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Liquid chromatography–tandem mass spectrometric quantification of the dehydration product of tetranor PGE-M, the major urinary metabolite of prostaglandin E_2 in human urine

Jason R. Neale*, Brian J. Dean

Department of Drug Metabolism, Array BioPharma Inc., Boulder, CO 80301, United States

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ABSTRACT

A LC–MS/MS method has been developed to analyze tetranor PGE-M, the major urinary metabolite of PGE_2, that involves the acid-catalyzed dehydration of tetranor PGE-M and its deuterated (d_6) analog followed by LC–MS/MS measurement of the dehydrated tetranor PGE-M product (tetranor PGA-M). We also report a method for quantification of creatinine in urine by LC–MS/MS to normalize tetranor PGE-M concentrations with that of urinary creatinine. These methods were used to study the effect of aspirin on urinary tetranor PGE-M levels in healthy male volunteers. Aspirin did not affect urinary creatinine concentrations but decreased urinary tetranor PGE-M concentrations by approximately 44%.

1. Introduction

Considerable attention has been devoted to studying the role of prostaglandin (PG) E_2 in inflammation and tumor progression, the role of which is supported by high concentrations of the major urinary metabolite of PGE_2, tetranor PGE-M (9,15-dioxo-11α-hydroxy-2,3,4,5-tetranor-prostan-1,20-dioc acid; Fig. 1), in smokers [1] and in patients with lung cancer [2] and colorectal cancer patients [3,4]. PGE_2 may be used as a biomarker of inflammation, disease state, and therapeutic effectiveness. However, direct measurement of PGE_2 in biological fluids is often difficult due to the chemical instability of PGE_2, therefore, analysis of degradants of PGE_2 frequently are performed.

The dehydration and stability of PGE_2 have been well-characterized. The chemical instability of PGE_2 involves the dehydration of the 9,11-ketol to PGA_2 in acidic and alkaline solutions according to first order kinetics [5,6]. At higher pH, PGE_2 also forms the chemically stable bicyclic compound 11-deoxy-13,14-dihydro-15-keto-11,16-cyclo-PGE_2, which has been suggested as a suitable marker for measurements instead of the labile parent compound. However, the bicyclic product exists in at least three epimerically distinct forms [7], making quantification difficult.

Tetranor PGE-M can be used as a biomarker for PGE_2, but robust, high-throughput methods for sample preparation and analysis are lacking. Common methods used to quantify PGE_2 and its metabolites in biological fluids include immunological assays, gas chromatography, high performance liquid chromatography, and mass spectrometry. Although immunoassays are sensitive and can accommodate high-throughput applications, specificity of the antibody is a concern regarding selectivity for the desired antigen, which can lead to variable results and an overestimation of analyte levels. The prostanoids have very similar structures, so cross-reactivity of the antibody may occur, especially with PGE_1, PGE_3, $PGF_{1\alpha}$, and $PGF_{2\alpha}$ when analyzing PGE_2. Recent developments in detection methods for PGE_2 and tetranor PGE-M include chromatographic and spectrometric techniques [2,8–11].

Urine volumes can affect urinary concentrations of excreted compounds. Since the development of the Jaffe reaction and

Abbreviations: PG, prostaglandin; NSAID, non-steroidal anti-inflammatory drug; Tetranor PGE-M, 9,15-dioxo-11α-hydroxy-2,3,4,5-tetranor-prostan-1,20-dioc acid; Tetranor PGA-M, 9,15-dioxo-10-ene-2,3,4,5-tetranor-prostan-1,20-dioic acid; LC–MS/MS, liquid chromatography–tandem mass spectrometry; MRM, multiple reaction monitoring.

* Corresponding author. Tel.: +1 303 386 1338; fax: +1 303 386 1129.
E-mail address: jason.neale@arraybiopharma.com (J.R. Neale).

