DETERMINATION OF URINARY F_2-ISOPROSTANES AS AN OXIDAITVE BIOMARKER IN PATIENTS WITH DIABETES MELLITUS TYPE 2 BY LIQUID CHROMATOGRAPHY-ELECTROSPRAY TANDEM MASS SPECTROMETRY

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ABSTRACT: A liquid chromatography tandem mass spectrometry (LC-MS/MS)-ion trap coupled with electrospray ionization was described for determination of trace biological molecular, urinary F_2-Isoprostanes (F_2-IsoPs), a sensitive biomarker of oxidative stress *in vivo*, in Thai patients with diabetic mellitus (DM) type 2 and compare with normal subjects. Spot urine samples were collected from 28 DM patients and 19 normal subjects and extracted by solid-phase extraction prior to injection onto the LC system. The analytical method was validated with limit of quantification (LOQ) of 200 pg/ml, good linearity was obtained in the range of 200-20,000 pg/ml (r = 0.9992) and recoveries at 400-10,000 pg/ml were 77-102%. The levels of F_2-IsoP in urines from DM patients were 720.37 $\pm$ 454.89 pg/mg creatinine (mean $\pm$ SD) whereas the levels in normal subjects were 375.08 $\pm$ 440.64 pg/mg creatinine (mean $\pm$ SD). It is worth noting that urine samples from 8 out of 19 normal subjects contained urinary F_2-IsoPs at the level below LOQ (200 pg/ml). This study demonstrated an alternative method by LC-MS/MS ion trap method for determination of F_2-IsoPs in urine as a sensitive biomarker.

Keywords: LC-MS/MS ion trap, F2-Isoprostanes, oxidative stress, diabetes

INTRODUCTION: Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) is the high technology instrument that used widely for qualitative and quantitative analysis in research and development of new drug, structural elucidation of drugs and metabolites, bioavailability, bioequivalence, food and environmental toxicology including measuring ultra-trace concentrations of small molecules (biomarkers) in biological samples that occurred from oxidative stress such as F_2-Isoprostanes (F_2-IsoPs)[1-4]. The measurement of F_2-IsoPs in biological fluids represents oxidative stress in vivo caused by free radicals and provides a reliable marker of lipid peroxidation *in vivo*. Importantly, the formation of F_2-IsoPs has been shown to increase dramatically in well-established animal models of oxidant injury[5]**.** Furthermore, Montero *et al*. demonstrated a link between F_2-IsoPs and high glucose in Streptozotocin-induced diabetic rats that had marked increase in plasma levels and urinary excretion rates of F_2-IsoPs[6]. Recent study, they found higher levels of F_2-IsoPs in onset of diabetes mellitus (DM) type 1[7] and in DM type 2[8] than non diabetic subjects. Various analytical methods have been used to measure F_2-IsoPs, including Enzyme Immunoassay (EIA), radio-immunoassay (RIA), gas chromatography mass spectrometry (GC-MS) and liquid chromatography mass spectrometry (LC-MS). Analysis of F_2-IsoPs in urine samples by GC-MS has been accepted by most researchers as the "gold standard technique". However, extensive sample preparation procedures are required for this method, including C18 and silica solid phase extractions, thin layer chromatography purification,

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two derivatization steps and several drying and reconstitution steps before samples can be inject onto the GC-MS column. Theses complex and time consuming sample preparation procedures make this technique less than ideal to handle large number of samples for determination of F_2-IsoPs in a clinical laboratory. Another analytical method has been used to measure F_2-IsoPs was Enzyme-linked immunosorbent assay (ELISA). ELISA have been developed and made commercially available by manufacturers. Liang *et al*. reported the ELISA assay, without sample purification steps, could generate results, which appeared to be over 1000-fold higher than they should be[3]. A recent article compared measurement of F_2-IsoPs in urine by ELISA and mass spectrometry and found considerable inconsistencies. Thus, at present, measurement of F_2-IsoPs by mass spectrometry remains the method of choice[5].

This study then showed the application of LC-MS/MS for trace biological molecular quantification and the results of method validation for detection of F_2-IsoPs in urine sample, employing normal subjects and DM type 2 patients by electrospray ionization (ESI) - LC-MS/MS ion trap detection.

