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Fast Evaluation of Oxidative DNA Damage by Liquid Chromatography-Electrospray Tandem Mass Spectrometry Coupled With Precision-cut Rat Liver Slices[1]

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Objective To establish a fast and sensitive method for the detection of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in precision-cut rat liver slices by HPLC-MS/MS and to investigate isoniazid (INH) -induced oxidative DNA damage. **Methods** Precision-cut liver slices (300 μm) were prepared from male rats, and incubated with INH (0.018 mol/L) for 2 h after 1 h preincubation. DNA in the slices was extracted and digested into free nucleosides at 37℃. The samples were injected into HPLC-MS/MS after the proteins were removed. The level of oxidative DNA damage was estimated using the ratio of 8-OHdG to deoxyguanosine (dG). **Results** The limit of detection of 8-OHdG was 1 ng/mL (S/N=3) and the intra-assay relative standard variation was 3.38% when one transition 284.3/168.4 was used as a quantifier and another two transitions 284.3/140.2, 306.1/190.2 as qualifiers. 8-OHdG and dG were well separated, as indicated by elution at 10.02 and 7.37 min, respectively. INH significantly increased the ratio of 8-OHdG to dG in rat liver slices ($P<0.05$). **Conclusion** 8-OHdG in precision-cut liver slices could be sensitively determined by HPLC-MS/MS. HPLC-MS/MS coupled with precision-cut tissue slices is a fast and reliable analytical technique to evaluate oxidative DNA damage of target tissues caused by procarcinogens and cytotoxins.

Key words: Isoniazid; 8-Hydroxy-2'-deoxyguanosine; HPLC-MS/MS; Precision-cut liver slices

INTRODUCTION

During cellular metabolism, the production of reactive oxygen species (ROS) is generally related with electron transfer reactions and can be mediated by metabolizing enzymes such as cytochrome P450 and peroxisomes[1]. ROS can potentially cause damage to biomolecules (e.g. DNA, protein, and lipid), and are widely identified as a significant contributors to the underlying pathophysiological mechanisms of toxins and procarcinogens. DNA is vulnerable due to its limited chemical stability, and DNA damage caused by ROS can directly trigger cell death by apoptosis or necrosis[2]. Hence, it is essential to accurately quantify DNA damage and assess the risk to target tissues posed by xenobiotics. The nucleophilic sites on DNA bases, such as purine and pyrimidine bases, can be covalently altered by electrophilic compounds. The modified DNA bases referred as DNA adducts represent early biomarkers that can indicate the extent of oxidative damage to genetic materials[3-4].

Presently, more than 20 different DNA oxidative products have been described. Among these DNA adducts, 8-hydroxy-2'-deoxyguanosine (8-OHdG) is probably one of the most well-documented adducts and has been shown to cause a G:C to T:A transversion. 8-OHdG has been identified as an excellent biomarker of oxidative damage, which is attributed to its considerable stability as it cannot be metabolized once generated by free radicals[5]. Owing to its very low levels, the measurement of 8-OHdG is a challenging analytical problem. HPLC with electrochemical detection (ECD) and GC-MS are two widely used techniques to quantify 8-OHdG. HPLC-ECD analysis demands multiple column switching techniques, and the timing of 8-OHdG fraction elution changes on different days[6]. GC-MS can provide the structural evidence. However, the bases hydrolyzed from DNA by acid must be

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derivatized before the analysis by GC-MS. Artificial formation of DNA adducts might occur during derivatization. Thus, a tedious and time-consuming HPLC procedure has been proposed to remove the intact bases in order to avoid the artificial formation of adducts[7-8].

Measurement of 8-OHdG in urine has been used to assess the extent of oxidative damage to DNA by xenobiotics. However, no information about the originally injured tissue can be obtained[9]. The liver is a significant target organ since it is chiefly responsible for the activation or deactivation of endogenous and exogenous compounds. Precision-cut liver slices have been well-accepted as an alternative *in vitro* model since the complexity of liver structure and all cell types are maintained in them[10]. There is strong evidence that both parenchymal and nonparenchymal cells have the ability to metabolize exogenous chemicals[11].

