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Quantitative determination of 8-isoprostaglandin $F_{2\alpha}$ in human urine using microfluidic chip-based nano-liquid chromatography with on-chip sample enrichment and tandem mass spectrometry

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ABSTRACT

Urinary 8-isoprostaglandin $F_{2\alpha}$ (8-iso$PGF_{2\alpha}$) has been reported as an important biomarker to indicate the oxidative stress status *in vivo*. In order to quantitatively determine the low contents of 8-iso$PGF_{2\alpha}$ (in sub- to low ng mL^{-1} range) in physiological fluids, a sensitive detection method has become an important issue. In this study, we employed a microfluidic chip-based nano liquid chromatography (chip-nanoLC) with on-chip sample enrichment coupled to triple quadrupole mass spectrometer (QqQ-MS) for the quantitative determination of 8-iso$PGF_{2\alpha}$ in human urine. This chip-nanoLC unit integrates a microfluidic switch, a chip column design having a pre-column (enrichment column) for sample enrichment prior to an analytical column for separation, as well as a nanospray emitter on a single polyimide chip. The introduction of enrichment column offers the advantages of online sample pre-concentration and reducing matrix influence on MS detection to improve sensitivity. In this study, the chip-nanoLC consisting of Zorbax 300A SB-C18 columns and Agilent QqQ Mass spectrometer were used for determining 8-iso$PGF_{2\alpha}$ in human urine. Gradient elution was employed for effective LC separation and multiple reaction monitoring (MRM) was utilized for the quantitative determination of 8-iso$PGF_{2\alpha}$ (m/z 353 → 193). We employed liquid–liquid extraction (LLE)/solid-phase extraction (SPE) for extracting analyte and reducing matrix effect from urine sample prior to chip-nanoLC/QqQ-MS analysis for determining urinary 8-iso$PGF_{2\alpha}$. Good recoveries were found to be in the range of 83.0–85.3%. The linear range was 0.01–2 ng mL^{-1} for urinary 8-iso$PGF_{2\alpha}$. In addition, the proposed method showed good precision and accuracy for 8-iso$PGF_{2\alpha}$ spiked synthetic urine samples. Intra-day and inter-day precisions were 1.8–5.0% and 4.3–5.8%, respectively. The method accuracy for intra-day and inter-day assays ranged from 99.3 to 99.9% and 99.4 to 99.7%, respectively. Due to its rapidity, enhanced sensitivity, and high recovery, this chip-nanoLC/QqQ-MS system was successfully utilized to determine the physiological biomarkers such as 8-iso$PGF_{2\alpha}$ in human urine for clinical diagnosis.

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1. Introduction

F_2-isoprostanes (F_2-IsoPs), chemically stable biomarkers of oxidative stress status *in vivo*, have been found to be associated with various diseases [1] including neurodegenerative diseases such as Alzheimer's disease [2,3], heart disease [4], diabetes [5], and cancer [6]. Previous publications have reported that the quantitative determination of plasma or urinary 8-isoprostaglandin $F_{2\alpha}$ (8-iso$PGF_{2\alpha}$, also called $iPF_{2\alpha}$-III or 15-F_{2t}-IsoP), a common F_2-IsoP isomer, was a reliable method to indicate the oxidative stress status *in vivo* [7–9]. Therefore, a sensitive analytical method became an important issue to quantitatively determine the low contents of 8-iso$PGF_{2\alpha}$ (in sub- to low ng mL^{-1} range) in physiological fluids, such as human urine, for clinical diagnosis.

A number of previous studies described various analytical methods for the determination of urinary 8-iso$PGF_{2\alpha}$, including immunoassays such as enzyme-linked immunosorbent assay (ELISA) and radio-immunoassay (RIA) [10–13], gas chromatography–mass spectrometry (GC–MS) [3,7,11,12,14], and liquid chromatography–tandem MS (LC–MS/MS) [8,15–17]. Although immunoassays showed potentially high selectivity to target analytes, cross reactions to other F_2-IsoPs and/or potential influences in biological fluids were frequently observed [11,12]. MS provides the advantages of sensitive detection for quantitative analysis and specific structural characterization for molecular iden-

