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Development and validation of a LC/MS/MS method for 6-keto $PGF_{1\alpha}$, a metabolite of prostacyclin (PGI_2)

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ABSTRACT

A sensitive liquid chromatography/tandem mass spectrometry (LC/MS/MS) method was developed for the quantitation of 6-keto $PGF_{1\alpha}$ in human urine and plasma. Prostacyclin (PGI_2) is a locally acting prostanoid, which mediates vasorelaxation and inhibition of platelet aggregation. 6-Keto $PGF_{1\alpha}$ is the most-immediate metabolite of PGI_2.

Samples were spiked with an internal standard (6-keto $PGF_{1\alpha}$-d_4), purified by immuno-affinity chromatography and selected reaction monitoring (SRM) was performed.

Analytical validation of the 6-keto $PGF_{1\alpha}$ assay was performed in urine. This included an assessment of assay precision, recovery, stability, sensitivity and linearity. Urinary 6-keto $PGF_{1\alpha}$ concentrations were also correlated to urinary 2,3-dinor-6-keto $PGF_{1\alpha}$ (PGIM) concentrations using urine samples collected from 16 healthy volunteers. The mean concentration of 6-keto $PGF_{1\alpha}$ in urine (mean ± SD) was 92 ± 51 pg/ml or 168 ± 91 pg/mg creatinine. Overall, there was a statistically significant correlation between urinary 6-keto $PGF_{1\alpha}$ and PGIM ($r^2 = 0.55$, $p \leq 0.001$; slope = 2.7; y-intercept = 130). However, PGIM was approximately 3-fold more abundant than 6-keto $PGF_{1\alpha}$ in urine. In addition, 6-keto $PGF_{1\alpha}$ concentrations were measured in EDTA plasma samples obtained from 7 healthy donors. The mean concentration of 6-keto $PGF_{1\alpha}$ in plasma was 1.9 ± 0.8 pg/ml (±SD).

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1. Introduction

PGI_2 is a prostanoid generated by the metabolism of arachidonic acid by cyclooxygenases (COXs) and PGI_2 synthase [1]. It is produced primarily by the vascular endothelium and it is known to inhibit platelet aggregation and induce vasorelaxation. PGI_2 is a local mediator (autocoid), is short lived, and is rapidly converted into its more stable metabolites. The most immediate hydration by-product of PGI_2 is 6-keto $PGF_{1\alpha}$ (Fig. 1), which is further metabolized into PGIM. Historically, urinary PGIM has been considered a biomarker of systemic production of PGI_2, whereas urinary 6-keto $PGF_{1\alpha}$ has been considered a marker of renal production [2]. However, there is evidence that urinary PGIM could also be of renal origin [3]. Thus, urinary PGIM may not entirely represent the systemic production of PGI_2. Immuno-based methods for the detection of 6-keto $PGF_{1\alpha}$ in plasma have been developed to assess the systemic PGI_2 production [4–6]. Notably, immunoassay-based methods for small molecules such as 6-keto $PGF_{1\alpha}$ are often complicated by cross-reactivity or non-specific binding that could affect the accurate measurements of these molecules in a complex matrix such as plasma. Therefore, measurement of the most immediate PGI_2 metabolite by a sensitive and specific LC/MS/MS method could prove to be a useful analytical tool that can be used to better understand the physiology of PGI_2, as well as interventions affecting its *in vivo* production.

2. Experimental methods

2.1. Reference materials

6-Keto $PGF_{1\alpha}$ standard and anti-6-keto $PGF_{1\alpha}$ affinity resin were purchased from Enzo Life Science (*formerly Assay Designs Inc.* Ann Arbor, MI, USA). The internal standard (IS), 6-keto $PGF_{1\alpha}$-d_4, was obtained from Cayman Chemical Inc. (Ann Arbor, MI, USA). The chemical structures of 6-keto $PGF_{1\alpha}$ and 6-keto $PGF_{1\alpha}$-d_4 are shown in Fig. 1.