MATERIAL AND METHODS:

Reagents and Chemicals

All organic solvent and deionized water were HPLC grade and obtained from Merck. Ammonium acetate was obtained from Fisher Scientific. Bond Elut 18 solid phase extraction (SPE) columns (500 mg, 3 ml) were obtained from Varian. F_2-IsoPs was obtained from Sigma.

Preparation of Standard Solution

The primary stock solution of F_2-IsoPs was prepared by dissolving 1.0 mg of F_2-IsoPs in 1 ml dimethylsulfoxide, producing a concentration of 1.0 mg/ml and was stored at -80 °C. Working solutions of F_2-IsoPs for calibration curve were prepared by spiking the stock solution with blank urine samples at the concentrations of 200, 1000, 2000, 5000, 10,000 and 20,000 pg/ml. For quantification of F_2-IsoPs in urine samples, external standard was used.

Equipment

LC-MS/MS experiments were carried out with an Agilent 1100 LC system consisting of degasser, binary pump, auto sampler, and column heater. The column outlet was coupled to an Agilent MSD Ion Trap XCT mass spectrometer equipped with ESI source. Data acquisition and mass spectrometric evaluation was carried out with Data Analysis software (Bruker). For the chromatographic separation a Zorbax XDB-C18 column (2.1x50, 3.5 µm) with an identical guard column (4x10, 5 µm) was used. The following parameters were employed throughout all MS experiments for ESI with negative ion polarity the capillary voltage was set to 4.0 kV, the drying temperature to 350 °C, the nebulizer pressure to 45 psi, and drying gas flow to 10 L/min. The maximum accumulation time was 400 msec, the scan speed was ultra scan mode and the fragmentation time was 40 ms. To determine the product ions of F_2-IsoPs, the deprotonated ion ($[M-H]^-$) at *m/z* 353 was isolated, helium gas introduced into the trap to induce collision with analyte ions and the fragments detected over a scan range of *m/z* 150-400. The most intensive product ion was *m/z* 193. Throughout all measurement, F_2-IsoPs was detected by multiple reaction monitoring (MRM).

Chromatographic Analysis

The mobile phase consisted of 5 mM ammonium acetate (pH 6) (A) and methanol: acetronitrile (5:95 v/v) (B). Both solutions were filtered by vacuum through a 0.22 µm cellulose acetate membrane. The HPLC separation was carried out with a solvent gradient program of 15 % B at time 0, a linear increase to 70% B at 6 min, a linear increase to 100% B at 11 min, then a linear decrease from 100 to 15% B within 1 min. The sample was delivered at flow rate of 200 µl/min. A switch valve was used to inject only the components eluted between 7.0-9.0 min into the mass spectrometer chamber. The total HPLC running time was 12 min.

Subjects

Random urine samples from 19 normal subjects were collected and 28 DM type 2 patients were recruited from the Diabetics Clinic, Warinchamrap hospital, Ubonratchani. Study protocol was clearly informed and subjects were asked to sign the inform consent prior to the initiation of the study. Urine samples were collected during regular follow ups.

Sample preparation

All urine samples were aliquoted and stored at -70 °C until analysis. The freshly thawed urines were mixed for a few seconds on a vertex shaker and centrifuged at 3500 ×g for 10 min to precipitate solids. Three milliliter of urine supernatant was applied on a Varian's Bond Elut C18 cartridge previously conditioned with 5 ml of ethanol and equilibrated with 5 ml of deionized water. After washing with 5 ml of water, 5 ml of ethanol: water (5:95 v/v) and 2 ml of hexane the cartridge was then elute with 4 ml of ethyl acetate. The sample eluent was evaporated to dryness under a stream of nitrogen gas and reconstituted in 50 µl of acetronitrile: water (20:80 v/v) solution. Ten microlites of the reconstituted urine sample were injected onto a column.

Method Validation

The validation analytical parameters were described in the Guidance for Industry: Bioanalytical Method Validation[9].

1. Specificity/ Selectivity

Chromatographic interference from urine component was investigated using pooled blank urine samples (1:10 dilution with distilled water) as well as sample from healthy subjects.