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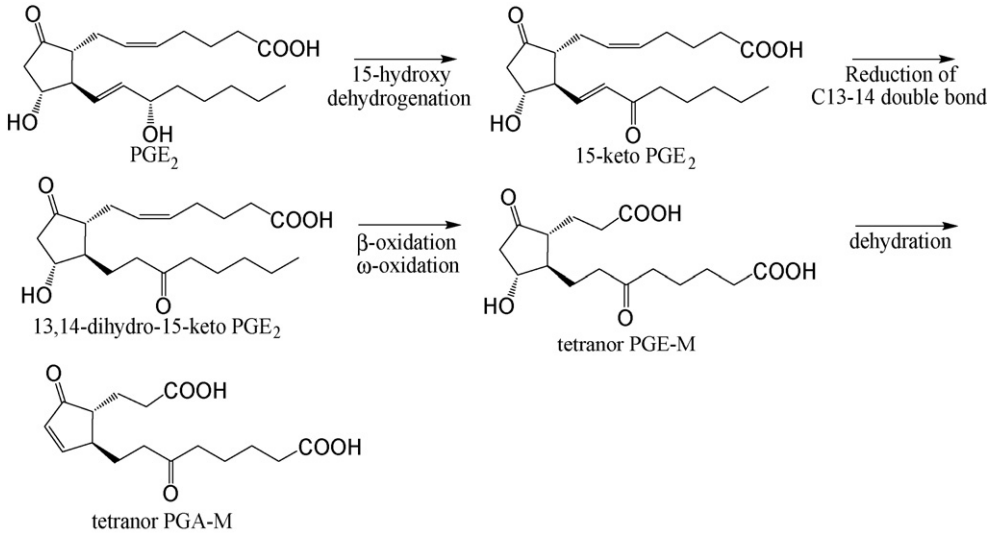

Fig. 1. Reactions involved in the formation of tetranor PGE-M, the major urinary metabolite of PGE_2, and its dehydration product tetranor PGA-M used to assess PGE_2 biosynthesis.

colorimetric assays, creatinine has been analyzed by HPLC and capillary electrophoresis and detected by enzymatic detection methods, fluorescence after post-column derivatization, and mass spectrometry [12]. The presence of interfering substances in the sample matrix and complicated sample preparation procedures make these afore mentioned procedures difficult. However, the specificity of analyte detection afforded by tandem mass spectrometry overcomes many of these problems.

Therefore, we have developed a facile and robust sample preparation method along with a rapid liquid chromatography–tandem mass spectrometric (LC–MS/MS) method utilizing the measurement of acid-catalyzed dehydration of tetranor PGE-M and its deuterated (d_6) analog to tetranor PGA-M as a surrogate for tetranor PGE-M concentrations in urine. Also, we have developed a high-throughput LC–MS/MS method for the urinary analysis of creatinine concentrations in order to normalize urinary tetranor PGE-M concentrations. These validated assays demonstrated a marked reduction in urinary tetranor PGE-M levels in normal, healthy male volunteers after the oral administration of aspirin.

2. Experimental

2.1. Chemicals and reagents

9,15-Dioxo-11α-hydroxy-2,3,4,5-tetranor-prostan-1,20-dioc acid (tetranor PGE-M; MW = 328.4) and 9,15-dioxo-11α-hydroxy-2,3,4,5-tetranor-prostan-1,20-dioc-17,17,18,18,19,19-d_6 acid (tetranor PGE-M-d_6; MW = 334.4) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Formic acid (96%), anhydrous creatinine (MW = 113.12), and creatinine-methyl-d_3 (MW = 116.14) were obtained from Sigma–Aldrich (St. Louis, MO). Water, acetonitrile, and isopropanol were purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ).

2.2. Preparation of standard solutions

Serial dilutions of tetranor PGE-M and tetranor PGE-M-d_6 (1–1000 ng/ml) were prepared from standard stock solutions (500 μg/ml) supplied in methyl acetate, which was evaporated to dryness under nitrogen gas and reconstituted in 10% aqueous formic acid. Serial dilutions of creatinine and creatinine-d_3 (0.1–10,000 μg/ml) were prepared in water from stock solutions (40 mg/ml).