In our previous study, isoniazid (INH), a highly effective drug in the chemotherapy of tuberculosis, was deduced to produce ROS mediated by cytochrome P4502E1 (CYP2E1)[12]. CYP2E1 is primarily expressed in the liver and contributes to the metabolic bioactivation of many procarcinogens (e.g. tobacco-derived nitrosamines and benzene) and cytotoxins (e.g. carbon tetrachloride)[13]. CYP2E1 also plays a principal role in the production of high levels of ROS leading to oxidative damage of cellular proteins, lipids, and DNA.

In the present study, a fast and sensitive method for the determination of 8-OHdG is established using HPLC-MS/MS combined with precision-cut tissue slices. Subsequently, INH-induced DNA damage in rat liver slices is investigated.

MATERIALS AND METHODS

Reagent

INH was from Sanjiu Wanrong Pharmaceutical Co., DMEM and newborn calf serum were from Gibco Co., DNase I and snake venom phosphodiesterase were purchased from TaKaRa Co., and alkaline phosphatase was obtained from Worthington Co. 8-OHdG and deoxyguanosine (dG) were the products from Sigma. Methanol and acetonitrile were of HPLC-grade from Fisher Co, and all the other chemicals and reagents were of analytical grade.

Animals

Male Wistar rats (135-145 g, Certificate No. 19-084) were supplied by Experimental Animal Center, Hubei Province. Before the study, the animals were housed in temperature-controlled rooms in a 12 h light-dark cycle with free access to food and water.

Precision-cut Liver Slice

Rats were euthanized by cervical dislocation, and the liver was quickly harvested and placed into ice-cold Krebs–Henseleit buffer (pH 7.4) pregassed with 95% O_2/5% CO_2. Liver slices (300 μm thick) were prepared from the tissue cylinders (1 cm outer diameter) as previously described[14]. The first and last slices from each tissue were discarded. Slices were placed into a 24-well plate with DMEM containing 10% newborn calf serum on an orbital shaker and preincubated with 95% O_2/5% CO_2 at 37℃ for 1 h. To investigate the effect of preincubation on the slices viability, ALT and Na^+, K^+-ATPase activities in the medium were assessed at 0, 0.5, 2, 4, and 6 h with or without 1 h preincubation. ALT activities were measured by standardized enzymatic procedures using kits from Shanghai Rongsheng Biotechnology Co. Na^+, K^+-ATPase activities were analyzed using test kits from Nanjing Biotechnology Co.

Following 1 h preincubation, liver sections were incubated in fresh medium with or without INH (0.018 mol/L), respectively, for another 2 h. At the end of incubation, slices were homogenized in 10 mmol/L ice-cold Tris-Cl buffer (pH 8.0) containing 1 mmol/L EDTA.

DNA Isolation and Digestion

Homogenized slices were incubated with 40 mg/mL proteinase K, 0.5% SDS, and 3 mg/mL RNase for 8 h at 37℃. Samples were extracted by phenol and chloroform-isoamyl alcohol (24:1). DNA was dried in vacuum following precipitation by ethanol prestored at -20℃. As previously described, DNA was dissolved in Tris-HCl (40 mol/L, pH8.5) containing 10 mol/L $MgCl_2$ and digested to free nucleosides by incubation overnight at 37℃ with a mixture of DNase Ⅰ, snake venom phosphodiesterase and alkaline phosphatase[15]. After the proteins were removed, 10 μL of the collected fraction was analyzed by HPLC-MS/MS.

Separation Condition

Samples were separated on Allsure C18 column (150 mm×2.1 mm, 2 μm). The mobile phase composition was ammonium acetate (20 mmol/L, pH 6.5) -methanol (90:10) at a flow-rate of 0.2 mL/min. The HPLC (Agilent Series 1100) was fully controlled by the mass spectrometer (API 3000 triple quadrupole mass spectrometer) equipped with a turboionspray source. Electrospray ionization was performed in multiple reaction-monitoring (MRM)

mode with positive ionisation. Nitrogen was used as nebulizing (7 Psi), curtain (9 Psi), collision (7 Psi), and auxiliary (8 L/min) gas. The electrospray probe was heated to 400℃, and the spray needle potential was set at 5200 V. The pseudo molecular ion $[M+H]^+$ and the sodium adduct $[M+Na]^+$ of 8-OHdG were selected by the first mass filter, and two intensive fragment ions from each of them were respectively used for quantification and qualification after collision activation. The protonated molecular ion for dG and its daughter ions were also analyzed.

