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RESEARCH ARTICLE

Development and Validation of a Fast and Simple LC-ESI MS/MS Method for Quantitative Analysis 8-Hydroxyl-2′-Deoxyguanosine (8-OHdG) in Human Urine

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Development and Validation of a Fast and Simple LC-ESI MS/MS Method for Quantitative Analysis 8-Hydroxyl-2′-Deoxyguanosine (8-OHdG) in Human Urine

SUMMARY

8-Hydroxyl-2′-Deoxyguanosine (8-OHdG) is an extensively used biomarker of oxidative DNA damage, which is formed by oxidative stress factors such as chemical carcinogens, cigarette smoke. The main aim of this study was to develop a fast, sensitive and easy method for the quantitative determination of 8-OHdG in human urine samples. In this study, we developed and validated an accurate, fast, sensitive, and robust liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for determination of 8-OHdG concentrations in human urine samples. The levels of the 8-OHdG in urine samples were determined quantitatively with LC-MS/MS by using positive electrospray ionization (ESI) in the multiple reaction monitoring (MRM) mode and a Hilic Plus column. 8-Hydroxy-2'-Deoxyguanosine [15N5, 98%] was used as an internal standard in this study. Our validated method was successfully applied to the analysis of spot urine samples collected from randomly selected healthy human cigarette smoker and non-smoker subjects. With the help of this validated LC-ESI MS/MS method we can determine the presence of 8-OHdG at very low concentrations in human urine samples.

Key Words: *Urine, LC-MS/MS, 8-Hydroxyl-2′-Deoxyguanosine, DNA damage, Biomarker, Tobacco smaoke*

İnsan İdrarında 8-Hidroksil-2′-Deoksiguanozin (8-OHdG) Kantitatif Analizi için Hızlı ve Basit LC-ESI MS/MS Yöntemi Geliştirilmesi ve Doğrulanması

ÖZ

8-Hidroksil-2′-Deoksiguanozin (8-OHdG), oksidatif DNA hasarının yaygın olarak kullanılan bir biyolojik belirleyicisi olup, kimyasal kanserojenler, sigara dumanı gibi oksidatif stres faktörlerine bağlı olarak oluşur. Bu çalışmanın amacı, insan idrarında 8-OHdG'nin kantitatif analizi için hızlı, hassas ve basit bir prosedür geliştirmektir. Bu çalışmada, insan idrarında 8-OHdG konsantrasyonlarını ölçmek için doğru, hızlı, hassas ve sağlam bir sıvı kromatografisi-tandem kütle spektrometresi (LC-MS/MS) metodu geliştirilmiş ve valide edilmiştir. İdrar numunelerindeki 8-OHdG seviyeleri, MRM modunda ve bir Hilic Plus kolonunda pozitif elektrosprey iyonizasyonu kullanılarak LC / ESI-MS / MS ile kantitatif olarak belirlendi. Bu çalışmada iç standart olarak 8-Hidroksi-2'-Deoksiguanozin [15N5,% 98] kullanılmıştır. Doğrulanmış yöntem, rastgele seçilen sağlıklı sigara içen ve sigara içmeyen deneklerden toplanan spot idrar örneklerinin analizine başarıyla uygulanmıştır. Valide edilmiş bu LC-ESI MS / MS metodu yardımıyla insan idrar numunelerinde 8-OHdG varlığı çok düşük seviyelerde tespit edilebilmektedir.

Anahtar Kelimeler: *İdrar, LC-MS/MS, 8-Hidroksi-2′-Deoksiguanosin, DNA hasarı, Biyobelirteç, Sigara içme*

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INTRODUCTION

In our daily lifes, one of the most important health risks posed by exogenous or endogenous reactive oxygen species (ROS) composed from various biochemical reactions throughout different physiological processes and the exposure to toxic xenobiotics such as polycyclic aromatic hydrocarbons (PAHs), heavy metals and cigarette smoke are creating alterations in DNA. We need to identify these alterations with the help of sensitive biomarkers. 8-Hydroxy-2'-deoxyguanosine (8-OHdG), a product of DNA base alteration made by the oxidation of deoxyguanosine, is extensively used for biomarker of oxidative DNA damage, since C-8 position of 8-OHdG is vulnerable to ROS and its mutagenic capacity.