2.2. Reagents

Water with 0.1% formic acid and acetonitrile with 0.1% formic acid were purchased from Fisher Scientific (Fair Lawn, NJ, USA). 1 M Tris–HCl pH 7.4 and 5 M NaCl were purchased from Accu-GENE (Walkersville, MD, USA). Ethanol and phosphate buffered saline (PBS) were purchased from Sigma–Aldrich (St. Louis, MO,

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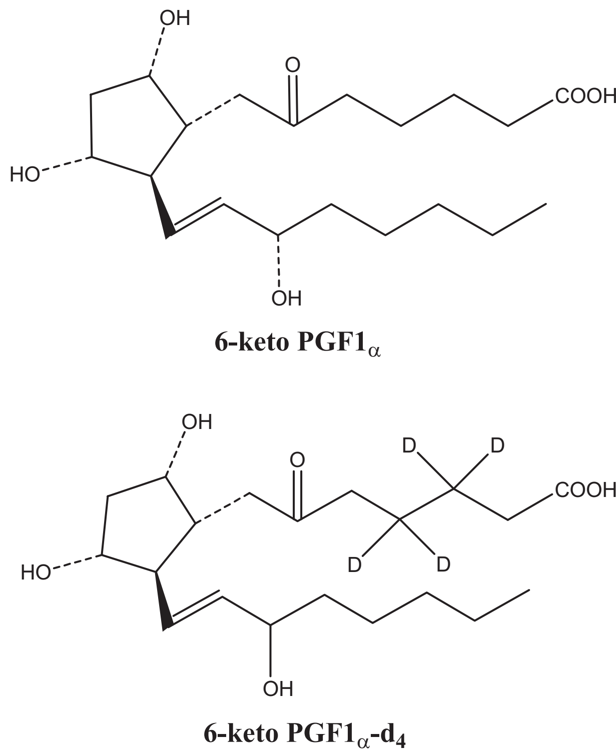

Fig. 1. Chemical structure of 6-keto $PGF_{1\alpha}$ and the IS, 6-keto $PGF_{1\alpha}$-d_4.

USA). Polypropylene columns (3 ml, 0.20 μm) were purchased from Varian, and the Atlantis column (dC18, 3 μm, 2.1 mm × 50 mm) from Waters Corporation. Antigen binding buffer (ABB) consisted of 50 mM Tris–HCl pH 7.4, 0.1 M NaCl.

2.3. Liquid chromatography–tandem mass spectrometry

The Shimadzu LC system (Columbia, MD, USA) consisted of a CBM-20A system controller, 2 LC-20AD pumps (A and B), a DGU-20A$_3$ degasser, a SIL-20AC auto sampler, a CTO-20A column oven, and a FCV-11AL valve unit. The column used was dC18 Atlantis 2.1 mm × 50 mm with a 3 μm particle size. A gradient elution program is shown in Fig. 2. Mobile phase A consisted of water with 0.1% formic acid and mobile phase B consisted of acetonitrile with 0.1% formic acid. The retention time for 6-keto $PGF_{1\alpha}$ and the IS was approximately 4.5 min (Fig. 2). A sample volume was 25 μl was injected at a flow rate of 400 μl/min and a column temperature of 30 °C. Sample was diverted to waste from 0.0 min to 3.6 min, and switched to the mass spec from 3.6 min to 5.0 min to reduce contamination of the instrument. The cycle time of the method was 5 min per injection.