2.3. Biological sample preparation

Written informed consent was obtained from human subjects prior to participation. The healthy male volunteers (age 23–37 years) were instructed to take no anti-inflammatory medications for 48 h before pre-dose urine collection. Pre-dose urine was collected, without preservatives, and pooled for 0–8 h, keeping the samples refrigerated (4 °C) during collection. Subjects took 650 mg of buffered aspirin (Xpect First Aid™, Cintas First Aid and Safety, Mason, OH) at both 8 and 22 h. Post-dose urine was collected, without preservatives, and pooled for 24–32 h, keeping the samples refrigerated (4 °C) during collection. Total urine volume (by weight) at each interval was recorded, and two 40 ml aliquots of the pooled urine were stored at −20 °C until analysis. For the analysis of tetranor PGE-M, 1 ml of urine was spiked with 100 ng tetranor PGE-M-d_6 followed by 100 μl of 96% formic acid. Samples were incubated in capped 1.4 ml ScreenMates™ tubes (Matrix Technologies Corp., Hudson, NH) at 60 °C for 24 h, allowed to return to room temperature, and then applied to Oasis® HLB extraction cartridges (60 mg; Waters, Milford, MA) previously preconditioned with 1 ml of methanol, 1 ml of acetonitrile and, finally, with 1 ml water. Loaded cartridges were washed with 1 ml of 5% aqueous acetonitrile and eluted with 1 ml acetonitrile. Eluants were dried under a stream of dry nitrogen gas and reconstituted in 100 μl of 5% aqueous acetonitrile containing 0.1% formic acid.

Creatinine concentrations were determined by spiking 1 ml of urine with 100 μg creatinine-d_3 as the internal standard. Spiked urine was diluted 100-fold with water before analysis by LC–MS/MS.

2.4. Analysis of tetranor PGE-M

Reversed-phase HPLC of tetranor PGE-M and tetranor PGA-M was performed using Agilent 1200 series quaternary pumps and degasser (Santa Clara, CA) and a CTC PAL autosampler (LEAP Technologies, Carrboro, NC). Mobile phase A was water containing 0.1% formic acid. Mobile phase B was acetonitrile containing 0.1% formic acid. For the qualitative analysis of tetranor PGE-M, analytes were separated at room temperature on a 75 mm × 2.0 mm I.D., 4 μm

particle size, Synergi™ Hydro-RP column (Phenomenex, Torrance, CA) at a flow rate of 0.6 ml/min using a linear gradient of 5–95% B over 10 min. The injection volume was 20 μl. Mass spectrometric detection was performed on an Applied Biosystems 4000 Q Trap® mass spectrometer (Foster City, CA). Zero-grade air was used as the nebulizing and curtain gases at a temperature of 700 °C. The Turbo V™ ion source was operated in negative EMS mode. The ionization energy was set to −4000 V, and the declustering potential was −30.0 V. The scan rate was 4000 amu/s with a range from 100 to 500 amu. Complete system control, data acquisition, and processing were performed using Analyst 1.4.2 software.