Statistical Analysis

The results were presented as $\bar{x} \pm s$ and statistically compared using two-tailed *t*-test. A probability level of significance was taken as $P<0.05$. Correlation was analyzed with linear regression.

RESULTS

Effects of Preincubation on the Viability of Precision-cut Liver Slices

In non-preincubated and preincubated liver slices, ALT activities were 10.05±2.1 and 10.1±1.1mmol/min/g protein at 0 h, respectively. Na^+, K^+-ATPase activities were 6.4±0.4 and 4.1±0.7 mmol Pi/h/g protein in non-preincubated and preincubated liver slices at 0 h, respectively. At 6 h, ALT and Na^+, K^+-ATPase activities were decreased by 5% and 15% in preincubated liver slices, whereas the activities were significantly decreased by 63% and 30% in the slices without preincubation ($P<0.05$) (Fig. 1). The results indicated that ALT and Na^+, K^+-ATPase activities were comparatively stable from 0 h to 6 h in preincubated liver slices.

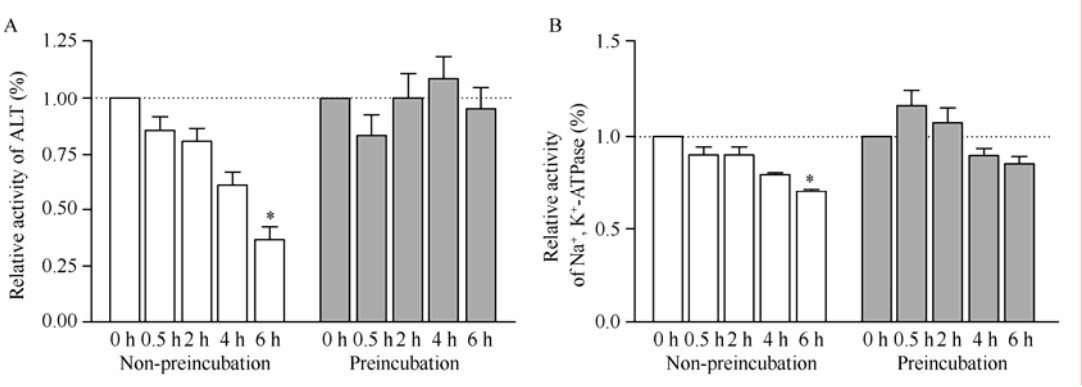

FIG. 1. Effects of preincubation on ALT (A) and Na^+, K^+-ATPase activities (B) in cultured precision-cut rat liver slices. The data are expressed relative to the activity measured at 0 h. n=5. $\bar{x} \pm s$. $^*P<0.05$ *vs* 0 h.

Mass Spectrometry Characterization of 8-OHdG and dG

Positive electrospray ionization (ESI) mode was used for the analysis of 8-OHdG and dG. The full-scan MS spectrum showed that the most abundant molecular ion of 8-OHdG was $[M+H]^+$ at *m/z* 284.3, and the other intensive ion was the sodium adduct $[M+Na]^+$ at *m/z* 306.1 (data not shown), which is consistent with previous reports[16-17]. The most intensive fragment produced from the pseudo molecular ion $[M+H]^+$ in MRM mode was *m/z* 168.4 which was selected for the quantification of 8-OHdG (Fig. 2). Another fragment ion at *m/z* 140.2 from the protonated ion $[M+H]^+$ was used for the qualification of 8-OHdG. In addition, two main fragments at *m/z* 190.2 and *m/z* 262.1 corresponding to protonated ion $[M+Na]^+$ were also recorded as qualifiers for 8-OHdG.

For dG, the most intensive pseudo molecular ion $[M+H]^+$ in full-scan MS spectrum was at *m/z* 268.5, and its intensive fragment ion at *m/z* 152.2 in MRM mode was selected as a quantifier ion. The other two transitions 268.5/117.3 and 268.5/135.3 were recorded for qualification (Fig. 3).

A typical MRM chromatogram of the standard solutions for 8-OHdG (150 mg/L) and dG (50 mg/L) is shown in Fig. 4. The limit of detection (LOD) of 8-OHdG, determined as 3-fold of the signal-to-noise ratio using the standard solution, was 1 ng/mL. The linear range for 8-OHdG was from 2 to 20 ng/mL (r=0.9997). The intra-assay variation of repeated injections of standards (5 ng/mL, n=5) was 3.38% which was determined as the instrumental precision.