Lately, 8-OHdG has been extensively used as a key marker in a number of studies for determination of the endogenous oxidative DNA damage. Besides, it is also as a risk factor for a lot of diseases such as Parkinson (Hirayama, et al.,2019), cancer (Ohtake, et al., 2018), neurodegenerative diseases (Long, et al., 2012), infectious diseases (Wu, et al., 2004), cardiomyopathy (Chen, et al., 2017) and cardiovascular diseases (Mendes et al., 2013), and diabetes (Nakanishi, et al., 2004).

Due to its very low levels in organisms, the development of an easy, highly accurate and reliable analytical procedure for the measurement of 8-OHdG is crucial. During the last two decades, several analytical techniques have been developed for the identification and determination of 8-OHdG in biological samples such as urine, blood, and salivary. During the last decade, important advances have been achieved about the detection limits of these techniques or methods. The most widely used detection methods for quantitative analysis of 8-OHdG in human bio-specimens include high-pressure liquid chromatography/electrochemical detection (HPLC-EC) (Li, et al., 2018) and enzyme-linked immunosorbent assay (ELISA) (Han, et al., 2010). In recent years, a number of methods have been established for attempting to assess 8-OHdG with the aid of solid-phase extraction by high-performance liquid chromatography (SPE-HPLC) (Hu, et al., 2010), high pressure liquid chromatography-tandem mass spectrometry (HPLC-MS-MS) (Chen, et al., 2010; Kataoka, et al., 2016), high performance liquid chromatography-positive electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) (Weiman, et al., 2002) gas chromatography mass spectrometry (GC–MS) (Dizdaroğlu, 1994), capillary electrophoresis with UV detection (CE-UV) (Kvasnicova, et al., 2003), carbon fiber microelectrode (Koide, et al., 2010) and electrochemical biosensors (Fan, et al., 2016).

The main aim of this study was to develop a fast, sensitive and easy method for the quantitative determination of 8-OHdG in human urine that can be use in the investigations of the potential a biomarker of oxidative stress and carcinogenesis.

MATERIALS AND METHODS

Materials and Chemicals

All chemicals used in this study were of analytical grade. 8- Hydroxyl-2′-Deoxyguanosine (8-Oxo-dG, >98% purity) (Figure 1) was purchased from Sigma-Aldrich (USA). Internal standard (IS), 8-Hydroxy-2'- Deoxyguanosine [15N5, 98%], was customized from Cambridge Isotope Laboratories (USA). HPLC-grade methanol and acetonitrile were purchased from Sigma–Aldrich (USA). Water for chromatography (LC-MS Grade) was from Merck, Darmstadt, Germany.

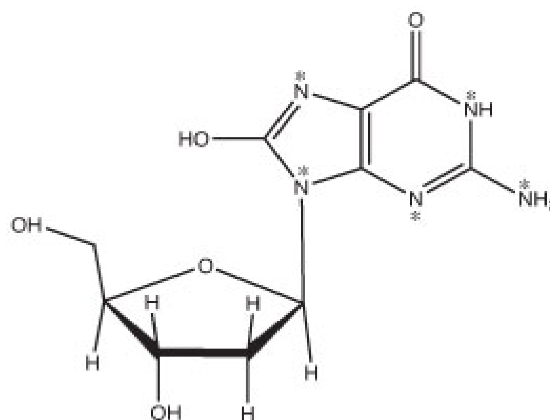

Figure 1. Chemical structure of 8-Hydroxy-2'-deoxyguanosine

Sample collection

For urinary 8-OHdG analysis, spot urine samples were taken from randomly selected smoker (n = 15; 34 ± 5 years) and healthy non-smoker volunteers (n =

15; 32 ± 6 years). In this study, smoker was preferred to be smoking for at least 10 years and at least 15 cigarettes in a day. Urine samples were collected in glass (PTFE-lined) screw cap bottles that had been previously cleaned with hexane. In this study, the code of ethics established by the Helsinki Declarations of 1964 and revised in 2000 were followed. After receiving a detailed description of the study and possible results, each participants gave informed consent and signed an informed consent form. The study protocol was reviewed and approved by the Mersin University Clinical Research Ethical Committees in Mersin (ethical committee no: 2015/36).

