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Rapid and simple one-step membrane extraction for the determination of 8-hydroxy-2′-deoxyguanosine in human plasma by a combination of on-line solid phase extraction and LC–MS/MS

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ABSTRACT

A quantitative analytical method using automated on-line solid phase extraction (SPE) and liquid chromatography–electrospray tandem mass spectrometry (LC–ESI-MS/MS) for the determination of 8-OHdG (8-hydroxy-2′-deoxyguanosine) in human plasma was developed and validated. A one-step membrane extraction method for the plasma sample preparation and a C18 SPE column with simple extraction and purification were used for the on-line extraction. A C18 column was employed for LC separation and ESI-MS/MS was utilized for detection. $^{15}N_5$-8-OHdG ($^{15}N_5$-8-hydroxy-2′-deoxyguanosine) was used as an internal standard for quantitative determination. The extraction, clean-up and analysis procedures were controlled by a fully automated six-port switch valve as one strategy to reduce the matrix effect and simultaneously improve detection sensitivity. Identification and quantification were based on the following transitions: m/z 284 → 168 for 8-OHdG and m/z 289 → 173 for $^{15}N_5$-8-OHdG. Satisfactory recovery was obtained, and the recovery ranged from 95.1 to 106.1% at trace levels in human plasma and urine, with a CV lower than 5.4%. Values for intraday and interday precision were between 2.3 and 6.8% for plasma and between 2.7 and 4.5% for urine, respectively. Values for the method accuracy of intraday and interday assays ranged from 93.0 and 100.5% for plasma and 110.2 and 119.4% for urine, respectively. The limits of detection (LOD) and LOQ were 0.008 ng/mL and 0.02 ng/mL, respectively.The applicability of this newly developed method was demonstrated by analysis of human plasma samples for an evaluation of the future risk of oxidative stress status in human exposure to nanoparticles and other diseases.

1. Introduction

Reactive oxygen species can react with many biological structures, including protein, lipids and DNA [1]. A specific type of ROS-induced DNA modification is the C-8 hydroxylation of deoxyguanosine, and the resulting 8-hydroxydeoxyguanosine (8-OHdG) is well known as a biomarker of oxidative stress [2]. 8-OHdG has been associated with several diseases, such as aging [3], cancer neurodegenerative diseases [1], diabetes [4], Alzheimer's disease [5], cardiomyopathy [6] and cardiovascular or infectious diseases [7]. Recently, quantification of 8-OHdG has been suggested as a reliable indicator of oxidative stress in biological fluids including plasma [8], serum [9], urine [10–12], saliva [13], and in tissue [14–16]. It can be regarded as a sensitive and stable biomarker of DNA oxidative damage to estimate the toxicity of internal and external factors.

Various traditional analytical approaches with different sensitivities and specificities can be used to measure 8-OHdG in biological fluids. These include chromatographic techniques such as high-performance liquid chromatography/electrochemical detection (HPLC/ECD) [9], capillary electrophoresis/ultraviolet detection (CE/UV) [17], gas chromatography/mass spectrometry (GC/MS) [18], liquid chromatography/tandem mass spectrometry (LC/MS/MS) [19], or immunoassays such as enzyme-linked immunosorbent assay (ELISA) [20]. However, HPLC-ECD has a disadvantage in its possible interference from biological matrixes and in the time required for analysis of a large samples [21]. Immunological methods are convenient and popular, but have cross-reactivity with related compound, thus leading to overestimation [22]. LC–MS/MS is a powerful technique, with good selectivity and sensitivity for the quantitative analysis of a variety biological matrixes such as urine [11], serum [9] and leukocytes [23]. But, in many cases it is now the sample pre-treatment process that has become the bottleneck in method development and sample analysis. Most published methods for the determination of 8-OHdG in plasma involves a labor-intensive and time-consuming

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procedure that requires purification by solid-phase extraction [24] or liquid–liquid extraction [25]. When the concentration is quite low, a large sample volume and endogenous components (salts, amine, fatty acid and triglycerides, etc.) are needed [8,24]. Therefore, we developed a new one-step membrane extraction for a sample preparation method and combined it with on-line SPE-LC–MS/MS, which was rapid, automated, a small sample volume with a decreased matrix effect that provided accurate, precise measurements of 8-OHdG in human plasma samples. The aim of the current study was to offer significant benefits, from a simple membrane filter and from ultracentrifuge steps that can save time and reduce the solvent needed for sample extraction, purification, and analysis of 8-OHdG in plasma samples. This new method is potentially applicable to the evaluation of the future risk of oxidative stress status in human exposure to nanoparticles and other diseases.