For the quantitative analysis of tetranor PGA-M, the HPLC column used at room temperature was an XTerra® C_{18} column (30 mm × 3.0 mm I.D., 3.5 μm particle size; Waters, Milford, MA) with the following gradient: 5–35% B over 0.1 min; 35–45% B over 1.7 min; and 45–95% B over 0.1 min. The flow rate was 0.75 ml/min, and the injection volume was 5 μl. An Applied Biosystems 4000 Q Trap® mass spectrometer was used for detection. Zero-grade air was used as the nebulizing and curtain gases at a temperature of 700 °C. The Turbo V™ ion source was operated in negative multiple reaction monitoring (MRM) mode. Ionization energy was set to −4000 V. Transitions monitored for tetranor PGA-M were *m/z* 309 → *m/z* 291 with a declustering potential set at −55.0 V and a collision energy of −25.0 V and *m/z* 309 → *m/z* 143 with a declustering potential of −60.0 V and a collision energy of −30.0 V. For tetranor PGA-M-d_6, the transitions monitored were *m/z* 315 → *m/z* 297, (declustering potential −60.0 V; collision energy −25.0 V) and *m/z* 315 → *m/z* 149 (declustering potential −50.0 V; collision energy −35.0 V). The on-column lower limit of detection of tetranor PGA-M was approximately 20 pg (signal/noise ratio of 4:1), and the on-column lower limit of quantitation was approximately 100 pg (signal/noise ratio of 10:1). Complete system control, data acquisition, and processing were performed using Analyst 1.4.2 software. The paired Student *t*-test was used to determine statistical significance ($p < 0.05$).

2.5. Analysis of creatinine

Analysis of creatinine was performed at room temperature using a CTC PAL autosampler and Agilent 1200 series quaternary pumps and degasser. Chromatography was achieved using a PVA-Sil™ column (50 mm × 4.6 mm I.D.; 5 μm particle size; Waters, Milford, MA). The injection volume was 2.5 μl, and the flow rate was 2 ml/min. Mobile phase A was water containing 0.1% formic acid. Mobile phase B was acetonitrile containing 0.1% formic acid. Chromatography was achieved at room temperature using the linear gradient of 90–5% B over 1.2 min. An Applied Biosystems 4000 Q Trap® mass spectrometer was used to detect creatinine and its deuterated internal standard. Zero-grade air was used as the nebulizing and curtain gases at a temperature of 700 °C. The Turbo V™ ion source was operated in positive the MRM mode. The ionization energy was set to 3000 V; the declustering potential was 45.0 V, and the collision energy was 20.0 V. Transitions monitored were *m/z* 114 → *m/z* 86 for creatinine and *m/z* 117 → *m/z* 89 for creatinine-d_3. Complete system control, data acquisition, and processing were performed using Analyst 1.4.2 software. The paired Student *t*-test was used to determine statistical significance ($p < 0.05$).

2.6. Intra- and inter-day variability and recovery

Standard solutions of tetranor PGE-M were prepared in 10% aqueous formic acid and spiked with 100 ng/ml of tetranor PGE-M-d_6 in five replicates and incubated at 60 °C for 24 h on 3 consecutive days at concentrations of 10, 30, 100, 300, and 1000 ng/ml. The acceptable accuracy and precision were each 10%. Recovery was tested by analysis of tetranor PGE-M concentrations before and after solid phase extraction as described above.

Creatinine standard solutions were prepared in water in five replicates spiked with 10 μg/ml of creatinine-d_3 on 3 consecutive days at the following concentrations: 1, 3, 10, 30, and 100 μg/ml. The acceptable accuracy and precision were each 10%.