Extraction procedure

Different organic solvents (water, methanol and acetonitrile) and buffers (ammonium acetate, ammonium formate, formic acid and ammonium fluoride) were tested in different proportions to ensure the best possible extraction conditions. Therefore the extraction procedure of 8-OHdG was a simple 0.1% formic acid in acetonitrile dilution/precipitation at 1:2 ratio was used to minimize potential sources of contamination by 8-OHdG. With the help of this extraction procedure, less interferences in blank matrices were detected at the transition of 8-OHdG.

Method application

Urine samples were stored at −20°C prior to analysis. After adding 50 µl (2, 5 ppm) of 8-Hydroxy-2'-deoxyguanosine [15N5, 98%] as internal standard, 1.0 ml of urine was mixed 950 µL of acetonitrile containing 0.1% formic acid. It was centrifuged at 8000 rpm for 10 min. Supernatants were taken and filtered through a 0.45 µm PTFE filter and transferred into 2 mL vials. The urinary 8-OHdG values that were achieved in our study were presented after being corrected for creatinine (Park, et al., 2008) and presented as ng/g creatinine and presented as ng/g creatinine.

Instrumentation and Chromatographic conditions

Instrumental analyses of 8-OHdG and (Figure 1) and 8-OHdG [15N5, 98%] (IS) were performed by LC–MS/MS system consisted of an Agilent Technologies 1200 series 6460 triple quadrupole (QqQ) HPLC/MS/MS with Jet-StreamW (ESI-MS/MS; USA). Five microliters (µL) of the extract was injected onto an 150 × 3.0 mm, 3.5 µm particle size Hilic Plus column. The tandem mass spectrometry (MS-MS) was runed with positive electrospray ionization in the multiple reaction monitoring mode (MRM). Nitrogen was used as the collision and drying gas. Optimum operating ESI conditions were programmed as follows: sheath gas and auxiliary gas (N_2, 99.995%) flow rates were 12 L/min; sheath gas temperature was 400°C; capillary voltage was 2000 V, positive; nozzle voltage was 2000 V, positive. The column temperature was hold at 25°C. The optimized parameters for each compound are listed together with the MRM transitions in Table 1. The peak areas of 8-OHdG and 8-OHdG [15N5, 98%] were determined with R2≥ 0.999.

In this study, for analysis MS 2 segment was used. The mobile phases A and B consisted of 1 mM ammonium fluoride in water and acetonitrile, respectively. The analysis for 8-OHdG and 8-OHdG [15N5, 98%] were completed by using the following gradient program: at a flow rate of 0.7 ml/min the gradient was initially started at 97% acetonitrile and maintained at that level for 3 min. The gradient was then decreased to 55% acetonitrile between 6 and 8 min, increased to 97% acetonitrile at 8.10 and maintained at this percentage between 8.10 and 13 min.

RESULTS

Optimization of HPLC/MS/MS assays

When evaluating the separation efficiency of tree columns, Agilent C_{18} liquid chromatography columns (150 × 2.1 mm, 5 µm particle size and 150 × 2.1 mm, 3 µm particle size), and Hilic Plus column (150 × 3.0 mm, 3.5 µm particle size), it is found that the Hilic Plus column was superior to the others. In this study the initial mobile phase which consisted of a gradient mixture of an ammonium acetate aqueous solution as solvent A and pure methanol as solvent B were modified. As an initial step, the effect of substituting methanol for acetonitrile was analysed. As acetonitrile peak shapes and resolution were observed to be better than methanol, acetonitrile was picked for further experiments. Following this step, formic acid, ammonium acetate and ammonium chloride were tested as

additives and the pH of the mobile phase was evaluated. It was studied that whether the addition of ammonium fluoride to distilled water improved the peak shapes and sensitivity for the optimum ionization of the 8-OHdG and IS. The best separation, peak shapes and ionization of the compounds were obtained by a mixture of ammonium fluoride aqueous solution as solvent A and acetonitrile as solvent B. For the determination of the optimum injection volume, a range from 3 to 25 µL was studied and 5 µL was chosen as the injection volume after observed that no extra broadening of the peaks even at the maximum value.