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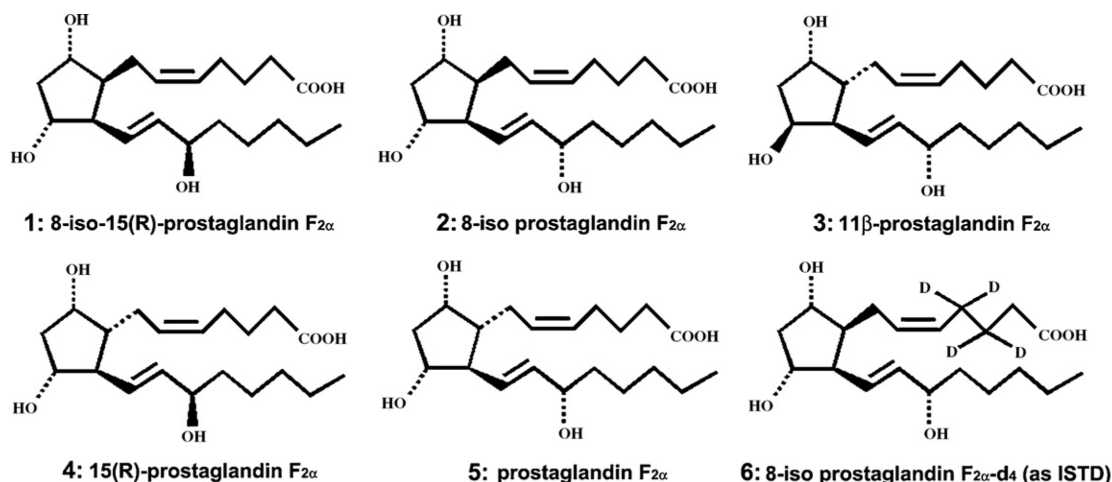

Fig. 1. Chemical structures of F_2-isoprostanes and the ISTD.

tification. GC–MS has been used for determining F_2-IsoPs; however, derivatization of 8-isoPGF$_{2\alpha}$ to achieve efficient GC separation and sensitive MS detection was required [3,7,11,12,14]. HPLC–MS has been utilized for direct analysis of 8-isoPGF$_{2\alpha}$ in human urine [8,16,18,19]. In these reports, the concentration of 8-isoPGF$_{2\alpha}$ found in healthy adults were in a broad range and the reported LOQs (limit of quantitation) were in the proximity of measured urinary 8-isoPGF$_{2\alpha}$ concentration in healthy adults. Thus, a more sensitive analytical method is desired to access the dynamic urinary 8-isoPGF$_{2\alpha}$ contents for clinical study. Recently, Agilent developed a microfluidic chip-nanoLC interface (chip-nanoLC), which incorporated a microfluidic switch, a chip column design having a pre-column (enrichment column) for sample enrichment prior to an analytical column for separation, as well as a nanospray emitter on a single polyimide chip [20,21]. Similar to most home-made or commercial microchip LC systems, this chip-nanoLC unit has been used commonly on analyzing peptides and proteins for proteomic research [22–27]. Only a few publications reported the analysis of small molecules such as anthocyanins on grape skins [28], oligosaccharides in human milk [29], and active pharmaceutical ingredients for drug discovery [30].

In this study, we employed an Agilent chip-nanoLC/triple quadrupole MS (QqQ-MS) system to determine 8-isoPGF$_{2\alpha}$ in human urine. The optimal conditions for MS detection and chip-nanoLC analysis were investigated prior to quantitative analysis. The experimental conditions of MS detection, the effect of additive in mobile phase, the loading capacity and reproducibility of the chip-nanoLC, as well as developing a suitable sample preparation strategy were characterized. The quantitative features of the proposed method, including linearity, method precision and accuracy, limit of detection (LOD), and limit of quantitation (LOQ) were validated under the optimal conditions. Clinical urine samples from a local hospital demonstrated the applicability of the chip-nanoLC/QqQ-MS method for clinical diagnosis.