2. Experimental

2.1. Chemicals and reagents

Sigma–Aldrich (St. Louis, MO, USA) supplied the 8-OHdG, dG, salts and all HPLC grade solvents. Cambridge Isotope Laboratories (Andover, MA) supplied the $^{15}N_5$-8-OHdG and $^{15}N_5$-dG. The Amicon Ultra-0.5 centrifugal filter was purchased from Millipore.

2.2. Stock solutions and working solutions

A stock standard solution of 8-OHdG (100 ng/mL) was prepared in 5% MeOH with 0.1% formic acid, and was then stored in polypropylene screw-top tubes at −20 °C. In establishing a linear calibration curve, the linear range determined for plasma was 0.02–0.5 ng/mL (0.02, 0.05, 0.1, 0.2 and 0.5 ng/mL) in neat solvent (5% MeOH (v/v) with 0.1% formic acid) and for plasma that was pooled from volunteers, and the linear range that was determined for urine was from 0.1 to 10 ng/mL (0.1, 0.2, 0.5, 1, 5 and 10 ng/mL) in neat solvent; each calibrator contained a fixed amount of 1 ng/mL $^{15}N_5$-8-OHdG as an internal standard (IS). In this study, real plasma samples were concentrated to fit into the range of concentrations used in standard curve from 0.02 to 0.5 ng/mL. The concentration factor was 5.

2.3. Subjects and sample collection

The present study was approved by the Institutional Review Board of the National Health Research Institutes. Spot urine and plasma samples were stimultaneously collected from 55 healthy subjects (37 males and 18 females) and the following characteristics were included in the analysis: the mean age was 38.6 ± 8.9 years; the mean creatinine was 122.5 ± 57.8 mg/dL; and, all participants were nonsmokers. Creatinine was determined using a method established by Jaffe at the Union Clinical Laboratory of Taipei. Well-trained interviewers collected information through a face-to-face interview based on a structured questionnaire. Information collected included the following: demographic and socioeconomic characteristics; general potential malignancy risk factors such as lifestyle, alcohol consumption, cigarette smoking (quantified), and occupational history; and, personal and family histories of disease.

2.4. Sample preparation and purification

Blood samples were collected from 55 healthy humans for plasma sample analysis using commercially available ethylenediaminetetraacetic acid (EDTA)-containing tubes. Samples were processed for isolation of plasma, erythrocytes, and leukocytes within 2 h of blood collection, then frozen at −80 °C and stored until analysis. The plasma was collected and filtered using a 0.45 μm nylon membrane, and 0.25 mL plasma was added into a Millipore Amicon Ultra-0.5, 3 K centrifuge tube (as the membrane extraction method), spiked with 50 μL of the $^{15}N_5$-8-OHdG internal standard (1 ng/mL) and 200 μL of deionized water, then centrifuged at 14,000 × *g* for 60 min at 4 °C. The analytes were added to 500 μL ACN and dried by nitrogen at 15 °C for 10 min, then rinsed tube with 200 μL ACN and dried by nitrogen at 15 °C for 5 min, total time of 15 min. Finally, 50 μL of 5% MeOH (v/v) solvent with 0.1% formic acid was added and the solution was mixed by vortexing for subsequent LC–MS/MS analysis

For urine sample analysis, urine from 55 healthy humans was collected in glass bottles and processed immediately or stored at −20 °C until use, then 20 μL of each urine sample was diluted 20-fold with 5% MeOH (v/v) and 0.1% formic acid, spiked with 50 μL of $^{15}N_5$-8-OHdG (1 ng/mL), and was mixed by vortexing for subsequent LC–MS/MS analysis.

