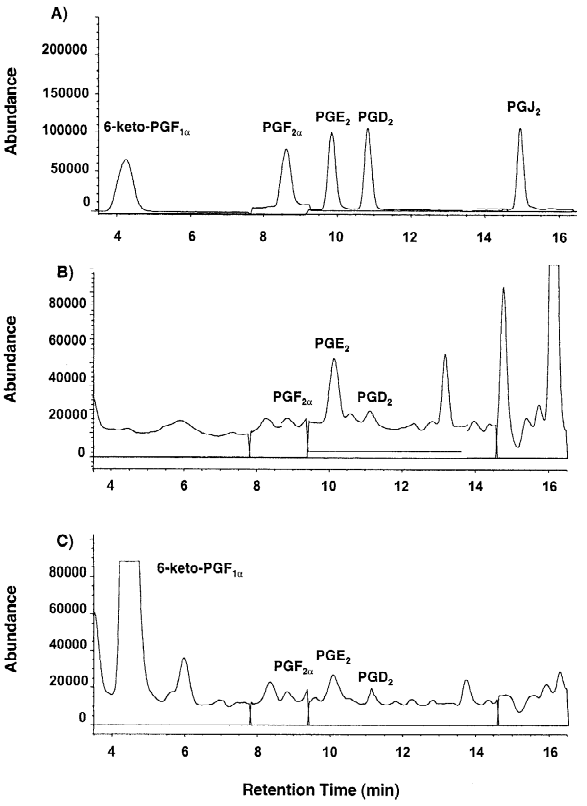

Fig. 3. LC–ESI-MS selected ion chromatograms of 6-keto-$PGF_{1\alpha}$, PGD_2, PGE_2, $PGF_{2\alpha}$, and PGJ_2. The m/z 353, 335, 335, 319, and 317 were detected for 6-keto-$PGF_{1\alpha}$, PGD_2, PGE_2, $PGF_{2\alpha}$, and PGJ_2, respectively. (A) The selected ion chromatograms of prostaglandin standards at 500 pg each. (B) The selected ion chromatograms of PC-3-S (invasive) cells indicating the peaks co-migrated with PGD_2, PGE_2, and $PGF_{2\alpha}$ standards. These peaks correspond to 32.8, 204.5, and 19.4 pg for PGD_2, PGE_2, and $PGF_{2\alpha}$, respectively. (C) The selected ion chromatograms of ECs stimulated with bradykinin (10^{-6} M) indicating the peaks co-migrated with 6-keto-$PGF_{1\alpha}$, PGD_2, PGE_2 and $PGF_{2\alpha}$ standards. These peaks correspond to 1958, 28.4, 97.1, and 21.6 pg for 6-keto-$PGF_{1\alpha}$, PGD_2, PGE_2, and $PGF_{2\alpha}$, respectively.

The recoveries for 6-keto-$PGF_{1\alpha}$, PGD_2, PGE_2, $PGF_{2\alpha}$ and PGJ_2 are 99.2±7.6, 98.4±4.2, 95.7±6.2, 91.2±5.1, and 69.6±6.1%, respectively. The low recovery of PGJ_2 using 15% ethanol in the samples for solid-phase extraction is similar to the recoveries of other hydrophobic eicosanoids such as epoxy-eicosatrienoic acids [79]. The amount of ethanol in the samples was optimized for the extraction of most prostaglandins except PGJ_2. Increase in ethanol content in the samples for solid-phase extraction will

Table 1
Statistical analysis parameters of the calibration curves from 5 to 1000 pg ($N=6$)

Parameters	6-keto-$PGF_{1\alpha}$	PGD_2	PGE_2	$PGF_{2\alpha}$	PGJ_2
Standard error of slope	6.326×10^{-6}	9.11×10^{-6}	4.517×10^{-6}	7.225×10^{-6}	7.635×10^{-6}
Standard error of y intercept	2.553×10^{-3}	2.680×10^{-3}	1.823×10^{-3}	2.916×10^{-3}	3.081×10^{-3}
Correlation coefficient	0.9999	0.9997	0.9999	0.9998	0.9996
Variance at 5 pg[a]	18%	16%	17%	13%	22%
Variance at 50 pg[a]	8%	9%	7%	9%	8%
Variance at 500 pg[a]	3%	2%	3%	2%	4%

[a] Calculated from six samples.

result in a decrease in the recoveries for other more polar prostaglandins [77]. Since no deuterated PGJ_2 was available and $[^2H_4]PGD_2$ was used as internal standard, the calculated concentrations of PGJ_2 should be corrected for its recovery.