Method validation

The quantification method for 8-OHdG was validated according to the US Food and Drug Administration (US FDA) guidelines in terms of selectivity, repeatability, linearity and reproducibility (US Department of Health and Human Services, 2001).

In order to control and test for our method, we spiked urine samples containing 5 and 10 ng/mL of 8-OHdG. The quantification method for 8-OHdG was validated for linearity, selectivity, repeatability reproducibility, precision and accuracy.

Selectivity and speciftiy

To test the selectivity of developed method, the IS in blank urine samples was used to identify the 8-OHdG peak in a chromatogram and under the described conditions no interferences from endogenous substances were observed at the retention time of 8-OHdG at 5.81 min. As shown in Table 1, specificity was achieved by using MRM transitions pairs. Figure 2 shows the MRM mode chromatogram of the 8-OHdG in a spiked urine sample in ESI positive mode.

Table 1. MS/MS transitions and instrument parameters used to quantify 8-OHdG and 8-OHdG [15N5, 98%] in urine

Compound	Transition	Fragmentor voltage	Collision energy
8-OHdG (quantifier)	284–168	90	8
8-OHdG (qualifier)	284–140	90	36
8-OHdG [15N5, 98%]	289–173	80	4

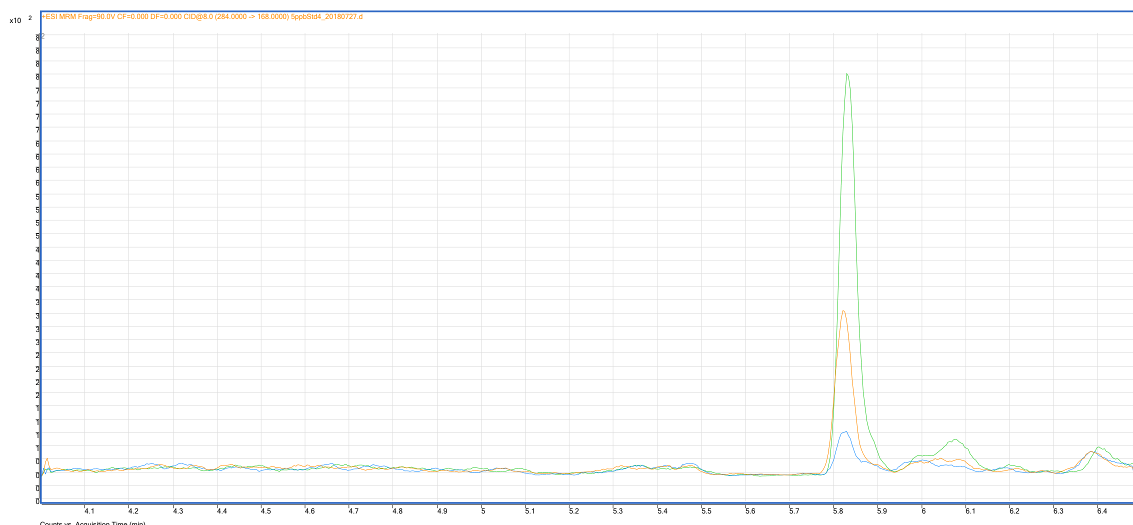

Figure 2. Representative chromatogram of spiked uring sample 8-OHdG (0.25, 1.0, 5.0 ng/mL).

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

Calibration curve was constructed by plotting the analyte/internal standard peak area ratio against concentration of analyte. The six standard solutions of 0.25, 0.5, 1, 5, 10 and 20 ng/mL of 8-OHdG in urine were evaluated for linearity. The analysis was performed in triplicate and very good calibration curve and correlation coefficient were obtained for the analyte ($R^2 \geq 0.998$) (Figure 3). In this developed study, the limit of detection (LOD) and the limits of quantification (LOQ) were found to be 0.019 ng/mL and 0.062 ng/ml respectively. Chromatogram of standards of 8-OHdG and 8-Hydroxy-2'-deoxyguanosine [15N5, 98%] (IS) in urine is shown in Figure 4.