2.5. Method validation

2.5.1. Accuracy and precision

The precision and accuracy of intraday and interday experiments were accomplished by analyzing replicates (n = 5) spiked with 0.01 ng/mL, 0.02 ng/mL and 0.05 ng/mL each of 1 ng/mL of $^{15}N_5$-8-OHdG internal standard in pooled plasma and calculating the %Bias and the CV (coefficient of variation), as described below. Aliquots (0.25 mL) of mixed plasma samples were transferred to Eppendorf tubes. There was a total of 4 samples each from healthy humans, which were separated into 4 groups. Group I was spiked with 50 μL $^{15}N_5$-8-OHdG internal standard (1 ng/mL) and 200 μL of deionized water; Group II was spiked with 50 μL $^{15}N_5$-8-OHdG internal standard (1 ng/mL), 50 μL 8-OHdG (0.01 ng/mL), and 200 μL of deionized water; Group III was spiked with 50 μL $^{15}N_5$-8-OHdG internal standard (1 ng/mL), 50 μL 8-OHdG (0.02 ng/mL), and 200 μL of deionized water; and, Group IV was spiked with 50 μL $^{15}N_5$-8-OHdG internal standard (1 ng/mL), 50 μL 8-OHdG (0.05 ng/mL), and 200 μL of deionized water. The solutions were mixed in a Millipore Amicon Ultra-0.5, 3 K centrifuge tube, followed by centrifugation at 14,000 × *g* for 60 min at 4 °C, and the analytes were dried by nitrogen at 15 °C for 15 min. Finally, 50 μL of solvent 5% MeOH (v/v) with 0.1% formic acid were added and the solution was mixed by vortexing for LC–MS/MS analysis.

For urine sample analysis, 20 μL of urine was diluted 20-fold by 5% MeOH (v/v) with 0.1% formic acid from urine samples spiked with 50 μL of the $^{15}N_5$-8-OHdG internal standard (1 ng/mL). The intraday accuracy and precision of the assay was determined by analyzing replicates (n = 5) spiked with 1 ng/mL, 5 ng/mL and 10 ng/mL in pooled urine samples and calculating the %Bias and the CV (coefficient of variation). The interday variation was assessed by analyzing replicates of pooled urine (n = 5) obtained on different days from the same subjects.

2.5.2. Matrix effect and recovery

Matrix effects were determined from the peak areas of the $^{15}N_5$-8-OHdG internal standard ($A_{IS(neat\ solvent)}$), as 1 ng/mL was added to the neat solvent and compared with the peak areas of the internal standard that was added to plasma ($A_{IS(plasma)}$) from the pre-extraction and urine ($A_{IS(urine)}$) from diluted method. The relative matrix effect was established according to the following formula: $R_1 = (A_{IS(plasma,\ pre\text{-}extraction)} - A_{IS(neat\ solvent)})/A_{IS(neat\ solvent)} \times 100\%$ and $R_2 = (A_{IS(urine,\ diluted\ method)} - A_{IS(neat\ solvent)})/A_{IS(neat\ solvent)} \times 100\%$.

To determine the extraction recovery of 8-OHdG was obtained by adding 8-OHdG to plasma and urine, at 0.05, 0.1 ng/mL and 2.5, 10 ng/mL, respectively, that already contained 1 ng/mL IS. The comparsion of the peak areas determined in the pre-extraction and

post-extraction spiked plasma samples indicated the extraction recovery of 8-OHdG and IS. The ratio (post-extraction spiked peak area/pre-extraction spiked peak area) × 100% was then calculated. In the urine, the ratio (spiked peak area in diluted urine/spiked peak area in neat solvent) × 100% was then calculated.

2.5.3. Artifactual formation of 8-OHdG during sample preparation

The artifactual formation of 8-OHdG during the sample preaparation was checked by spiking $^{15}N_5$-dG at concentrations of 1 ng/mL and 10 ng/mL in plasma. The samples were treated as described for the sample preparation and purification.