The detector used was an Applied Biosystems (MDS Sciex, Concord, Ontario, Canada) API 5000^{TM} triple quadrupole mass spectrometer operating in selected reaction monitoring (SRM) mode. Samples were ionized via electrospray ionization (ESI) in the negative mode. The ESI source was operated with ion spray voltage at −4500 V and heater temperature at 650 °C. Gas settings were as follows: curtain gas 35, collision gas 8, nebulizer gas 50, and heating gas 60. Dwell time per transition was 200 ms. Unit mass resolution was used in Q1 and Q3 to select the following SRM transitions with collision energies given in parenthesis: m/z 369.2→245.1 (−35 eV)

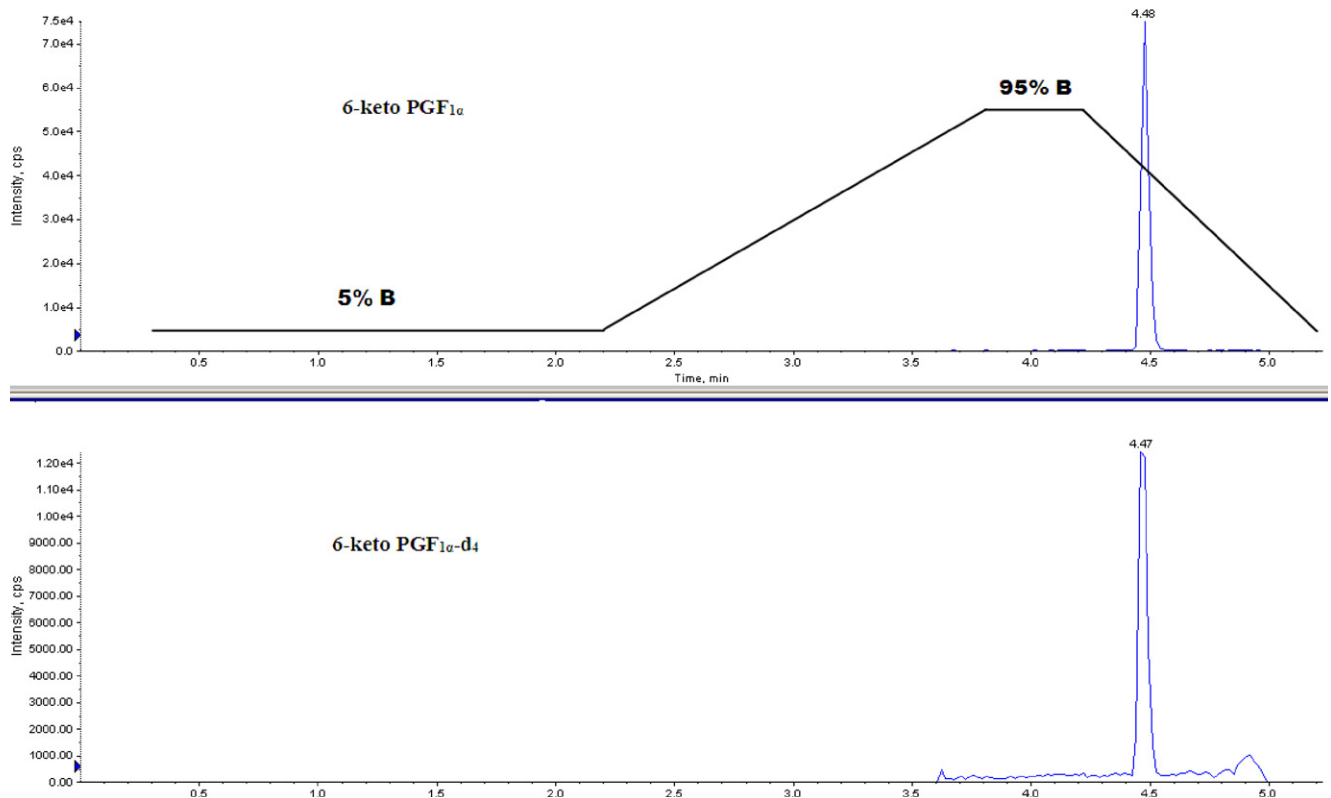

Fig. 2. Peak profile of 6-keto $PGF_{1\alpha}$ and IS with LC gradient.

Table 1
Elution buffer optimization. Bold denotes the condition ultimately selected.