2. Linearity

Calibration curve was prepared by spiking blank urine samples with proper volume of one of the above-mentioned working solution to produce the calibration curve point's equivalent to 200, 1000, 2000, 5000, 10,000 and 20,000 pg/ml of F_2-IsoPs. The samples were assayed using the method described above. The standard calibration curve for F_2-IsoPs was constructed using the analyte peak area versus the nominal concentrations of the analytes. Linear least-squares regression analysis with weighting factor of $1/x^2$ was performed to assess the linearity as well as to generate the standard calibration equation: $y=ax+b$, where y is analyte peak area, x the concentration, a the slope and b is the intercept of the regression line.

3. Accuracy

The accuracy of the procedure was demonstrated by spiking urine samples with three levels of F_2-IsoPs standard, 400, 2000 and 10,000 pg/ml. The spiking urine samples in three replicates were analyzed on the same day.

4. Precision

The precision of the procedure was demonstrated by spiking urine samples with two levels of F_2-IsoPs standard, 400 and 10,000 pg/ml. The spiking urine samples in three replicates were analyzed on the same day to determine the intra-day precision, and nine samples of each concentration on separate days to determine inter-day precision.

5. Sensitivity

The lower limit of quantification (LLOQ) was determined for F_2-IsoPs, base on the criteria that : (1) the analyte response at LLOQ is five time of baseline noise; (2) the analyte response at LLOQ can be determined with sufficient precision and accuracy, i.e. precision of 20% and accuracy of 80-120%.

RESULTS:

Method Validation

1. Specificity/ Selectivity

A negative ion electrospray mass scan spectrum of F_2-IsoPs is shown in Figure 1. The major ions observed were $[M\text{-}H]^-$, m/z = 193. Figure 2 shows the representative chromatograms of blank urine and urine sample spiked with 5000 pg/ml of F_2-IsoPs. The lack of interference at the same retention time represented acceptable specificity of the assay. Figure 3 shows the representative chromatogram of full scan MS-MS mode (Total ion chromatogram; TIC) and the chromatogram of extraction mode (Extract ion chromatogram; EIC). The extraction mode is known to be the most sensitive

MS setup of an ion trap detector, thus permitting the quantitative analysis of analytes in complex matrices with a good sensitivity.

2. Linearity

Good linearity of the calibration were plotted between standard F_2-IsoPs concentration and peak area, was observed over the concentration range from 200 to 20,000 pg/ml (r = 0.9992) (Figure 4).

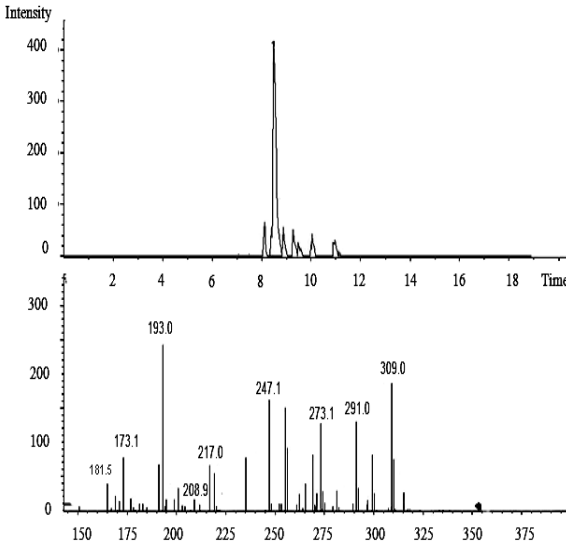


Figure 1 Negative ion electrospray mass scan spectra of F_2-IsoPs

3. Accuracy

The accuracy of the assay was demonstrated by spiking urine samples with three levels of F_2-IsoPs standard, 400, 2000 and 10,000 pg/ml. The recovery of F_2-IsoPs from urine samples was determined to be 102.00 %, 77.49 % and 98.10 %, respectively (Table 1).

4. Precision

The precision of the assay, presented as percentage of RSD was determined as the intra- and inter-day variation of standard F_2-IsoPs spiked in urine sample with two levels, 400 and 10,000 pg/ml. The intra-day precisions were 19.33% and 17.33%, respectively and the inter-day precisions were 20.20% and 17.48%, respectively (Table 2).