2.6. Automated on-line SPE

The column-switching system used in the present study is described in detail elsewhere. This system consisted of a switching valve (10-port, 2-position microelectric actuator from Valco Instrument Co., Ltd.) and an Inertsil ODS-3 (33 mm × 4.6 mm × 5 μm) column. The switching valve function was controlled by Analyst 1.4.2™ software (AB SCIEX, Canada). The column-switching operation, including the LC gradients, used during the on-line cleanup and the analytical procedures are summarized in Table 1. When the switching valve was at position A, 40 μL of prepared plasma sample was loaded on the cartridge by an autosampler (Agilent 1200 SL, Agilent Technology, U.S.A.), and a binary pump (Agilent 1200, Agilent Technology) delivered 100% solvent A (5% MeOH (v/v) with 0.1% formic acid) at a flow rate of 1 mL/min as the loading and washing buffer (Eluent I). After the column was flushed with the loading buffer for 4 min, the valve was switched to the injection position (position B) to inject the sample into the LC system. At 5.5 min after injection, the valve was switched back to position A, and the column was eluted with solvent A (Eluent I) using a linear gradient from 100% solvent A to 100% solvent B (90% ACN (v/v) with 0.1% formic acid) for 4.5 min., followed by 100% solvent A for 2 min for equilibration of the trap column and preparation for the next analysis. The total run time was 12 min.

2.7. Liquid chromatography

The HPLC system consisted of a quaternary pump, an autosampler (Agilent 1200SL, Agilent Technology, U.S.A.), and an Inertsil 5 μm, ODS-80A, 150 mm × 4.6 mm. Chromatography elution using Eluent II was used to separate the analytes. After automatic sample cleanup for 4 min, the sample was automatically eluted from the trap column into the analytical column. The mobile phase (solvent A) was 5% ACN (v/v) with 0.1% FA, solvent B was 95% ACN (v/v) with 0.1% FA, and each was delivered at a flow rate of 1 mL/min.

2.8. Electrospray ionization MS/MS

The sample eluted from the HPLC system was introduced into a Turbo V™ source using an ESI probe installed on an API 4000™ triple-quadrupole mass spectrometer (AB SCIEX. Canada), operated in positive mode with a needle voltage of 5.5 kV. Nitrogen gas was used as the nebulizing, heating, curtain and collision gas, and the heater gas temperature was set at 500 °C. Data acquisition and quantitative processing were accomplished using Analyst 1.4.2™ software (AB SCIEX, Canada). The optimized source parameters multiple reaction monitoring mode (MRM) transition pairs of 8-OHdG, $^{15}N_5$-8-OHdG, dG and $^{15}N_5$-dG were set as m/z 284 → 168, m/z 289 → 173, m/z 268 → 152 and m/z 273 → 157 for the quantitative pair and m/z 284 → 140 and m/z 289 → 145 for the 8-OHdG and $^{15}N_5$-8-OHdG qualitative pair, respectively. The dwell times per channel were set at 150 ms for both analyte and internal standard. Nebulizer gas (Gas 1), heater gas (Gas 2) and curtain gas were set at 55, 65 and 10 psi, respectively. The collision-assisted dissociation gas (CAD) was set at 6. The collision energy (CE) and collision exit potential (CXP) were set at 19 V and 12 V for both 8-OHdG and 8-OHdG $^{15}N_5$. The resolution was set as the unit resolution for Q1 and Q3.

2.9. Statistical analysis

Spearman correlation was used to assess the relationship of 8-OHdG concentration in plasma and urine samples. All analyses were performed using the Statistical Package of SPSS 17.

3. Results and discussion

3.1. Optimization of the extraction procedure for the on-line SPE system

The object of the present study was to develop a simple, novel, rapid method with demonstrated validity for the detection/identification/quantification of 8-OHdG in plasma samples. Therefore, extensive assay validation and careful assessment of LC–MS/MS specificity were important factors, including studies of the matrix effect, ion suppression and chromatographic separation. Many variables affect separation and extraction performance and signal intensities, such as the type column, organic solvents, pH and buffer and wash time. To achieve maximum sensitivity and optimize the peak shape, the composition of the mobile phase included an on-line C18 SPE trap column and an analysis column, as shown in Table 1. For MS operation, ESI positive ion mode was elvated for the determination of 8-OHdG and $^{15}N_5$-8-OHdG (internal standard). The optimized source parameters multiple reaction monitoring mode (MRM) transition pairs of 8-OHdG and $^{15}N_5$-8-OHdG were set as m/z 284 → 168 and m/z 289 → 173 for the quantitative pair and m/z 284 → 140 and m/z 289 → 145 for the qualitative pair, respectively.