2. Experimental

2.1. Materials and reagents

HPLC-grade methanol (MeOH) and ethyl acetate (EA) were purchased from Mallinckrodt Baker, Inc. (Paris, KY, USA). Deionized water was obtained from a Milli-Q Integral 5 water purification system (Millipore, Bedford, MA, USA). The F_2-isoprostanes, as shown in Fig. 1, and the internal standard (ISTD; 8-isoPGF$_{2\alpha}$-d$_4$), were obtained from Cayman Chemicals (Ann Arbor, MI, USA). Each working standard was prepared by spiking a stock solution into synthetic urine at a desired concentration. Synthetic urine was prepared using a method described by Miró-Casas et al. [31]. Synthetic urine was prepared by dissolving $CaCl_2$ (0.49 g), NaCl (4.6 g), $MgCl_2{\cdot}6H_2O$ (1.01 g), KCl (1.6 g), Na_2SO_4 (2.3 g), KH_2PO_4 (2.8 g), Na_3·citrate·$2H_2O$ (0.65 g), NH_4Cl (1.0 g), urea (25 g), and creatinine (1.1 g) in 1 l of water. Ammonium hydroxide (NH_4OH), Na_2SO_4, and KH_2PO_4 were from J.T. Baker (Phillipsburg, NJ, USA). $CaCl_2$ was purchased from Merck (Darmstadt, Germany) and KCl was from Fluka (Steinheim, Germany). HCl, $MgCl_2{\cdot}6H_2O$, NaCl, Na_3·citrate·$2H_2O$, NH_4Cl, urea, creatinine, and ammonium acetate (NH_4OAc) were obtained from Sigma–Aldrich (Steinheim, Germany). All urine samples were filtered through a 0.22 μm Durapore membrane filters (Millipore, County Cork, Ireland) prior to extraction. Urine samples were either collected from voluntary healthy adults (age: 22–30 years) or from Alzheimer's disease-diagnosed patients (no age, gender, or other personal information were disclosed), which were kindly provided by the Taipei Veterans General Hospital (Taipei, Taiwan).

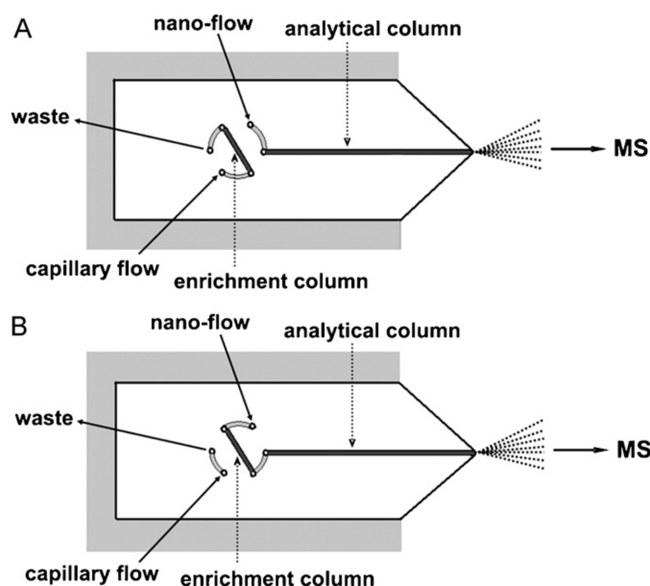

Fig. 2. Schematic graphs of the nanoLC-chip: (A) the flow path under enrichment mode for online sample concentration; (B) the flow path under analysis mode for chromatographic separation.

2.2. Instrumentation and conditions

An Agilent 6410 series Triple Quad LC/MS mass spectrometer (Agilent, Waldbronn, Germany) was utilized for MS detection. The nanospray source on chip-nanoLC was operated under the negative mode with the spray voltage set at 1850 V. A heated gas mixture (N_2:air/3:7; 4 L min^{-1} at 325 °C) was introduced to evaporate solvent from the ionization chamber and obtain stable responses. Agilent Mass Hunter Workstation (version B.02.01) was utilized for system control, data acquisition, and data processing. Multiple reaction monitoring (MRM) was performed to quantitatively determine 8-isoPG$F_{2\alpha}$ in sample solutions. The MRM settings included the following transitions: m/z 353 → 291, 247, 193 for 8-isoPG$F_{2\alpha}$ and m/z 357 → 295, 251, 197 for 8-isoPG$F_{2\alpha}$-d_4. A mini centrifuge (Costar, USA) was used for the study. Oasis HLB 96-well plates from Waters (Milford, MA, USA) were applied for solid phase extraction.

An Agilent 1100 capillary HPLC system was utilized for sample loading onto the enrichment column (Fig. 2) and an Agilent 1200 nano-HPLC system was used to provide nano-flow mobile phase into analytical column for chromatographic separation (Fig. 2). An Agilent micro well-plate auto-sampler with a temperature control module (Agilent 1200 G1330B FC/ALS Therm) was utilized for injection and to keep sample solutions at 4 °C during the analysis. Agilent nanoLC-chips, consisting of reversed-phase Zorbax 300A SB-C18 packed stationary phase (5 μm), included a 500-nL enrichment column and an analytical column (150 mm × 75 μm) for chip-nanoLC/QqQ-MS analysis.