5. Sensitivity

The LLOQ was 200 pg/ml for F_2-IsoPs. The percent recovery of LLOQ was 103.33 and the precision, presented as percentage of RSD was 13.97.

Clinical Application

To show the usefulness of the optimized method, we evaluted urinary F_2-IsoPs levels in normal subjects and patients with DM type 2. The peak of F_2-IsoPs was identified by retention time and the levels of urinary F_2-IsoPs were quantified by the six-point calibration curve and then were transformed to pg/mg creatinine. The

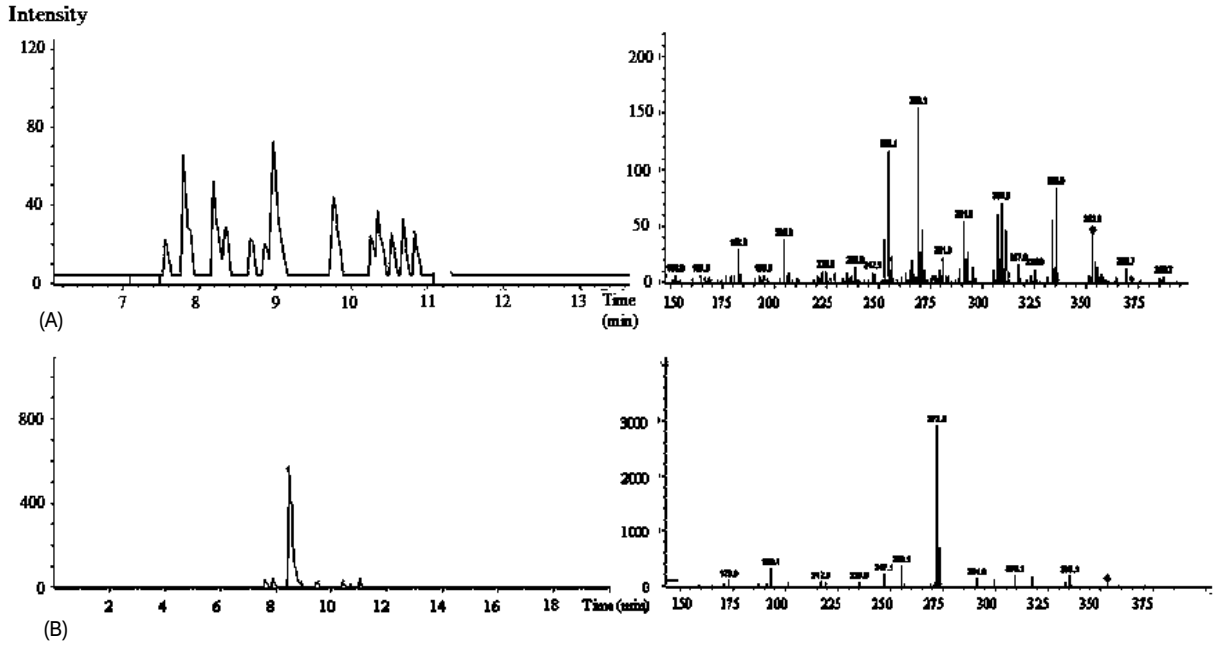
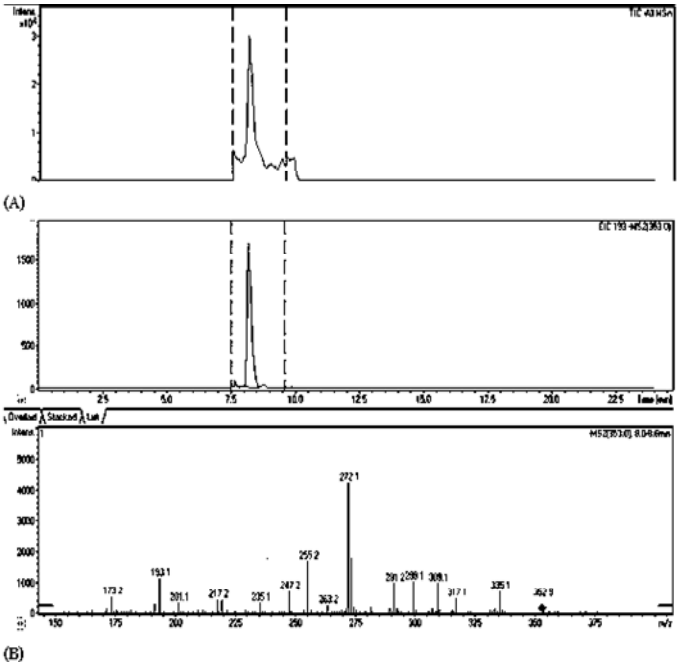