Nevertheless, it is well known that plasma samples contain large amounts of endogenous matrix [26–28] components that can decrease sensitivity and specificity. Extraction and purification methods are often critical and time-consuming, requiring successive chromatographic steps, which can lead to a major loss of target compounds such as SPE cartridges and liquid–liquid extraction to isolate 8-OHdG from the plasma samples. In the present study, two major analytical approaches were developed for 8-OHdG measurement in plasma: a one-step membrane extraction method and the on-line C18 SPE column-switch technique. By combining one-step membrane extraction sample preparation and the column switch technique, we reduced the contamination of the mass spectrometer interface and made this method more robust and unique. Under optimal conditions, the chromatograms of the 8-OHdG and $^{15}N_5$-8-OHdG had well-defined peak shapes and maximal sensitivity, as shown in Fig. 1A. However, when the on-line C18 SPE column-switch technique and the off-line (by direct injection to the analytical coulmn) chromatogram were compared, the latter resulted in matrix effects that led to either suppression or enhancement of the analyte response, as shown in Fig. 1B. To evaluate both performances, two different concentrations (0.01 and 0.02 ng/mL) were spiked in human plasma. The on-line C18 SPE column-switch technique was 5-fold (signal to noise ratio) more sensitive than the off-line method, as shown in Table 2. In the one-step membrane extraction method, the efficiency of extraction was studied with different solvents such as deionized water, 50% ACN (v/v) with 0.1% FA and ACN. Results indicated that deionized water was the best extraction solvent for satisfactory recovery in the analysis of plasma.

Table 1
Timetable for the column-switching procedure.

Time (min)	Flow rate (mL/min)	Eluent I (trap)[a]		Flow rate (mL/min)	Eluent II (analysis)[b]		Time (min)	Valve position	Remarks
		Solvent A	Solvent B		Solvent A	Solvent B			
0	1	100	0	1	100	0	0	A	load and wash samples on trap column
5.5	1	100	0	1	100	0	4	B	inject samples into analytical column
10	1	0	100	1	0	100	5.5	A	end of injection; clean up and recondition trap column
10.1	1	100	0	1	100	0	12	A	
12	1	100	0	1	100	0			

[a] Eluent I: Solvent A: 5% MeOH (v/v) with 0.1% FA; Solvent B: 90% ACN (v/v) with 0.1% FA.
[b] Eluent II: Solvent A: 5% ACN (v/v) with 0.1% FA; Solvent B: 95% ACN (v/v) with 0.1% FA.

With this new method, the sample throughput increased significantly (more than 200 samples could be processed daily) due to the employment of rapid on-line SPE and LC–MS/MS procedures, optimal removal of the plasma matrix, and minimal contamination of the curtain plate. Moreover, by reducing the extent of manual SPE operations, this procedure also reduces the potential for human error [9,24]. This technique offers a diagnostic approach to the identification of biomarkers associated with oxidative stress and to evaluation of the risk of occupational and environmental exposure in human studies.

3.2. Method validation

3.2.1. Selectivity, specificity and matrix effect

To test the selectivity of this method, the IS in blank plasma and urine samples was used to identify the 8-OHdG peak in a chromatogram and to compensate for the possible fluctuations in retention time. Specificity was achieved using two MRM transitions pairs, 8-OHdG and $^{15}N_5$-8-OHdG, set as m/z 284 → 168, m/z 289 → 173 for the quantitative pair and m/z 284 → 140 and m/z 289 → 145 for the qualitative pair, respectively.

To evaluate the matrix effects, calculations of the peak areas of the 1 ng/mL internal standard were added to the neat solvent and compared with the peak areas of the 1 ng/mL internal standard that was added to plasma (pre-extraction) and urine (diluted method). The calculations of the effects of the relative matrixs, R_1 and R_2, were less than 15%, and CV ranged from 1.85 to 1.38% for IS in both plasma and urine samples, so it was conclued that the matrix effects did not influence method reliability. Therefore, the use of a stable istotope-labelled internal standard compensated for the different matrix effects. The low matrix effect achieved in this study ensures that the method has high sensitivity.