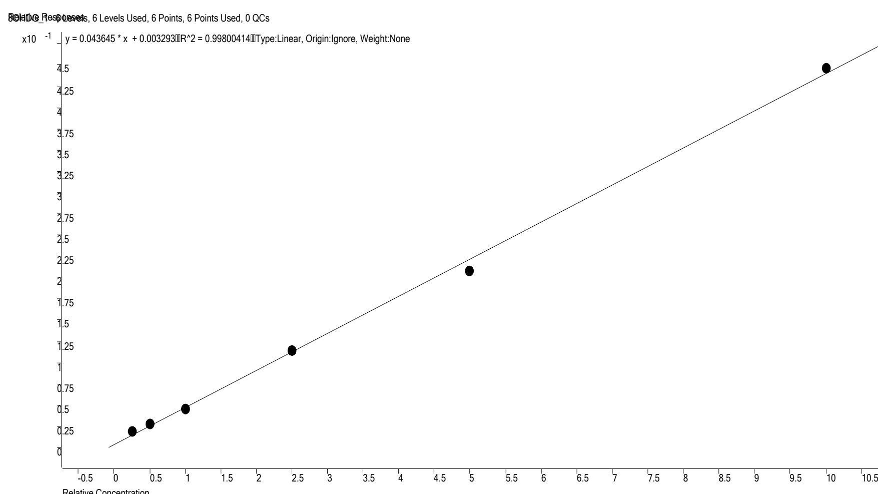

Figure 3. Calibration curve obtained by plotting the ratio between the area of the 8-OHdG peak divided by the area of the internal standard peak (8-Hydroxy-2'-deoxyguanosine [15N5, 98%]) as a function of the 8-OHdG concentration.

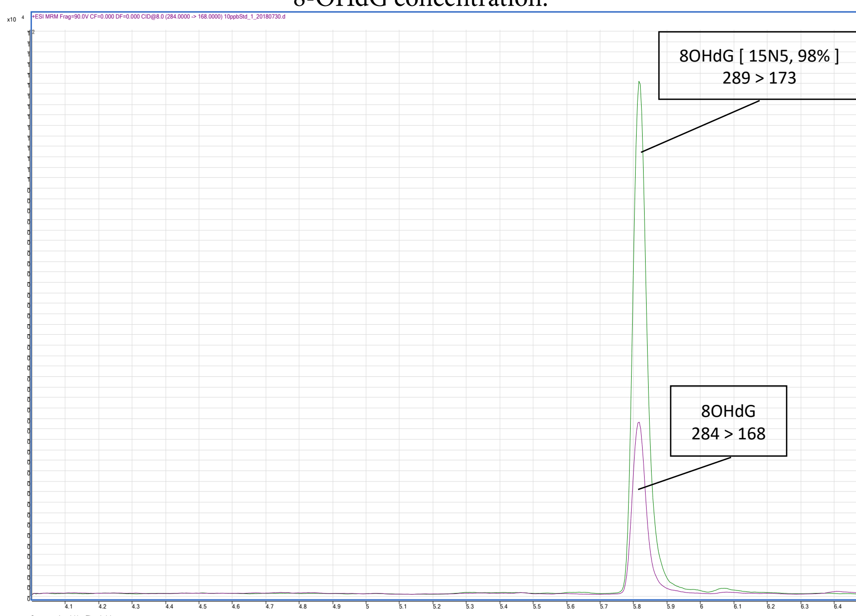

Figure 4. Chromatogram of standards of 8-OHdG and 8-Hydroxy-2'-deoxyguanosine [15N5, 98%] (IS).

Precision and accuracy

Precision was expressed as the percentage of the relative standard deviation (RSD, %). Intra- and inter-day precision of our method were within the acceptable limits that ranged from 0.32 to 6.16% and from 1.85 to 3.24%, respectively. To estimate the precision of the method, laboratory repeatability and reproducibility were analysed at two different concentrations for each urine sample (five, 10 ng/mL 8-OHdG). The precision study was also performed in triplicate on the same day to estimate intra-day variability and was repeated on three consecutive days to establish inter-day variability. Our precision and accuracy data showed that the extraction procedure of 8-OHdG from urine was highly reproducible and robust. The precision and accuracy data for the analytical procedures are represented in Table 2.