Figure 2 Chromatogram of (A) blank urine; (B) urine sample spiked with 5000 pg/ml of F_2-IsoPs

urinary F_2-IsoPs levels in 19 normal subjects and 28 patients with DM type 2 were 375.08 ± 440.64 pg/mg creatinine and 720.37 ± 454.89 pg/mg creatinine, respectively (mean ± SD) (Figure 5).

DISCUSSION AND CONCLUSION: F_2-IsoPs can be used to evaluate local or systemic lipid peroxidation in vivo, since they are detected in body fluids. Increased F_2-IsoPs levels were found in several experimental and clinical condition associated with free radical mediated oxidant damage, Consequently, F_2-IsoPs are becoming popular oxidative marker. As importance of investigating biomarker of oxidative stress has increased, novel methods of analysis have been explored to facilitate their measurement; however, LC-MS/MS ion trap detection can be used to measure F_2-IsoPs levels in urine. We have employed ion trap MS for quantification of F_2-IsoPs, although it is not the first choice instrument, it can also meet the demand of quantitative analysis through method validation. And because of the condition limited, we had no choice to use the better instrument for analysis.



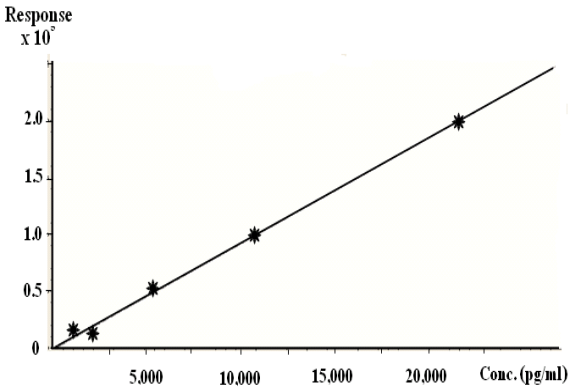


Figure 3 (Left) Total ion chromatogram; TIC (A) extract ion chromatogram; EIC (B) of F_2-IsoPs in urine sample

Figure 4 (Above) Calibration curve for F_2-IsoPs, 200 – 20,000 pg/ml urine matrixes

Figure 5 (Below) Levels of urinary F_2-IsoPs in 28 type 2 diabetic patients (◆), 11 healthy subjects (○) and 8 healthy subjects contain urinary F_2-IsoPs at the level below LOQ (●). Symbols represent individual measurements; horizontal bars represent mean value for each group

Table 1 Percent recovery of F_2-IsoPs from urine samples

Urine sample (pg/ml)	Spiked Amount (pg/ml)	Urine ± Spiked (pg/ml)	Recovery (%)
271.09 ± 0.03	400	679.07 ± 0.13	102.00
	2000	1820.92 ± 0.22	77.49
	10,000	10,080.82 ± 1.75	98.10

Results presented as mean ± SD, *n* = 3

Table 2 Intra- and inter-day precision of F_2-IsoPs spiked in urine sample

Urine sample (pg/ml)	Spiked Amount (pg/ml)	Parameters	Intra-day [a]	Inter-day [b]
271.09 ± 0.03	400	Mean	679.07	621.87
		RSD (%)	19.33	20.00
	10,000	Mean	10,080.82	10,548.69
		RSD (%)	17.33	17.48