3.2.2. Artifactual formation of 8-OHdG during sample preparation

In the present study, when samples were pretreated with one-step membrane extraction followed by drying under nitrogen, use of the column switch technique could not induce artifactual formation of $^{15}N_5$-8-OHdG from $^{15}N_5$-dG at concentrations of 1 ng/mL and 10 ng/mL in plasma.

3.2.3. Linearity, limits of detection(LOD) and limits of quantification (LOQ)

In general, the two major considerations in the development of any analytical method are its linearity range and its sensitivity. Calibration curves were constructed by plotting a peak-area ratio of the reference and internal standards against concentration. For plasma analysis, the calibration curves ranged from 0.02 to 0.5 ng/mL, and contained a fixed amount of 1 ng/mL IS in 5% MeOH (v/v) with 0.1% FA (neat solvent) and plasma pooled from volunteers. The calibration curves had correlation coefficients higher than 0.999. A comparsion of the slopes and the intercepts showed no evidence of a significant matrix effect, as the slopes and intercepts of curves in neat solvent and plasma were similar (slopes were 0.89 and 0.94, respectively). For urine analysis, the calibration curves ranged from 0.1 to 10 ng/mL, and contained a fixed amount of 1 ng/mL $^{15}N_5$-8-OHdG in 5% MeOH (v/v) with 0.1% FA (neat solvent). The limit of detection (LOD) was defined by a signal-to-noise ratio of 3, that was found to be 0.008 ng/mL (on column 1.1 fmol in an injection volume of 40 μL). The LOQ was defined as the lowest concentration in the linear range (signal/noise ratio is about 7).

3.2.4. Accuracy and precision

The accuracy of the procedure was demonstrated by spiking three concentrations of 8-OHdG standard – 0.01, 0.02 and 0.05 ng/mL for pooled plasma and 1, 5 and 10 ng/mL for urine – to evaluate interday and intraday assay precision. These preparations were also used as quality control samples to monitor the day-to-day performance of the assay. The intra- (n = 5) and inter- (n = 5) assay variations were 2.3–6.8% for plasma, as shown in Table 2. The intra- (n = 5) and inter- (n = 5) assay variations were 2.7–4.5% for urine, as shown in Table 3. All validation parameters were within an acceptable range. More than 200 plasma samples have been assayed continuously without significant contamination of the curtain plate (data not shown). The variation of the signal intensities

Table 2
Intra-assay and inter-assay accuracy and precision of QC samples prepared in plasma at different concentration levels of 8-OHdG using a one-step membrane extraction method.

Spiked level (ng/mL)	On-line/off-line fold (S/N ratio)[a]	Intraday[b]		Interday[b]	
		Accuracy (%)[c]	Precision (%)[b]	Accuracy (%)[c]	Precision (%)[b]
Blank (0.015 ± 0.002)	–	–	2.3	–	4.3
0.01	4.2	93.0	6.8	95.2	5.6
0.02	5.4	100.5	5.4	99.7	3.8
0.05	–	100.0	4.4	99.8	4.2

[a] S/N: signal to noise ratio, n = 5.
[b] CV: coefficient of variation, n = 5.
[c] Expressed as [(measured concentraion − spiked concentration)/spiked concentration] × 100%.

Table 3
Intra-assay and inter-assay accuracy and precision of QC samples prepared in urine at different concentration levels of 8-OHdG using dilution method.

Spiked level (ng/mL)	Intraday[a]		Interday[a]	
	Accuracy (%)[b]	Precision (%)[a]	Accuracy (%)[a]	Precision (%)[a]
Blank (3.9 ± 0.6)	–	4.2	–	4.0
1	111.7	2.7	110.2	4.5
5	119.4	2.7	115.3	4.2
10	115.6	3.0	114.1	4.0

[a] CV: coefficient of variation, $n = 5$.
[b] Expressed as [(measured concentraion – spiked concentration)/spiked concentration] × 100%.

for the same standard, tested at the beginning and after 50 plasma samples was less than 5%.