Under enrichment mode (Fig. 2A), the capillary pump delivered a MeOH/H_2O mixture (5:95, v/v) as the initial mobile phase at 4 μL min^{-1} (injection volume of 5 μL) for sample loading onto the enrichment column and additional 4 μL of the initial mobile phase was applied to remove un-retained components from the system. The 6-way μ-switch valve was then switched to the analysis mode (Fig. 2B) for nanoLC separation and a capillary flow with MeOH/H_2O mixture (95:5, v/v) was applied for 5 min to wash the loading flow path and then back to the initial mobile phase composition until next run. The solvents delivered by the nano-HPLC pump for chromatographic separation included solvent C (Milli-Q water containing 0.1 mM NH_4OAc) and solvent D (MeOH). A linear gradient (25–70% D in 16 min) was applied for nanoLC separation and then kept at the final composition for another 2 min before equilibrating the analytical column at 25% D for additional 5 min. The flow rate was set at 300 nL min^{-1}. After complete elution of analytes, the μ-switch valve was switched back to the enrichment mode at 18 min for equilibrating the enrichment column using the capillary flow.

2.3. Extraction procedures

A 1-mL urine sample containing ISTD (8-isoPG$F_{2\alpha}$-d_4, 0.20 ng mL^{-1}) was placed in a test tube for LLE as described by Ohashi and Yoshikawa [32]. After the sample solution was adjusted to pH 3 using 1 M HCl, 3 mL EA were added into the tube, and then the tube was shaking for 2 min. The mixture was centrifuged (3000 rpm, 5 min) and the settled organic phase was transferred into a clean test tube, dried under N_2 gas, and reconstituted in 1 mL Milli-Q water prior to SPE. The SPE procedures were modified from a previous publication [33]. Firstly, the Oasis HLB 96-well plate was conditioned using 1 mL MeOH and 1 mL H_2O before sample loading. After loading, the SPE cartridge was washed consecutively by 1 mL of MeOH/H_2O (5:95, v/v), 1 mL of MeOH/2% NH_4OH (5:95, v/v), and 1 mL of MeOH/H_2O (15:85, v/v). Two milliliters of MeOH containing 0.5% NH_4OH (v/v) was then added to elute analytes from the cartridge. The extracted analytes were dried under N_2 gas, reconstituted in 1 mL Milli-Q water and centrifuged at 10000 rpm for 5 min. Then 5 μL of the sample solution was auto-injected into the chip-nanoLC/QqQ-MS system for determination. The recovery (R) was obtained using the following equation: $R = A_{syn}/A_{w,0} \times 100\%$, where A_{syn} = peak area of 8-isoPGF2$_\alpha$ spiked synthetic urine after extraction and $A_{w,0}$ = peak area of 8-isoPGF2$_\alpha$ spiked Milli-Q water without extraction.

Table 1
MRM parameters for QqQ-MS negative mode detection.

	Transition	Fragmentor (V)	Collision energy (V)
8-Iso prostaglandin $F_{2\alpha}$	353 → 291	144	16
	353 → 247	144	20
	353 → 193[a]	144	22
8-Iso prostaglandin $F_{2\alpha}$-d_4	357 → 295	144	16
	357 → 251[a]	144	20
	357 → 197	144	22

[a] Quantitative ion.

2.4. Method validation process

In order to validate the proposed method for quantitative analysis, the following experiments were performed. A calibration curve of peak area ratio (8-isoPG$F_{2\alpha}$/ISTD) versus 8-isoPG$F_{2\alpha}$ concentration was constructed to evaluate the linearity of the proposed method. Eight spiked synthetic urine solutions (0.01, 0.02, 0.06, 0.10, 0.15, 0.20, 1.00 and 2.00 ng mL^{-1}, each contained 0.20 ng mL^{-1} of ISTD) were prepared and used as calibration standards. Five measurements of each standard solution were performed for method validation. The LOD was defined as the signal-to-noise ratio of 3. The precision and accuracy were obtained by calculating the relative standard deviation (RSD). The precision and accuracy, including intra-day and inter-day experiments, were performed and evaluated using three different concentrations (0.01, 0.10, and 1.00 ng mL^{-1}, each contained 0.20 ng mL^{-1} of ISTD) of spiked synthetic urine samples. The quantitation of 8-isoPG$F_{2\alpha}$ were determined using the peak area ratios of 8-isoPG$F_{2\alpha}$/ISTD.