There is always a certain amount of water remaining in the extraction cartridge, even when vacuum is applied to pull it out. The SPE normally applies organic solvents such as petroleum ether or hexane to get rid of water as well as some non-polar interfering compounds from the sample [77,80]. However, we found that this resulted in high background interference in the LC–MS chromatograms. In this study, the sample was eluted from the SPE cartridge with ethyl acetate and the water layer residue in the sample tube was extracted by ethyl acetate as an additional step [78,79]. This approach gave a cleaner sample than the sample obtained from the direct liquid–liquid extraction or the water removal from the extraction cartridge by organic solvents.

3.3. Production of prostaglandins in human prostate cancer cells

Fig. 4 shows the prostaglandins produced by human prostate cancer cells. Both PC-3-S cells (Fig. 4A) and PC-3-U (Fig. 4B) cells produced predominantly PGE_2, slightly less PGD_2, and a small amount of 6-keto-$PGF_{1\alpha}$ and $PGF_{2\alpha}$. However, PC-3-S cells produced PGE_2 about threefold higher and PGD_2 about twofold higher than PC-3-U cells. Upon treatment of the cells with 10^{-5} *M* indomethacin (a non-specific COX inhibitor) and 10^{-5} *M* NS-398 (a specific COX-2 inhibitor), the concentrations of PGE_2 and PGD_2 were markedly decreased. The concentrations of prostaglandins agree with the results from the immunoblots indicating about three times higher COX-2 expression in PC-3-S cells than PC-3-U cells (Fig. 5). The COX-1 expression was not significantly different between the cells. This indicates that the difference in the production of prostaglandins was due mainly to the COX-2 enzyme. Interestingly, these cells produced very low concentration of 6-keto-$PGF_{1\alpha}$, the stable product of prostacyclin commonly found in many cell types. PGJ_2 was not detected in either of these cells. The peak at 14.82 min (Fig. 3B) was not PGJ_2 because PGJ_2 migrated at 15.56 min. This suggests that these cells produce less prostaglandins that may have antitumor and/or antimetastatic potential. PGD_2 and PGE_2 deserve further investigation for their role in these cells. These results indicate the ability of the technique to differentiate the effects of enzymatic inhibition as well as detect the presence and absence of peaks of the prostaglandins, and it is a useful tool in cancer research.

3.4. Production of prostaglandins by bovine coronary artery endothelial cells

Fig. 6 shows the prostaglandins produced by bovine coronary artery endothelial cells. Bradykinin (10^{-6} *M*) stimulated about two-, three-, and twofold increase in the production of 6-keto-$PGF_{1\alpha}$, PGE_2, and $PGF_{2\alpha}$, by endothelial cells. The production of PGD_2 was small and similar for both control and bradykinin-stimulated endothelial cells. Bovine cor-