3.2.5. Recovery of 8-OHdG in plasma and urine

The recovery rates of 8-OHdG in 0.25 mL of human plasma, as determined by the one-step membrane extraction method at two different concentrations (0.05 and 0.1 ng/mL) in the pre-extraction and post-extraction, are shown in Table 4. The mean recovery rates of 8-OHdG using one-step membrane extraction at 0.05 and 0.1 ng/mL were 102.0 and 95.1%, respectively. Thus, one-step membrane extraction was the best choice in the sample preparation process. To determine recovery rates in the urine sample analysis, 20 μL of urine was diluted 20-fold with 5% MeOH (v/v) that contained 0.1% formic acid. Spiked concentrations of 2.5 and 10 ng/mL, are shown in Table 4. The results obtained from the urine samples were satisfactory, with recoveries ranging between 106.1 and 106.0%.

For other parameters, it was necessary to establish a good extraction time. Comparison of different extraction times, 30 min ($n = 5$, CV = 25.5%) and 60 mins ($n = 5$, CV = 5.3%), indicated that 60 min produces good reproducibility results for plasma. This could possibly be explained by the equilibrium-based extraction procedure, and, therefore, would be a time-dependent process. Our method compares favorably with known methods in term of analytes applicable, sample volume, sample pretreament, LOD and detection levels, as shown in Table 5. Therefore, the combination of one-step membrane extraction sample preparation and column switch technique reduced the contamination of the mass spectrometer interface and made this method more robust.

3.3. Application to the analysis of plasma and urine samples

This method was applied to the analysis of plasma samples obtained from the one-step membrane extraction method and the on-line C18 SPE column-switch technique. Of the 55 healthy subjects (37 males and 18 females) included in the analysis, the mean age was 38.6 ± 8.9 years and all participants were non-smokers. The mean baseline levels of 8-OHdG in plasma were 14.3 ± 5.9 (pg/mL). Analysis of the relationship of plasma and urine 8-OHdG levels revealed a statistically significant positive correlation in healthy subjects ($r = 0.325$, $p = 0.016$), as shown in Fig. 2. The present study compares favorably with previously published data [24], indicating similar results in terms of the applicable analytes.

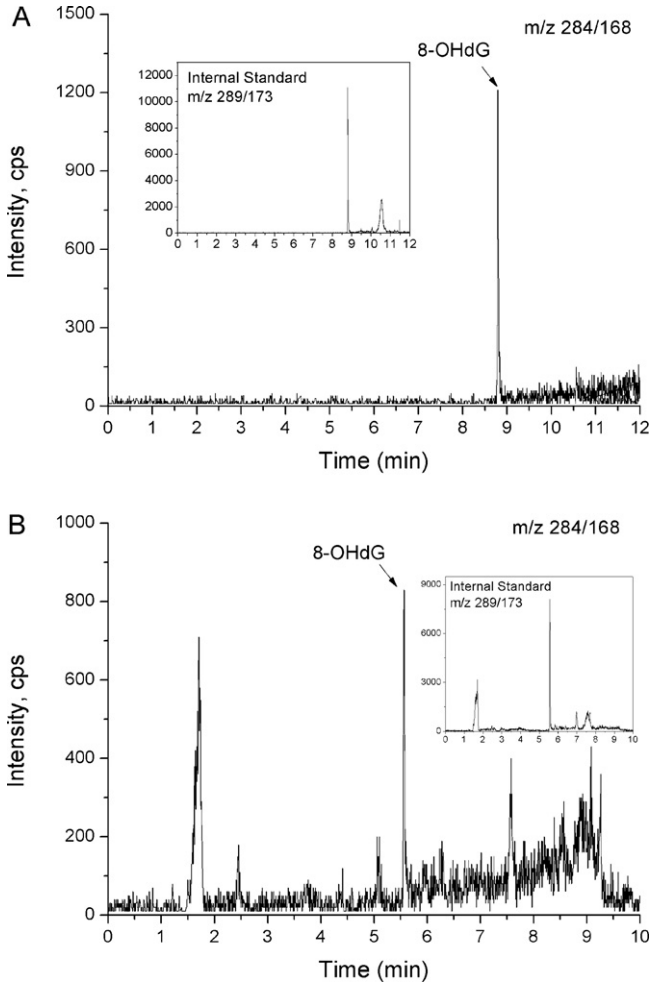

Fig. 1. LC–MS/MS chromatogram of reference compounds using MRM (multiple reaction monitor) mode. (A) on-line and (B) off-line, spiked with 1 ng/mL $^{15}N_5$-8-OHdG in the plasma sample. All compounds were dissolved in [5% MeOH + 0.1% FA].