Sample/elution buffer	IS peak area	% Recovery
IS Ctrl (no elution)	43,000	–
50% EtOH/2.5 M acetic acid	14,600	34
2.5 M acetic acid	23,000	53
Methanol	23,100	54
Ethanol	**33,400**	**78**
Methyl acetate	21,600	50
Ethyl acetate	10,400	24

for 6-keto $PGF_{1\alpha}$, and m/z 373.1→249.5 (−40 eV) for internal standard 6-keto $PGF_{1\alpha}$-d_4.

Data were processed using Analyst 1.4.2 software (MDS SCIEX). The calibration curve was generated by plotting peak area ratio between authentic (6-keto $PGF_{1\alpha}$) and IS (6-keto $PGF_{1\alpha}$-d_4) vs. the amount of authentic (pg/ml).

2.4. Preparation of standard curve and samples

Standard curves were generated by performing 2-fold serial dilutions of 500 pg/ml 6-keto $PGF_{1\alpha}$ in ABB to 7.8 pg/ml for the urine assay, or 2-fold serial dilutions of 50 pg/ml 6-keto in ABB to 0.78 pg/ml for the plasma assay. The standard curve is subjected to the same process as samples/QCs, including affinity purification.

On the day of analysis, IS (6-keto $PGF_{1\alpha}$-d_4: 40 pg/ml final concentration in urine; 20 pg/ml in plasma) was added to an aliquot of sample (2.5 ml urine or 5 ml EDTA plasma)/standards/QCs in a 15 ml conical tube, followed by the addition of 300 μl Tris–HCl (pH 7.4, 1.0 M), and 200 μl anti-6-keto $PGF_{1\alpha}$ antibody affinity resin (25%, v/v, suspension in ABB). The mixture was incubated at room temperature for 1 h on a rocker and centrifuged for 20 min at 600 rpm. The 6-keto $PGF_{1\alpha}$ bound to the resin was eluted using an automated RapidTrace (Caliper Life Sciences) instrument with the following procedure: 2× column wash with 2 ml ABB, and elution of 6-keto $PGF_{1\alpha}$ with ethanol. The RapidTrace automates solid phase extraction using positive pressure and precise flow rates for column conditioning, sample loading, and collection. The workstation consisted of ten modules, resulting in a maximum of 100 samples being analyzed in a single run. Eluted samples were dried down under nitrogen gas using a Turbo Vap (Caliper Life Sciences) and reconstituted with 100 μl PBS/EtOH (50:50). Reconstituted samples were injected in the LC/MS/MS.

2.5. Analytical validation study design

Elution efficiency was determined by comparing the areas of IS from samples that were extracted using different extraction buffers, shown in Table 1. Percent recovery was calculated by comparing the IS area from each extracted sample to an IS area of a non-extracted sample.

The optimal amount of resin volume used to extract 6-keto $PGF_{1\alpha}$ from urine samples was determined by spiking the same amount of IS into three identical samples, each processed with increasing amounts of antibody resin, (Table 2). IS area was monitored to determine the most efficient volume.

Table 2
Resin volume optimization. Bold denotes the condition ultimately selected.

Blank sample volume (ml)	Bead volume (μl)	Resin volume (25%, v/v, suspension)	IS peak area (counts)	% Change
2.5	**50**	**200**	**35,600**	–
2.5	100	400	34,400	−3.4
2.5	150	600	32,500	−8.7

In order for a run to be accepted, at least three-quarters of the standard points must have had accuracy within ±15% of the nominal value (±20% at the lower standard). Three quality control (QC) samples containing different levels of 6-keto $PGF_{1\alpha}$ were prepared for assay validation purposes: high (447 pg/ml), medium (162 pg/ml), and low (14 pg/ml). The high and medium QC samples were prepared by spiking 400 pg/ml and 100 pg/ml 6-keto $PGF_{1\alpha}$ into a pooled urine sample, respectively. The low QC consisted of a 1:2 dilution of pooled urine sample with ABB.