3. Results and discussion

3.1. Tetranor PGE-M stability

Tetranor PGE-M is chemically unstable, undergoing acid-catalyzed dehydration [13], similar to PGE_2, to a dehydrated product designated herein as tetranor PGA-M (9,15-dioxo-10-ene-2,3,4,5-tetranor-prostan-1,20-dioic acid; Fig. 1). The method described in this present paper does not directly quantify tetranor PGE-M but rather tetranor PGA-M. A chromatogram of authentic tetranor PGE-M (Fig. 2A) shows that tetranor PGE-M degrades to the dehydrated product tetranor PGA-M after 24 h in water at room temperature (Fig. 2B). The degradation of tetranor PGE-M also is dependent upon temperature and pH. Based on previously reported stability results for prostaglandins [5,6,14,15], samples in the current method were acidified with 10% formic acid to minimize the formation of multiple products observed at higher pH and to force the degradation of tetranor PGE-M to tetranor PGA-M. Greater than 93% of tetranor PGA-M was formed from tetranor PGE-M after 24 h in 10% aqueous formic acid at 60 °C (Fig. 2C). Further chemical degradation of tetranor PGA-M proceeds to at least four other products after 80 h in 10% aqueous formic acid at 60 °C (Fig. 2D). Although the degradation of PGE_2 proceeds beyond a single dehydrated product at high temperature and after extended sample incubation, the addition of deuterated tetranor PGE-M as the internal standard at the beginning of sample preparation ensures accurate results. A key technical point in the method described herein is that samples must be collected and stored correctly to produce accurate and reliable results. In-source dehydration of analytes prevented conclusive assignment of masses for other peaks using LC–MS/MS. The structure of tetranor PGA-M was determined by NMR analysis (Supplemental data). Based on these results, 10% formic acid was added to samples containing tetranor PGE-M samples, which were then incubated for 24 h at 60 °C to yield tetranor PGA-M.

3.2. Tetranor PGE-M method validation

The deprotonated tetranor PGA-M and tetranor PGA-M-d_6 were detected at *m/z* 309 and *m/z* 315, respectively, by negative ion electrospray mass spectrometry. Collision-induced dissociation of the $[M+H]^-$ ions produced abundant fragment ions of *m/z* 291 and *m/z* 143 for tetranor PGA-M and *m/z* 297 and *m/z* 149 for tetranor PGA-M-d_6. The fragment ions correspond to $[M-H-H_2O]^-$ and $[M-H-H_2O-C_9H_8O_2]^-$, respectively [10]. Chromatographic conditions were optimized to separate co-eluting peaks, and two mass transitions were monitored for both analyte (tetranor PGA-M) and internal standard (tetranor-PGAM-d_6). Two major peaks were present in the MRM transition *m/z* 309 → *m/z* 291 for tetranor PGA-M (Fig. 3A) and *m/z* 315 → *m/z* 297 for tetranor PGA-M-d_6 (Fig. 3C). However, only one predominant peak was detected in the MRM transition *m/z* 309 → *m/z* 143 for tetranor PGA-M (Fig. 3B) and *m/z* 315 → *m/z* 149 for tetranor PGA-M-d_6 (Fig. 3D). Although signal intensities were greater for the transitions monitoring the loss of 18 amu, the transitions monitoring the loss of 166 amu were more selective and had lower signal/noise ratios, thus, MRM transitions *m/z* 309 → *m/z* 143 and *m/z* 315 → *m/z* 149 were used for quantification.