3. Results and discussion

3.1. Mass spectrometry detection

In order to verify the suitability of using nanospray for ionization, we compared the MS/MS results obtained by this chip-nanoLC/QqQ-MS system (data not shown) with those results reported by other researchers using ordinary LC/ESI-MS [16,33]. Under the negative mode, the $[M-H]^-$ ion was obtained as the base ion for 8-isoPG$F_{2\alpha}$ and ISTD using the condition mentioned in Section 2.2 and Table 1. The same precursor ion and transitions shown in Table 1 as well as the similar fragmentations in MS/MS results were found in the previous publications [16,33]. Therefore, the $[M-H]^-$ ion was chosen as the precursor ion for QqQ-MS measurements and the MRM parameters were set at m/z 353 → 291, 247, and 193 for 8-isoPG$F_{2\alpha}$; m/z 357 → 295, 251, and 197 for 8-isoPG$F_{2\alpha}$-d_4 (ISTD) to perform further experiments for qualitative and quantitative analysis.

3.2. Optimization of chip-nanoLC conditions

Several F_2-isoprostane isomers (shown in Fig. 1), which came from the same metabolic pathway as 8-isoPG$F_{2\alpha}$, were frequently found in human urine along with 8-isoPG$F_{2\alpha}$. Since these F_2-isoprostanes are diastereomers of 8-isoPG$F_{2\alpha}$, same transitions, as shown in Table 1, would also be found in the MS measurements. Therefore, these isomers, together with 8-isoPG$F_{2\alpha}$ and ISTD, were included in the standard solutions to obtain appropriate LC separation conditions for reducing interferences from these isomers

Table 2
Retention time reproducibility of chip-nanoLC/QqQ-MS system.

	Chip 1[a]					Chip 2[a]				
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 1	Day 2	Day 3	Day 4	Day 5
Conc. (ng mL^{-1})										
0.02	15.8	16.0	16.0	16.1	16.1	16.1	15.9	15.7	15.6	15.6
0.06	15.8	15.9	16.1	16.2	16.3	16.0	15.9	15.6	15.6	15.7
0.10	15.8	15.9	16.1	16.1	16.3	16.0	15.8	15.6	15.5	15.6
0.15	15.8	15.9	16.0	16.1	16.0	15.9	15.9	15.6	15.5	15.7
0.20	15.9	15.9	15.9	16.1	16.2	15.9	15.8	15.6	15.5	15.7
1.00	15.9	15.9	16.0	16.1	16.2	15.9	15.7	15.6	15.5	15.6
2.00	15.9	15.9	15.9	16.0	16.1	15.8	15.7	15.7	15.5	15.5
Mean (min)	15.8	15.9	16.0	16.1	16.2	15.9	15.8	15.6	15.5	15.6
RSD (%)	0.35	0.23	0.39	0.35	0.61	0.54	0.52	0.15	0.26	0.32

[a] The retention time of individual 8-isoPGF$_{2\alpha}$-spiked synthetic urine sample on each day represents the average retention time of six injections at the same concentration.

Table 3
Numerical Results for the calibration of spiked Milli-Q water and spiked synthetic urine samples.

	Calibration equation	Regression (R^2)	RSD (%)[a]
Milli-Q H_2O	$y = 23.8114x - 0.0066$	0.9998	0.6–8.6
Synthetic urine	$y = 24.3714x - 0.0008$	0.9998	0.9–4.3

[a] The values represent the experimental variations among the eight concentration levels used for calibration.

prior to quantifying urinary 8-isoPGF$_{2\alpha}$ using chip-nanoLC/QqQ-MS method.

Under the enrichment mode (Fig. 2A), the analytes were retained and concentrated in the enrichment column. In addition to sample pre-concentration, excess mobile phase delivered by capillary pump was applied to remove un-retained components from the sample matrix prior to separation. This column design provides the advantage of online sample pre-concentration and reduces the ion-suppression effect. Afterward, the 6-way μ-valve was switched to the analysis mode (Fig. 2B) for chip-nanoLC separation. In the mean time, a gradient program (Section 2.2) of capillary flow was applied to avoid carry-over by washing the loading flow path. After complete elution of 8-isoPGF$_{2\alpha}$, the μ-switch valve was back to the enrichment mode to equilibrate the enrichment column using the capillary flow.