[a] *n* = 3, [b] *n* = 9

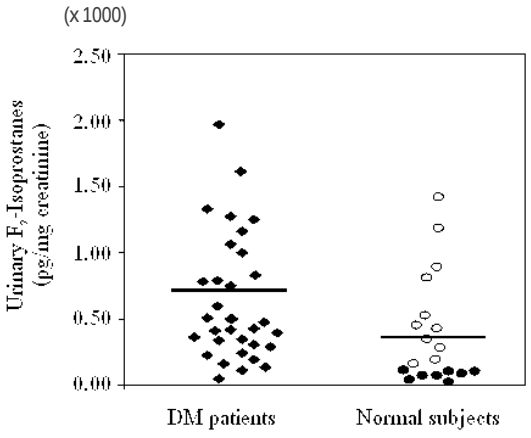


Figure 1 shows the negative ion ESI-MS spectrum of F_2-IsoPs. The main peak at *m/z* 353 is assigned to the deprotonated ion $[M-H]^-$ and the most intensive product ion was *m/z* 193. In addition, other products ions of lower intensity, at *m/z* 209, *m/z* 247, *m/z* 273 and *m/z* 309 were observed too. This result proved that fragment ions resulted from negative ionization mode in LC-MS/MS ion trap detection are mostly similar to those from ESI with the same mode in LC-MS/MS triple stage quadrupole detection, which also referred in review[3]. Quantification of F_2-IsoPs was performed in the full scan MS-MS mode. Post acquisition data processing of full scan MS-MS data permitted the "extraction" of analyses of interest by selecting specific product ions. This mode is known to be the most sensitive MS setup of an ion trap detector, thus permitting the quantitative analysis of analyses in complex matrices with a good sensitivity. In method validation, however, the precision of the assay presented high variation, due to manual sample clean up step. Presumably, an automate SPE sample preparation can be used to reduce human error.

Along with the LC separation, the method provided good sensitivity for the determination of F2-IsoPs. The single-step solid phase extraction was efficient for separating F_2-IsoPs from interfering urinary matrix components. The recovery of F_2-IsoPs in the extraction procedure in this study was found to be 77.49-102.00% and was constant over the concentration range from 200-20,000 pg/ml. The total LC running time was 12 min, and a switch valve was used to inject only the components eluted between 7.0-9.0 min into the mass spectrometer chamber. In addition to the waste-switched period of the first 7 min for eliminating the contamination of the ESI ion source, the MS data acquisition was divided into three segments. In urine samples, however, an unknown peak appeared on the chromatogram. Due to unknown matrix effect, retention time shift had been observed in some urine samples. Therefore, standards were used after every tenth injection to monitor the accuracy. According to the clinical application, the results showed the higher levels of urinary F_2-IsoPs in DM patients, demonstrating an oxidative status due to excessive blood glucose concentrations. These experiments demonstrate that F_2-IsoPs can be measured reliably and conveniently in human urine samples using LC-MS/MS ion trap.

In summary, a simple alternative method by LC-MS/MS ion trap method for determination of F_2-IsoPs in human urine has been established, which is specific, accurate, precise, and can cover very broad concentration range. The sample pretreatment procedure is based on a simple one-step solid phase extraction. This developed non-invasive method is suitable for the determination of F_2-IsoPs in human urine.

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REFERENCES:

1. Papac DI, Shahrokh Z. 2001. Mass spectrometry innovations in drug discovery and development. Pharmaceutical Research 18(2): 131-45.

2. Tozuka Z, Kaneko H, Shiraga T, Mitani Y, Beppu M, Terashita S, *et al*. 2003. Strategy for strugtural elucidation of drug and drug metabolites using $(MS)^n$ fragmentation in an electrospray ion trap. Journal of Mass Spectrometry 38: 793-808.

3. Liang Y, Wei P, Duke RS, Reaven PD, Harman M, Cutler RG, Heward CB. 2003. Quantification of 8-iso-prostaglandin-$F_{2\alpha}$ and 2,3-dinor-8-iso-prostaglandin $F_{2\alpha}$ in human urine using liquid chromatography tandem mass spectrometry. Free Radical Biology & Medicine 34: 409-18.

4. Baranczewski P, Stanczak A, Kautiainen A, Sandin P, Edlund PO. 2006. Introduction to early *in votro* identification of metabolites of new chemical entities in drug discovery and development. Pharmacological Reports 58: 341-52.

5. Robert LJ, Morrow JD. 2000. Measurement of F_2-isoprostanes as an index of oxidativestress *in vivo*. Free Radical Biology & Medicine 28: 505-13.

6. Montero A, Munger KA, Khan RZ, Valdivelso JM, Morrow JD, Guasch A, *et al*. 2000. F_2-isoprostanes mediate high glucose-induced TGF-β synthesis and glomerular prteinuria in experimental type I diabetes. Kidney International 58: 1963-72.