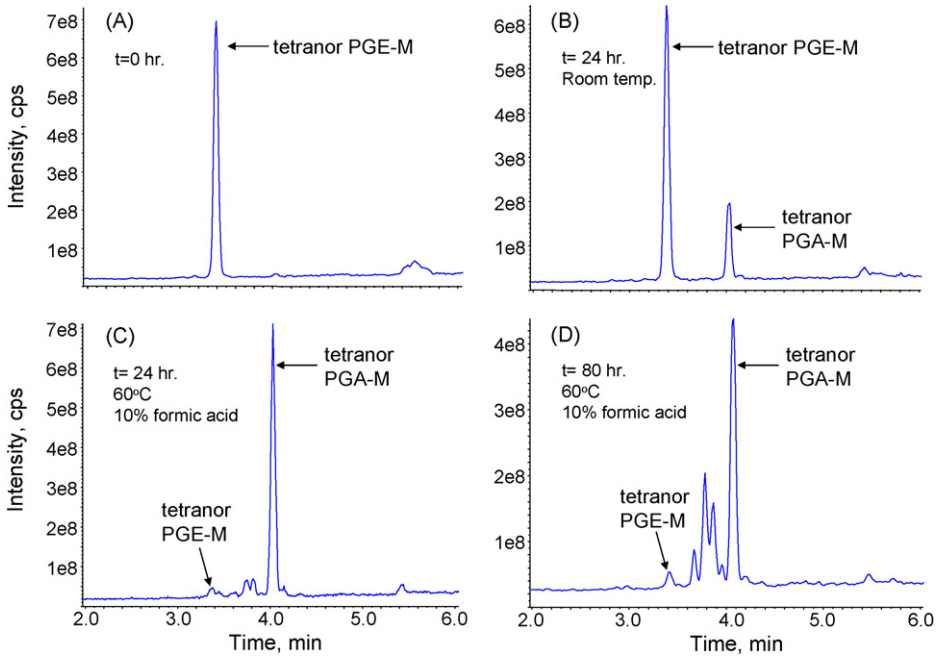

Fig. 2. Negative ion spray total ion chromatogram (TIC) (100–500 amu) LC–MS analysis of the acid-catalyzed degradation of tetranor PGE-M over time. Tetranor PGE-M (A) was incubated in water for 24 h at room temperature (B), 24 h at 60 °C in 10% aqueous formic acid (C), or 80 h at 60 °C in 10% aqueous formic acid (D).

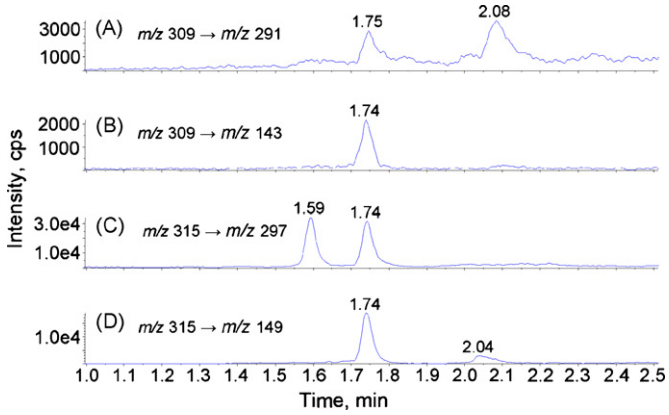

Fig. 3. Representative MRM chromatograms obtained in negative ion mode of tetranor PGA-M mass transitions mode m/z 309 → m/z 291 (A) and m/z 309 → m/z 143 (B) in a urine sample from a healthy male human volunteer after oral aspirin treatment and the internal standard tetranor PGA-M-d_6 mass transitions m/z 315 → m/z 297 (C) and m/z 315 → m/z 149 (D).

To assess the linearity of response for the assay, different concentrations of tetranor PGE-M (100, 300, or 1000 ng/ml) and tetranor PGE-M-d_6 (1–1000 ng/ml) were spiked in triplicate into normal human urine. These spiked samples were acidified and incubated for 24 h at 60 °C. The ratio of observed peak areas of mass transitions m/z 315 → m/z 149 to m/z 309 → m/z 143 were plotted against the spiked concentration ratio of tetranor PGE-M-d_6 to tetranor PGE-M. The lower limit of detection of tetranor PGA-M was approximately 20 pg (signal/noise ratio of 4:1), which is about 100-fold lower than the amount in normal human urine. The response was linear over a 1000-fold range. The linear equation representing the regression line was $y = 0.8078x + 0.0399$ ($r^2 = 0.998$).

Values for the intra- and inter-day precision, accuracy, and recovery for tetranor PGA-M are listed in Table 1. The intra-day precision ranged from 1.6% to 7.3%, and the intra-day accuracy ranged from 97.8% to 106.6%. The inter-day precision ranged from 2.4% to 9.9%, and the inter-day accuracy ranged from 103.9% to 107.7%. Recoveries of tetranor PGA-M following solid phase extraction ranged from 96.0% to 100.4%.