Table 2. Method validation parameters for 8-OHdG in human urine samples

	Day 1			Day 2			Day 3			Inter-day		
Spiked	Mean	Recovery	RSD	Mean	Recovery	RSD	Mean	Recovery	RSD	Mean	Recovery	RSD
(ng/ ml)	(n=6)[a]	(%)[b]	(%)[b]	(n=6)[a]	(%)[b]	(%)[b]	(n=6)[a]	(%)[b]	(%)[b]	(n=6)[a]	(%)[b]	(%)[b]
5	4,93	4,93	4,52	4,88	4,88	2,83	4,72	4,72	0,32	4,84	4,84	1,85
10	10,22	10,23	6,16	10,71	10,71	3,23	9,89	9,89	1,83	10,27	10,27	3,24

[a] Mean measured concentration is reported. [b] Urine samples spiked at three different levels of 8-OHdG were analysed in triplicate and injected in duplicate (n = 6) for each level on three consecutive days to estimate the intra-day and inter-day precision (percentage RSD) and accuracy (percentage recovery).

Human Results

The results obtained from urine samples of volunteers are presented in Table 3. The mean 8-OHdG levels have been found as 24.08±8.38 ng/g creatinine for cigarette smokers, and 7, 46±7, 77 ng/g creatinine for healthy nonsmokers. Mean 8-OHdG level of cigarette smoker group was statistically higher than those of nonsmokers (p<0,001).

Data analysis

Statistical analyses were performed using Statistical Package for Social Science (SPSS) version 11.5 (SPSS, Chicago, IL). Student's t-tests for independent samples and paired samples were applied to test the differences between study groups. Pairwise comparisons for the significance of the differences were performed using the Mann–Whitney U-test. p values equal or lower than 0.05 were accepted statistically significant.

Table 3. Urinary 8-OHdG concentrations of cigarette smokers and healthy nonsmokers volunteers (creatinine-corrected ng/g).

	Non Smokers	Smokers
1	7,54	24,22
2	19,27	26,33
3	7,33	14,75
4	7,33	29,85
5	<LOQ	28
6	7,25	17,47
7	3,93	19,67
8	<LOQ	29,57
9	6,31	16
10	12,15	20,4
11	4,3	23,22
12	2,9	13,4
13	<LOQ	29,25
14	4,61	22
15	29	47

<LOQ, Measured 8-OHdG concentration is below limit of quantitation (<0.062 ng/mL).

DISCUSSION

In this study, a fast LC–ESI–MS/MS analytic procedure was developed and described for the rapid separation and quantification of 8-OHdG in urine for routine applications. In recent decades, there has been an increased awareness in urinary 8-OHdG as an excellent biomarker of oxidative DNA damage in the pathogenesis of many diseases including aging, atherosclerosis, neurodegenerative diseases, parkinson, alzheimer, and early marker of different types of cancer. Thus, 8-OHdG is a marker not only as an indicator of endogenous oxidative DNA damage, but also for early detection and prediction of the susceptible population (Pylväs-Eerola, et al., 2015).

Up to date with the help of several studies more than 20 DNA base adducts or oxidative products have been determined in DNA exposed to oxidative agents. Among these, DNA adducts 8-OHdG is one of the most studied one and has been shown to cause a G: C to T: A transversion-type mutations if left unrepaired (Mei, et al., 2001; Dizdaroğlu, 1992). On the other hand, as because 8-OHdG is stable (Matsumoto et al., 2008) and is excreted in the urine without undergoing any type of metabolic transformation, its measurement in the urine seems to be a practical approach in diagnosis and the case.