In order to increase the MS responses of 8-isoPGF$_{2\alpha}$ under negative mode, an additive such as NH_4OH or NH_4OAc was commonly used in the mobile phase to deprotonate the carboxyl group. However, the pH values of NH_4OH solutions were higher than 8.5, which exceeded the pH range (pH 2–8) recommended by the manufacturer. Therefore, various concentrations of NH_4OAc (0, 0.1, 0.5, 1.0 mM) were added into solvent C delivered by the nano pump to evaluate the effect of NH_4OAc on the MS responses of 8-isoPGF$_{2\alpha}$. The highest signal of 8-isoPGF$_{2\alpha}$, at least 3-fold higher than the others, was obtained when 0.1 mM ammonium acetate was added into solvent C. Therefore, Milli-Q water containing 0.1 mM ammonium acetate was used as solvent C in gradient elution (Section 2.2) for chip-nanoLC separation. In addition, the loading capacity of enrichment column was investigated using 2.0 ng mL^{-1} of 8-isoPGF$_{2\alpha}$ containing 0.2 ng mL^{-1} of ISTD with different injection volumes (2–8 μL at a 1 μL interval). The MS signals increased proportionally as the injection volume increased up to 5 μL, and then remained at its maximum responses as the injection volume was 6 μL or higher. Therefore, the injection volume of 5 μL was applied for the subsequent experiments. The relatively large injection volume (5 μL), 10-fold higher compared to enrichment column volume (500-nL), was utilized to pre-concentrate analytes in the enrichment column prior to chip-nanoLC separation. The optimized LC condition was employed for the subsequent experiments.

3.3. Sample pre-treatment

Sample pre-treatment was usually involved in quantitative determination of target analytes in complex sample matrix, such as human urine, to reduce the matrix effect. In this study, 8-isoPGF$_{2\alpha}$ spiked human urine samples were first examined for the influence of urine matrix with different extraction strategies using the proposed chip-nanoLC/QqQ-MS method. The signals in human urine decreased to about 42% of that in Milli-Q water while only SPE was applied for sample pre-treatment, which indicated remarkable ion-suppression on MS detection caused by matrix components. LLE was therefore utilized prior to SPE to further reduce the matrix effect. The recoveries (define in Section 2.3) were obtained in the range of 83.0–85.3% with 3 different concentrations of 8-isoPGF2$_{\alpha}$ spiked synthetic urine samples (0.02, 0.15, and 2.00 ng mL^{-1}, each contained 0.20 ng mL^{-1} of ISTD; $n = 3$) using the proposed sample pre-treatment process. The observed ion-suppression on MS responses among the 8-isoPGF$_{2\alpha}$ spiked Milli-Q water, synthetic urine, and human urine samples was found to be less than 3%. The results justified the use of LLE followed by SPE for sample pre-treatment in the subsequent experiments.

3.4. Reproducibility and quantitative performance

Reproducibility of the chip-nanoLC analysis was evaluated by obtaining the variations on retention times as well as the MS responses of 8-isoPGF$_{2\alpha}$. Table 2 summarizes the results of the retention time reproducibility using this chip-nanoLC/QqQ-MS system. The variations on retention times (intra-day and inter-day) were obtained by calculating the RSDs of six injections per day on five consecutive days for 7 different concentrations (0.02, 0.06, 0.10, 0.15, 0.20, 1.00 and 2.00 ng mL^{-1}, each contained 0.20 ng mL^{-1} of ISTD) of 8-isoPGF$_{2\alpha}$ spiked synthetic urine samples using the same chip (chip 1). As shown in Table 2, the average retention time of chip 1 was 16.0 min among the total 210 injections during five consecutive days and the RSDs for intra-day and inter-day experiments were 0.23–0.61% and 0.90%, respectively. The chip-to-chip variations on retention time were also investigated by performing the same precision experiments using another chip (chip 2) with different lot numbers. For chip 2 (Table 2), the average retention time was 15.7 min among the total 210 injections during five consecutive days and the RSDs for intra-day and inter-day experiments were 0.15–0.54% and 1.00%, respectively. Based on our experience,

Table 4
Precision and accuracy of chip-nanoLC/QqQ-MS analysis.

Concentration (ng mL^{-1})	Intra-day[a]			Inter-day[a]		
	Found[b] (ng mL^{-1})	RSD (%)	Accuracy (%)	Found[b] (ng mL^{-1})	RSD (%)	Accuracy (%)
0.01	0.010 ± 0.000	5.0	99.9	0.010 ± 0.001	5.8	99.7
0.10	0.100 ± 0.003	2.8	99.7	0.099 ± 0.004	4.3	99.4
1.00	0.993 ± 0.018	1.8	99.3	0.996 ± 0.047	4.7	99.6

[a] $n = 5$.
[b] The found 8-isoPGF$_{2\alpha}$ concentrations in spiked synthetic urine were expressed as "mean ± SD".