Intra-assay precision was determined by measuring 6 individual replicates of the same sample in one run. Inter-assay precision was determined by measuring the same samples over $n = 5$ runs. The limit of quantitation (LOQ) was estimated based on the lowest concentration that can be measured with a signal to noise ratio (S/N) of at least 5. The S/N ratio was measured using the S/N script on Analyst Software (Applied Biosystems). Dilution linearity was measured by serially diluting a urine sample in 1× PBS up to 1:16. Stability was assessed in each QC level by comparing observed concentrations of QCs that have undergone 3 additional freeze/thaw cycles, to QC samples that have not.

2.6. PGIM and creatinine measurement

PGIM was measured by a LC/MS/MS method, which was previously described [7]. Both PGIM and 6-keto $PGF_{1\alpha}$ levels were normalized against creatinine. Creatinine was measured by PPD Laboratories (Wilmington, NC).

2.7. Study subjects

Study subjects used for urine and plasma collection were healthy males, 18–45 years of age. The 16 urine samples were collected over a period of 6 h in the morning. EDTA plasma was obtained from 7 normal healthy volunteers. All samples were obtained with informed consent.

3. Results and discussion

Ethanol was selected as the optimal extraction buffer since it resulted in the highest IS recovery (78%) when compared to the

Table 3
Summary statistics for back-calculated calibration standards and urine QC samples.

Conc (pg/ml)	STD 1 (7.80 pg/ml)	STD 2 (15.6 pg/ml)	STD 3 (31.3 pg/ml)	STD 4 (62.5 pg/ml)	STD 5 (125 pg/ml)	STD 6 (250 pg/ml)	STD 7 (500 pg/ml)	Low	Med	High (pg/ml)
Run 1	7.49	14.8	33.8	64.9	122	257	491	13.4	148	443
Run 2	8.30	16.9	32.7	60.6	132	248	493	13.0	171	460
Run 3	8.73	17.0	33.7	66.1	129	256	481	12.5	171	466
Run 4	7.45	17.1	31.4	63.3	126	250	497	14.3	161	417
Run 5	8.96	17.3	35.1	65.4	129	255	481	15.7	160	450
Mean	8.19	16.6	33.3	64.1	128	253	489	13.8	162	447
S.D.	0.70	1.03	1.38	2.19	3.78	3.96	7.27	1.26	9.52	19.07
% CV	8.5	6.2	4.1	3.4	3.0	1.6	1.5	9.1	5.9	4.3
% DIFF	5.0	6.4	6.4	2.6	2.4	1.2	−2.2	–	–	–

Table 4
Dilution linearity.

Dilution	Obs. conc. (pg/ml)	Conc. × dilution (pg/ml)	% Change from neat
Neat	202	–	
1:2	114	228	13
1:4	56	224	11
1:8	28	224	11
1:16	15	235	16

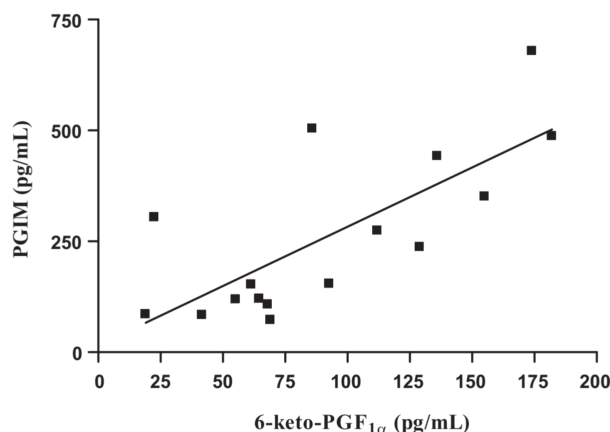

Fig. 3. Correlation between PGIM and 6-keto $PGF_{1\alpha}$ in human urine.