3.3. Creatinine method validation

In order to compare inter- and intra-individual tetranor PGE-M levels as measured by tetranor PGA-M, results have been normalized to the excretion of urinary creatinine. Collision-induced dissociation of the $[M-H]^+$ ions produced abundant fragment ions of m/z 86 and m/z 72 for creatinine and m/z 89 and m/z 75 for creatinine-d_3. The loss of 28 amu from their respective $[M+H]^+$ ions was chosen as the mass transition used in the MRM scans to quantify creatinine concentrations in urine. Representative MRM

Table 1
Inter- and intra-day accuracy, precision, and recovery for tetranor PGE-M measured as tetranor PGA-M

Theoretical concentration (ng/mL)	Inter-day			Intra-day			Recovery (%)
	Mean measured concentration (ng/mL)	Accuracy (%)	Precision (%CV)	Mean measured concentration (ng/mL)	Accuracy (%)	Precision (%CV)	
10	10.4	103.9	9.9	9.8	97.8	7.3	100.4
30	32.3	107.7	3.3	32.0	106.6	3.8	96.2
100	105.0	105.0	2.9	104.6	104.6	3.7	96.5
300	313.1	104.4	2.8	313.3	104.4	4.1	99.3
1000	1041.9	104.2	2.4	1051.7	105.2	1.6	96.0

Number of replicates = 5.

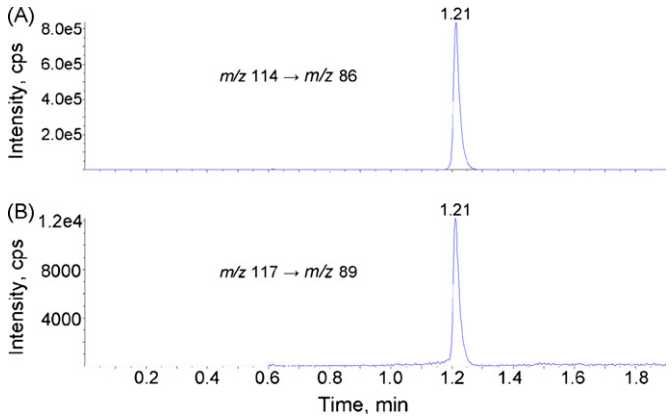

Fig. 4. Representative MRM chromatograms of creatinine mass transition m/z 114 → m/z 86 (A) in a urine sample from a healthy male human volunteer after oral aspirin treatment together with the internal standard creatinine-d_3 mass transition m/z 117 → m/z 89 (B).

chromatograms are shown in Fig. 4. Due to the high amounts of creatinine that may be excreted into the urine (greater than one gram in 24 h) and the matrix effect of undiluted urine causing poor and unreproducible chromatography and decreased signal intensity (data not shown), urine samples were diluted 100-fold with water.

The high endogenous urinary creatinine concentrations would have interfered with the assessment of the linearity of the assay, hence, water was spiked with different concentrations of creatinine (0.1, 1, 10, or 100 μg/ml) and varying concentrations of creatinine-d_3 (0.1–100 μg/ml). The ratio of observed peak areas of mass transitions m/z 117 → m/z 89 to m/z 114 → m/z 86 were plotted against the spiked concentration ratio of creatinine-d_3 to creatinine. The response was linear over a 1000-fold range, and the equation depicting the linear regression line was $y = 0.727x + 1.1991$ ($r^2 = 0.999$).

Intra- and inter-day precision and accuracy values for creatinine are listed in Table 2. The intra-day precision ranged from 0.4% to 1.3%, and the intra-day accuracy ranged from 94.4% to 106.9%. The inter-day precision ranged from 0.5% to 2.2%, and the inter-day accuracy ranged from 95.0% to 104.2%.

Reported values for creatinine are highly dependent upon the age, gender, lean body mass, physical activity, and diet of the patient and can be quite variable, ranging from 1 to 3.3 g of creatinine in a typical 24 h sample of urine [12]. Our measured concentrations (1.4–2.8 g/24 h) using the LC–MS/MS method lie within reported ranges. Measurement by LC–MS/MS provides a simple, accurate, and alternative method for those labs equipped with tandem mass spectrometers. The only sample processing steps are addition of deuterated internal standard and sample dilution.