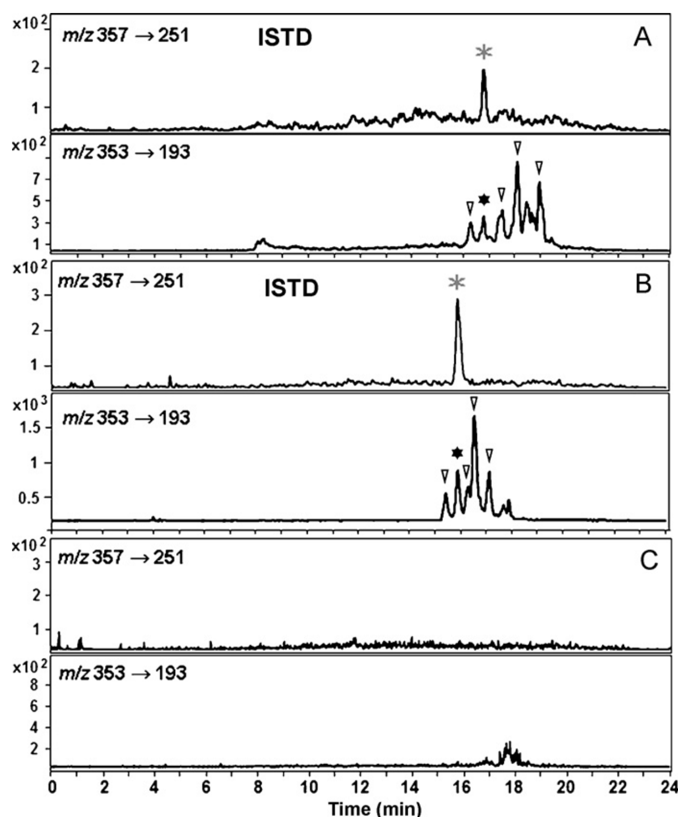

Fig. 3. Representative chromatograms of extracted (A) urine sample from a local hospital, spiked with ISTD (0.20 ng mL^{-1}); (B) F_2-isoprostanes spiked (0.10 ng mL^{-1} each and 0.20 ng mL^{-1} of ISTD) synthetic urine; (C) non-spiked synthetic urine. The following symbols were used to indicate the identified peaks in the figure: "✳" for ISTD, "★" for 8-isoPGF$_{2\alpha}$, and "▽" for F_2-isoprostane isomers (in the order of 8-iso-15(R)-prostaglandin F$_{2\alpha}$, 11β-prostaglandin F$_{2\alpha}$, 15(R)-prostaglandin F$_{2\alpha}$, and prostaglandin F$_{2\alpha}$).

a nanoLC-chip could approximately hold up to 500 urine sample injections.

The intra-day RSDs on MS responses of 8-isoPGF$_{2\alpha}$ were less than 10% among the 7 concentrations in chips 1 and 2; however, high inter-day variations (RSDs: 9.0–31.0%) on MS responses were found in both chips. The variations on MS responses were greatly reduced by the addition of ISTD and the utilization of peak area ratio (8-isoPGF$_{2\alpha}$/ISTD) for quantitative analysis. For chip 1, the RSDs of intra-day and inter-day assays on relative MS responses (8-isoPGF$_{2\alpha}$/ISTD) were 0.5–7.5% and 3.6–6.0%, respectively. For chip 2, the RSDs of intra-day and inter-day assays on relative MS responses (8-isoPGF$_{2\alpha}$/ISTD) were 0.4–5.7% and 4.3–6.0%, respectively.

In addition, the following quantitative features including linearity, LOD, LOQ, as well as precision and accuracy were examined using 8-isoPGF$_{2\alpha}$ spiked synthetic urine under optimal conditions to further validate the suitability of utilizing the chip-nanoLC/QqQ-MS system for quantitative analysis. A good linear relationship was obtained (0.01–2.00 ng mL^{-1}, each contained 0.20 ng mL^{-1} of ISTD) with a regression coefficient higher than 0.999 in synthetic urine. Moreover, an excellent correlation was observed between 8-isoPGF$_{2\alpha}$ spiked Milli-Q water and 8-isoPGF$_{2\alpha}$ spiked synthetic urine samples (Table 3). The LOD (signal-to-noise ratio of 3) and the LOQ (the lowest concentration in the linear range) were determined as 0.005 ng mL^{-1} and 0.01 ng mL^{-1}, respectively. As shown in Table 4, the intra-day and inter-day precisions of 8-isoPGF$_{2\alpha}$ were in the ranges of 1.8–5.0% and 4.3–5.8%, respectively. The ranges of accuracy for intra-day and inter-day assays were 99.3–99.9% and 99.4–99.7%, respectively. According to these results, the chip-nanoLC/QqQ-MS method was proved to be suitable for quantitatively determining the urinary 8-isoPGF$_{2\alpha}$.