other extraction solvents (Table 1). The most efficient volume of antibody beads was 50 μl, or 200 μl 25% (v/v) resin suspension (Table 2). The LOQ of the assay was determined to be 1.6 pg/ml based on a signal to noise ratio >5.0 for a buffer sample containing 1.6 pg/ml of 6-keto $PGF_{1\alpha}$ ($n = 7$). Intra-assay precision was found to be 11.2%. The inter-assay precision for the urine assay ranged from 4.3 to 9.1% (Table 3). The assay was observed to be linear up to at least a 1:16 dilution (Table 4). Stability of all QC levels was found to be acceptable after 3 freeze/thaw cycles (< 20% change). Spike recoveries for the medium and high QC samples were calculated to be 104% and 101%, respectively.

The concentrations of 6-keto $PGF_{1\alpha}$ in the urine of 16 healthy volunteers ranged from 19 to 182 pg/ml (27–129 pg/mg creatinine). There was a relatively poor correlation, although statistically significant, with urinary PGI-M ($r^2 = 0.55$, $p < 0.001$) (Fig. 3). The concentrations of PGIM in urine were approximately 3-fold higher than 6-keto $PGF_{1\alpha}$. The levels of 6-keto $PGF_{1\alpha}$ in the plasma of 7 healthy volunteers ranged from 0.9 to 3.2 pg/ml. The mean concentration was 1.9 ± 0.8 pg/ml (±SD).

In this study we analytically validated a 6-keto $PGF_{1\alpha}$ assay for human urine. All assay performance characteristics tested were found to be acceptable. This method can also be used to measure 6-keto $PGF_{1\alpha}$ in plasma.

PGIM has been considered a marker of the systemic production of PGI_2, whereas 6-keto $PGF_{1\alpha}$ has been thought to be a marker for the renal production of PGI_2 [8]. However, some PGIM may also be of renal origin since the kidney has been shown to have β-oxidation capability. For instance, isolated rabbit kidney perfused with prostacyclin was shown to produce PGIM, indicating that the kidneys have the necessary enzymatic machinery to produce PGIM from PGI_2 [9]. Thus, urinary PGIM likely reflects both systemic and renal PGI_2 generation [3]. The lack of a good correlation between the 6-keto $PGF_{1\alpha}$ and PGIM levels in urine seen in this study may suggest these two analytes may originate from different organs. One of the aims of this study was to also develop an assay with the sufficient sensitivity to measure 6-keto $PGF_{1\alpha}$ in human plasma. There is one previous report on the measurement of 6-keto $PGF_{1\alpha}$ in human urine using a GC/MS/MS method [10]. The sensitivity of this assay, which was performed by Taylor Technology, Inc. (Princeton, NJ), was not as low as the LC/MS/MS described here (10 pg/ml vs. 1.6 pg/ml). We were able to modify the assay for the quantitation of 6-keto $PGF_{1\alpha}$ in human plasma by using a larger volume of sample (5 ml). With this modification, the LOQ of the assay was reduced to 0.78 pg/ml, which allowed the quantitation of 6-keto $PGF_{1\alpha}$ in all 7 plasma samples tested. To the best of our knowledge, this is the first described mass-spectrometry based assay with the sufficient sensitivity to measure 6-keto $PGF_{1\alpha}$ in human plasma. There have been other studies which employed immuno-based assays to measure 6-keto $PGF_{1\alpha}$ in human plasma. For instance two recent reports, which employed two different assays, indicate that the concentration of 6-keto in human plasma ranged approximately from 200 to 1000 pg/ml. This is approximately 500 times higher than the concentration measured by the LC/MS/MS assay described here. These high levels may not necessarily represent 6-keto $PGF_{1\alpha}$, but could be due to non-specific binding and/or cross reactivity that is observed with the immuno-based methods. These findings further emphasize the need for assays with greater specificity, such as that offered by mass-spectrometry based methods.

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