3.4. Effects of orally administered aspirin on urinary tetranor PGA-M concentrations

An important application for these assays is the assessment of therapeutic effectiveness and progression of disease. Healthy males ($n = 10$) between the age of 23–37 years were recruited for the study and asked to refrain from strenuous physical activity and avoid eating more than 8 ounces of meat per meal during the study. Urine samples were collected and pooled for 8 h from volunteers before and after aspirin administration (two doses of 650 mg 14 h apart). No significant differences between pre- and post-aspirin treatment on urinary creatinine concentrations were observed (Fig. 5A). However, as shown in Fig. 5B, these assays demonstrate that oral treatment of volunteers with aspirin (2 × 650 mg, 14 h apart) decreased the urinary tetranor PGE-M concentration from 24.0 ± 3.97 ng/mg creatinine (mean ± SEM) to 13.4 ± 1.74 ng/mg creatinine. Seyberth and colleagues have shown that aspirin (2600 mg/day) and indomethacin (150 mg/day) decreased tetranor PGE-M excretion in humans by approximately 45% and 60%, respectively [13]. Murphy and colleagues found that ibuprofen (3200 mg/day) significantly reduced tetranor PGE-M excretion by 66% and that the selective COX-2 inhibitor rofecoxib (50 mg/day) decreased tetranor PGE-M excretion by 62% in healthy individuals [2].

Future studies will use these methods to explore alternative pathways of PGE_2 biosynthesis and to evaluate levels of urinary tetranor PGE-M in humans afflicted with pathophysiological disorders such as cancer and inflammation and the effectiveness of therapy on these diseases.

Table 2
Inter- and intra-day accuracy and precision for creatinine

Theoretical concentration (μg/mL)	Inter-day			Intra-day		
	Mean measured concentration (μg/mL)	Accuracy (%)	Precision (%CV)	Mean measured concentration (μg/mL)	Accuracy (%)	Precision (%CV)
1	1.0	101.9	2.2	1.0	101.4	1.0
3	3.1	104.2	2.1	3.2	105.2	0.4
10	10.4	104.2	1.2	10.7	106.9	1.3
30	30.7	102.5	1.1	31.1	103.7	0.7
100	95.2	95.0	0.5	94.4	94.4	0.7

Number of replicates = 5.

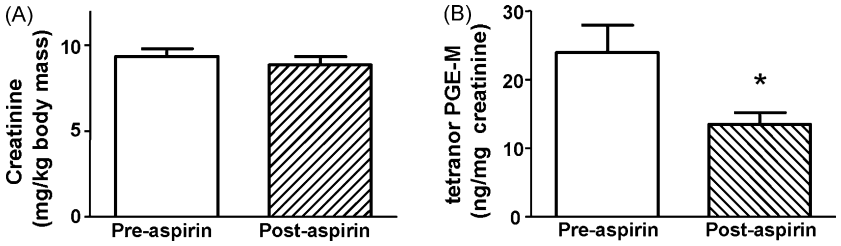

Fig. 5. Effect of oral treatment with aspirin on the urinary excretion of creatinine (A) and tetranor PGE-M (B). Values are mean ± SEM; $n = 10$; $^*p < 0.01$ vs. pretreatment.

4. Conclusions

We have developed novel, robust, and efficient methods for the analysis of urinary tetranor PGE-M as the dehydration product, tetranor PGA-M, and creatinine in human urine using LC–MS/MS. Advantages of the current method for the analysis of tetranor PGE-M over previously published methods include (i) simple sample preparation, (ii) inclusion of a stable isotope as an internal standard at the beginning of sample preparation, and (iii) a faster analytical run time. The application of these methods could greatly facilitate the evaluation of inflammatory disease state in humans as well as the effects of therapeutic intervention.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2008.06.042.

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