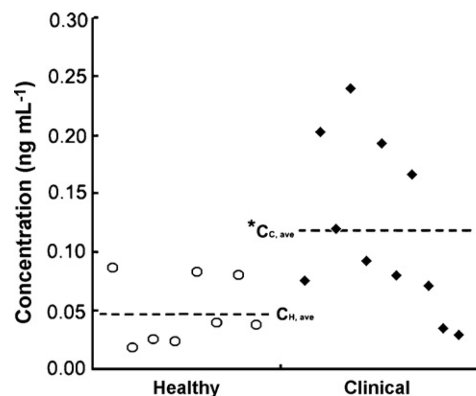

Fig. 4. Distribution of the 8-isoPGF$_{2\alpha}$ contents measured in human urine samples.

3.5. Application: analysis of 8-isoPGF$_{2\alpha}$ in urine samples

Eleven clinical urine samples from a local hospital and eight urine samples from healthy adults were analyzed by the newly developed chip-nanoLC/QqQ-MS system. The representative chromatograms of human urine sample, 8-isoPGF$_{2\alpha}$ spiked synthetic urine sample and non-spiked synthetic urine sample are shown in Fig. 3. In this study, several F_2-isoprostanes (diaseteromers of 8-isoPGF$_{2\alpha}$) together with 8-isoPGF$_{2\alpha}$ were found in all human urine samples, which resulted in the multiple peaks shown in Fig. 3. In addition to the transition (m/z 353 → 193) for quantifying 8-isoPGF$_{2\alpha}$, the other two transitions (m/z 353 → 291/247) were also utilized for qualitative confirmation. Negligible variations on retention time were observed in most clinical urine samples and all samples from healthy adults. Only a few clinical urine samples (Fig. 3A) presented noticeable retention time shifts, which might be caused by unknown matrix components in those urine samples due to individual differences such as sample storage and/or personal health conditions. Therefore, the ISTD in urine samples was used to identify the target 8-isoPGF$_{2\alpha}$ peak in a chromatogram and compensate the possible fluctuations on retention times as well as the MS responses for both qualitative analysis and quantitative analysis. The distribution of the determined 8-isoPGF$_{2\alpha}$ contents in different human urine samples is given in Fig. 4. The range of 8-isoPGF$_{2\alpha}$ concentration for 11 clinical urine samples was from 0.029 to 0.240 ng mL^{-1} in which the mean ($C_{C,ave}$) and the median values in this group were 0.118 and 0.092 ng mL^{-1}, respectively. For the 8 urine samples from healthy adults, the range of 8-isoPGF$_{2\alpha}$ concentration was from 0.017 to 0.084 ng mL^{-1} in which the mean ($C_{H,ave}$) and the median values in the group were 0.048 and 0.039 ng mL^{-1}, respectively. The symbol "*" appeared on the figure indicated a significant change ($P = 0.018 < 0.05$) between the average 8-isoPGF$_{2\alpha}$ contents in the two groups ($C_{C,ave}$ vs $C_{H,ave}$) using ANOVA. The 8-isoPGF$_{2\alpha}$ contents in healthy adults have been reported in a broad range [8,16,18,19]. For instance, the reported urinary 8-isoPGF$_{2\alpha}$ contents ranged approximately from 0.06 to 0.56 ng mL^{-1} of healthy control volunteers [16,18]. In this study, the relatively small urinary 8-isoPGF$_{2\alpha}$ contents found in healthy adults might be due to the individual differences (such as age and gender) compared with previous publications.

4. Summary

In this study, the use of chip-nanoLC/QqQ-MS for analyzing 8-isoPGF$_{2\alpha}$ showed good linearity in the range of 0.01–2.00 ng mL^{-1}. The chip-nanoLC/QqQ-MS method also offered good reproducibil-

ity on retention times as well as good intra-day/inter-day precision and accuracy for quantitative analysis of 8-isoPGF$_{2\alpha}$. Because of the dynamic range of urinary 8-isoPGF$_{2\alpha}$ contents [8,16,18,19], an analytical method with low LOQ becomes crucial for determining trace 8-isoPGF$_{2\alpha}$ in human urine. Compared with other LC/tandem MS methods for quantifying urinary 8-isoPGF$_{2\alpha}$, the chip-nanoLC/QqQ-MS method offered a LOQ as low as 0.01 ng mL^{-1}, while Saenger et al. stated a LOQ of 0.1 ng mL^{-1} [16] and Yan et al. reported a LOQ of 0.03 ng mL^{-1} [19]. Based on these facts, the chip-nanoLC coupled to QqQ-MS has been illustrated as a sensitive analytical technique for quantitative determination of 8-isoPGF$_{2\alpha}$ contents in human urine.

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