For determining the concentration of 8-OHdG in various biological samples including plasma, saliva, urine, and cerebrospinal fluid, several analytical methods have been developed. The first application of oxidized guanine determination based on HPLC-MS/MS was reported by Weimann et al. (2002). As other techniques used for this purpose such as GC-MS, HPLC-ECD, immunochemistry, ELISA, have either a low sensitivity or a non-specific defect; numerous studies have been performed to improve the performance of the HPLC-MS/MS system. In recent years, there have been several methods and studies in the literature attempted to assess 8-OHdG with the help of LC/MS-MS system (Chen, et al., 2016 ; Kataoka, et. al.,; 2016; Crow, et al., 2008; Zhanga, et.al., 2015). Liquid chromatography–mass spectrometry (LC–MS) method offers various advantages and provides a robust approach to identifying the unknown components in the biological samples through effective separation abilities of HPLC and exact structural characterization by MS. Today, mass spectrometry detection with electro spray ionization (ESI) is one of the best sensitive technique, and among the most successful interface used in LC–MS configurations. ESI is the one of the ionization modes used in the determination of fragmentation patterns of the ions and is capable of producing small fragmentation patterns through electrical energy, which allows the ions to transfer from liquid to gaseous phase before being analysed in mass spectrometer (Kumar, 2017; Ho, et al., 2003). Configuration of LC-MS with ESI provides fragmentation data for structural confirmation and represents a powerful method for the analysis of complex systems. The use of MRM mode reduces the noise caused by background ions, which increases the sensitivity of the HPLC-MS / MS system compared to HPLC-MS (Banerjee and Mazlumdar, 2012). The major disadvantages of mass spectrometric techniques are the high capital costs of these instruments and the need for specially trained individuals in the operation and maintenance stages. On the other hand, the strengths of mass spectrometry such as high sensitivity, detection time, specificity, accuracy, and the ability to determine several lesions in a single run, makes it prominently used in the analysis of oxidative damage of nucleic acids. Additionally, in order to remedy the false-positives obtained from other interferences from biological matrix during the analytical trials, to explain the loss during sample preparation and ion suppression, 8-OHdG [15N5, 98%] was used as an internal standard.

This newly developed method differs broadly from the other 8-OHdG analyses that have been developed thus far. Our method, which requires only membrane filter application without any extraction method including solid-phase extraction (SPE), is different from other methods particulary with this feature. This difference makes the application to be extremely fast and simple and prevents the loss of time to get the results. Due to these characteristics, our study is distinguished from similar studies (Hosozumi et al., 2012) in a positive way. With the help of our method not only routine measurements, but also in large-scale

population (large numbers samples) studies can be analysed in a reasonable time.

The LOD value in this study was calculated as 0.019 ng/ml for 8-OHdG. This value in our study method was more sensitive than the results of many other studies explained in the literature. When the studies performed with LC-ESI MS/MS were considered, for example, LOD values for 8-OHdG were determined as 0.2 ng/ml by Sabatini et al. (2005), 0.053 ng/ml by Hari et al (2007), 0.028 ng/ml by Hosozumi et al (2012), 0.17 ng/ml by Zhanga et al (2015), and 0.083 ng/ml by Kataoka et al (2016).

The choice of urine in biological monitoring is mostly preferred in which 8-OHdG is determined, because of being a noninvasive collection and allowing access to vulnerable groups such as children. To evaluate the efficacy of the developed method, we determined spot urinary 8-OHdG concentrations in randomly selected smoker and non-smoker healthy human subjects. As represented in Table 4, the mean urinary 8-OHdG concentrations of fifteen smokers (24.08±8.38 ng/g creatinine) were statistically higher ($p < 0.001$) in comparison to fifteen non-smoker subjects (7,46±7,77 ng/g creatinine) (Fig.3). Similar results have been observed in some other studies (Kataoka et al., 2016; Kanaya et al., 2004; Campos et al., 2011; Lu et al., 2014). Determined urinary 8-OHdG values were normalized to creatinine concentration. This study was carried out by using spot urine samples. Some studies have reported that 24 hour urine samples were needed to determine the amount of urine in the nucleic acid oxidative products, while others preferred random samples as we did in our study. After creatinine correction, the levels of nucleic acid oxidative products in random urine samples were found as consistent with those in 24 hour urine samples regardless of renal disease or age.

CONCLUSION

The findings of this study suggest that the LC-ESI MS/MS method can detect the presence of 8-OHdG at very low concentrations in human urine samples. The linearity, precision, and recovery were evaluated and these parameters all met the criteria in the literature. It is considered that initiating further studies in the shortest delay on other biological materials by using our developed method, which will facilitate and save time on LC/MS/MS analyses for 8-OHdG, will make significant contributions to biomonitoring studies.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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