7. Flores L, Rodela S, Abian J, Claria J, Esmatjes E. 2004. F_2-Isoprostane is already increased at the onset of type 1 diabetes mellitus: effect of glycemic control. Metabolism 53(9): 1118-20.

8. Davi G, Ciabattoni G, Consoli A, Mezzetti A, Falco A, Santarone S, *et al*. 1999. In vivo formation of 8-iso-prostaglandin $F_{2\alpha}$ and platelet activation in diabetes mellitus effects of improved metaboic control and vitamin E supplementation. Circulation 99: 224-9.

9. The United States Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Center for Veterinary Medicine. 2001. Guidance for Industry: Bioanalytical Method Validation. [online]. Available from http://www.fda.gov/CDER/GUIDANCE/4252fnl.htm [2005, Feb 8]

การตรวจวัดปริมาณตัวชี้วัดภาวะเครียดออกซิเดชัน F_2-Isoprostanes ในปัสสาวะผู้ป่วยเบาหวานชนิดที่ 2 ด้วยเทคนิค Liquid Chromatography - Electrospray Tandem Mass Spectrometry

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บทคัดย่อ: พัฒนาและตรวจสอบความถูกต้องวิธีวิเคราะห์ F_2-Isoprostanes (F_2-IsoPs) ตัวชี้วัดภาวะเครียดออกซิเดชัน ในปัสสาวะด้วยเทคนิค Liquid Chromatography-Electrospray Tandem Mass Spectrometry (ESI-LC-MS/MS) สกัดแยก F_2-IsoPs ออกจากปัสสาวะด้วยวิธี solid phase extraction จากนั้นตรวจวิเคราะห์ด้วยเทคนิค LC-MS/MS ผลการตรวจสอบความถูกต้องวิธีวิเคราะห์พบว่า ความเข้มข้นต่ำสุดของ F_2-IsoPs ที่สามารถหาปริมาณได้ (LOQ) คือ 200 pg/mg F_2-IsoPs ในช่วงความเข้มข้น 200-20,000 pg/ml มีความสัมพันธ์กับพื้นที่ใต้กราฟเป็นเส้นตรง โดยมีค่าสัมประสิทธิ์ความสัมพันธ์ (r) เท่ากับ r = 0.9992 ผลการทดสอบความแม่นของวิธีวิเคราะห์ตลอดช่วงการวิเคราะห์มีความแม่น (Accuracy) แสดงโดยค่าเฉลี่ยของ % recovery โดยการเติมสารมาตรฐาน 3 ระดับ อยู่ในช่วง 77-102% ผลการประยุกต์ใช้วิธีโดยการหาปริมาณ F_2-IsoPs ในปัสสาวะอาสาสมัครคนปกติจำนวน 19 คนและอาสาสมัครผู้ป่วยเบาหวานชนิดที่ 2 จำนวน 28 คน พบว่าอาสาสมัครคนปกติมีระดับ F_2-IsoPs น้อยกว่าอาสาสมัครผู้ป่วยเบาหวานชนิดที่ 2 คือ 375.08 $\pm$ 440.64 pg/mg creatinine และ 720.37 $\pm$ 454.89 pg/mg creatinine (mean $\pm$ SD) ตามลำดับ โดยในอาสาสมัครสุขภาพดีจำนวน 8 คนในจำนวน 19 คน มีระดับ F_2-IsoPs น้อยกว่า LOQ การตรวจวัดปริมาณ F_2-IsoPs ในปัสสาวะด้วยเทคนิค LC-MS/MS สามารถใช้ตรวจวัดปริมาณ F_2-IsoPs ในปัสสาวะคนปกติ ผู้ป่วยเบาหวาน และผู้ป่วยจากโรคที่เกิดจากอนุมูลอิสระได้ซึ่งการตรวจวัดปริมาณ F_2-IsoPs ในตัวอย่างปัสสาวะนี้แสดงให้เห็นถึงการเกิดภาวะเครียดออกซิเดชันที่เกิดจากอนุมูลอิสระ

คำสำคัญ: LC-MS/MS ion trap, F_2-Isoprostanes, oxidative stress, diabetes

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