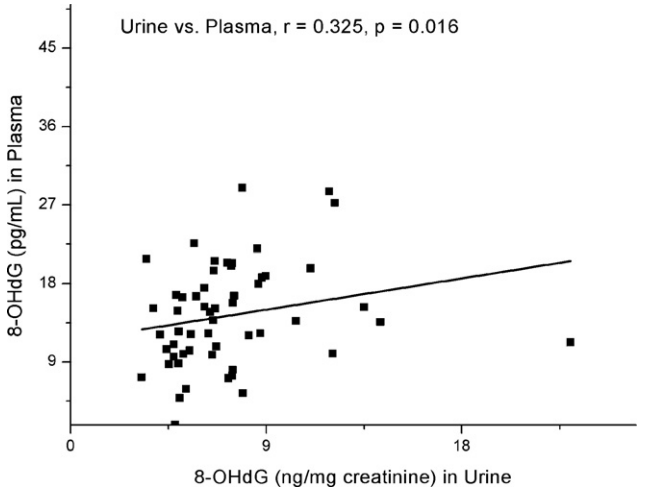

Fig. 2. Correlation between 8-OHdG concentrations in human plasma vs. urine by Spearman correlation.

Table 4
Recovery of the method in plasma and urine samples.

Analyte	Matrix	Spiked level (ng/mL)	Recovery (%)	CV% ($n=5$)[c]
8-OHdG	Plasma	Blank (0.015 ± 0.002)	–	2.3
		0.05	102.0[a]	4.0
		0.1	95.1[a]	4.4
	Urine	Blank (3.9 ± 0.6)	–	4.2
		2.5	106.1[b]	5.4
		10	106.0[b]	2.5

[a] The ratio (post-extraction spiked peak area/pre-extraction spiked peak area) × 100%.
[b] The ratio (spiked peak area in diluted urine/spiked peak area in neat solvent) × 100%.
[c] CV: coefficient of variation, $n=5$.

Table 5
Literature basal level of 8-OHdG in plasma samples from healthy subjects.

Methods	Sample volume	Pretreatment	Level[a] (pg/mL ± SD)	LOD	Reference
ELISA[b]	2 mL	Membrane filtration	500 ± 200 ($n=29$)	125 pg/mL	[29]
HPLC-ECD	5–10 mL	Protein precipitation, immunoaffinity column purification	19.5 ± 4.3 ($n=6$)	50 fmol	[21]
HPLC-ECD	0.5 mL	Manual SPE	13.4 ± 2.1 ($n=28$)	1.2 fmol	[30]
On-line SPE LC–MS/MS	0.5 mL	Manual SPE	21.7 ± 9.2 ($n=50$)	2.0 fmol	[24]
On-line SPE LC–MS/MS	0.25 mL	One step membrane extraction	14.3 ± 5.9 ($n=55$)	1.1 fmol	This study

[a] Healthy subjects.
[b] ELISA: enzyme linked immunosorbent assay.

4. Conclusions

A simple, rapid LC–MS/MS method combined with an on-line SPE step was developed for the detection, identification, and quantification of 8-OHdG in human plasma. Due to low sample consumption (0.25 mL of plasma), the pre-treatment procedure was extremely simple and did not involve a costly time- and labor-consuming sample preparation step. Satisfactory recovery was obtained with a precision rate that ranged from 95.1 to 106.1% at trace levels in human plasma and urine, with a CV lower than 5.4%. Intraday and interday precision were 2.3–6.8% for plasma and 2.7–4.5% for urine, respectively. The method accuracy for intraday and interday assays ranged from 93.0 to 100.5% for plasma and 110.2 to 119.4% for urine, respectively. The high selectivity and sensitivity of this method for simultaneous measurement of 8-OHdG would greatly improve future studies of indicators of oxidative stress for 8-OHdG relative to the role of oxidative stress in human disease and health assessment.

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