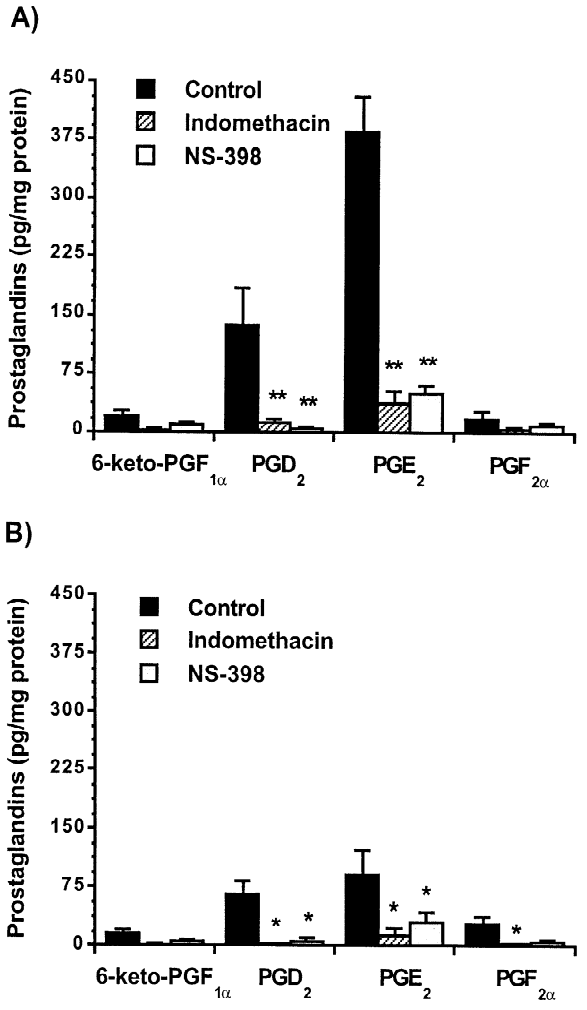

Fig. 4. The production of prostaglandins by human prostate cancer cells. (A) PC-3-S (invasive) cells and (B) PC-3-U (non-invasive) cells. PC-3-S cells produced about three-times more PGE_2 and about two-times more PGD_2 than PC-3-U cells. 6-Keto-$PGF_{1\alpha}$ and $PGF_{2\alpha}$ are about the same in these cells. PGE_2 was the major COX metabolite in these cells. Upon treatment of the PC-3-U cells and PC-3-S cells with indomethacin (10^{-5} *M*) or NS-398 (10^{-5} *M*), the concentrations of prostaglandins markedly decreased. The results are the mean±SEM ($n=25$; seven experiments of 3–4 samples). *Significantly different, $P<0.05$; **significantly different, $P<0.01$.

onary artery endothelial cells produced about 10 times more of 6-keto-$PGF_{1\alpha}$ compared to the other prostaglandins combined. This indicates that these cells have a high expression and/or activity of prostacyclin synthase.

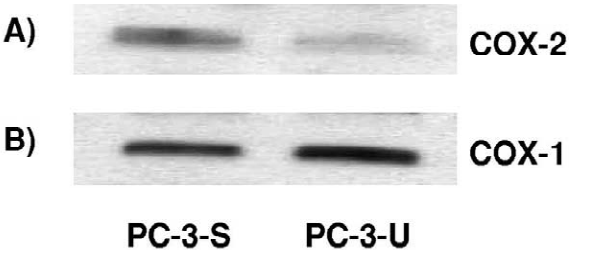

Fig. 5. Immunoblots of human prostate cancer cells. (A) The representative immunoblot of COX-2 in PC-3-S (invasive) and PC-3-U (non-invasive) cells. (B) The representative immunoblot of COX-1 in PC-3-S (invasive) and PC-3-U (non-invasive) cells.

In summary, the LC–ESI-MS in a positive ion detection mode was successfully used for simultaneous determination of 6-keto-$PGF_{1\alpha}$, PGD_2, PGE_2, $PGF_{2\alpha}$, and PGJ_2 in bovine coronary artery endothelial cells (ECs) and human prostate cancer cells (PC-3) with different degree of invasiveness. The method requires the same sample preparation as HPLC with fluorescence detection or GC–MS without sample derivatization. The results indicate that PC-3-S (invasive) cells produced PGD_2 and PGE_2 at

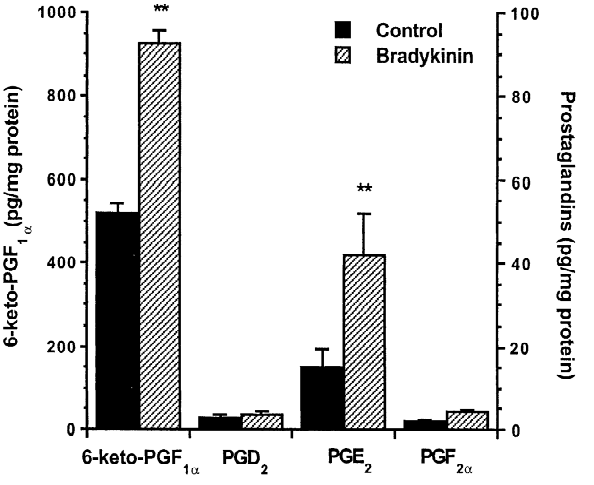

Fig. 6. The production of prostaglandins by bovine coronary artery endothelial cells. The concentration of 6-keto-$PGF_{1\alpha}$ is shown on the left *y*-axis and the concentrations of PGD_2, PGE_2, and $PGF_{2\alpha}$ are shown on the right *y*-axis. Bradykinin (10^{-6} *M*) stimulated about a twofold increase in the production of 6-keto-$PGF_{1\alpha}$, PGE_2, and $PGF_{2\alpha}$. The major COX metabolite of these cells was 6-keto-$PGF_{1\alpha}$. The results are the mean±SEM ($n=12$; three experiments of four samples). *Significantly different, $P<0.05$; **significantly different, $P<0.01$.

about three times higher concentrations than PC-3-U (non-invasive) cells. Bradykinin stimulated about a twofold increase in the production of 6-keto-$PGF_{1\alpha}$, PGE_2, and $PGF_{2\alpha}$ by ECs and 6-keto-$PGF_{1\alpha}$ was the major product of COX in ECs.

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