INH-induced DNA Oxidative Damage

The ratio of 8-OHdG to dG was $2.2\pm1.8/10^5$ in control liver slices (Fig. 5). The ratio was significantly increased by 3-fold in INH group ($P<0.05$), suggesting that ROS generated from INH caused DNA oxidative damage in rat liver slices (Fig. 6).

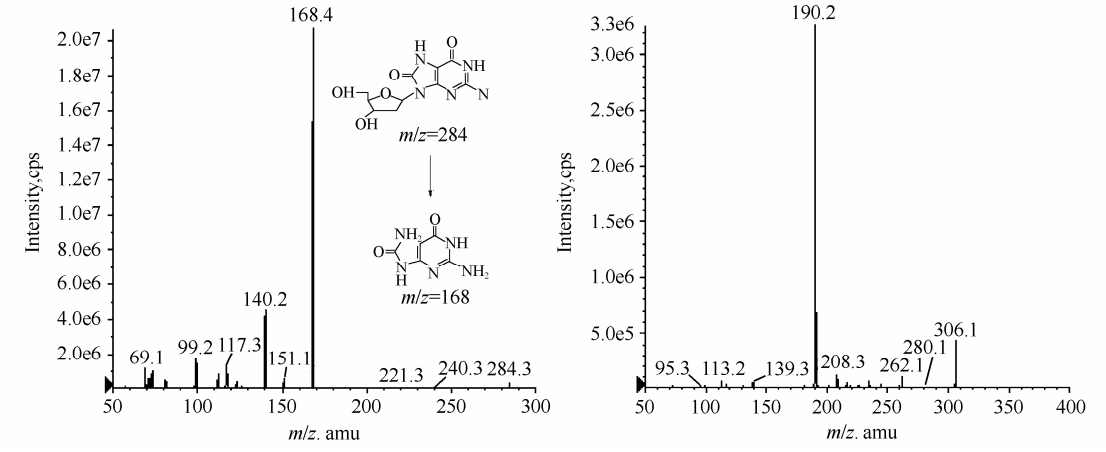

FIG. 2. Positive electrospay ionization mass spectrum (MS/MS) of 8-OHdG. A: product ion scan of $[M+H]^+$ (*m/z* 284.3); B: product ion scan of $[M+Na]^+$ (*m/z* 306.1).

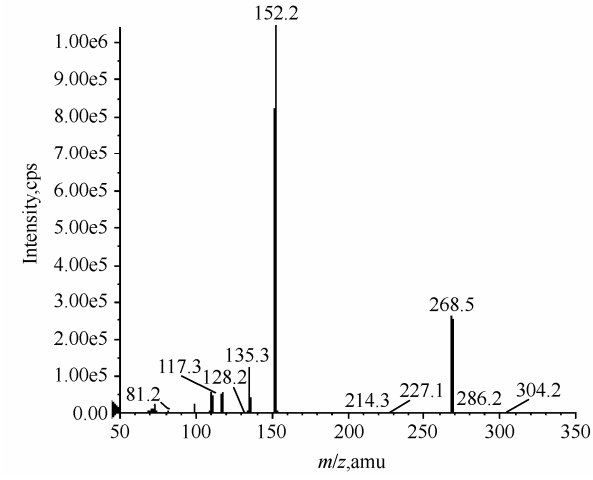

FIG. 3. Positive electrospay ionization mass spectrum (MS/MS) of dG molecule ion $[M+H]^+$ (*m/z* 268.5).

DISCUSSION

This is the first study to evaluate DNA adducts using HPLC-MS/MS coupled with precision-cut liver sections. DNA samples are analyzed by HPLC-MS/MS without derivatization as DNA is hydrolyzed to nucleosides. Under the HPLC conditions used, 8-OHdG and dG are eluted at 10.02 and 7.37 min, respectively, indicating that these biomolecules are well separated, which is fundamental for chromatographic methods. The LOD is 1 ng/mL in MRM mode when 10 μL of the sample is injected onto the HPLC column, which is equal to 35 fmol of 8-OHdG. However, the LOD of 8-OHdG is approximately 5 pmol when SIM mode is used to analyze the standard solution in the LC-MS/MS

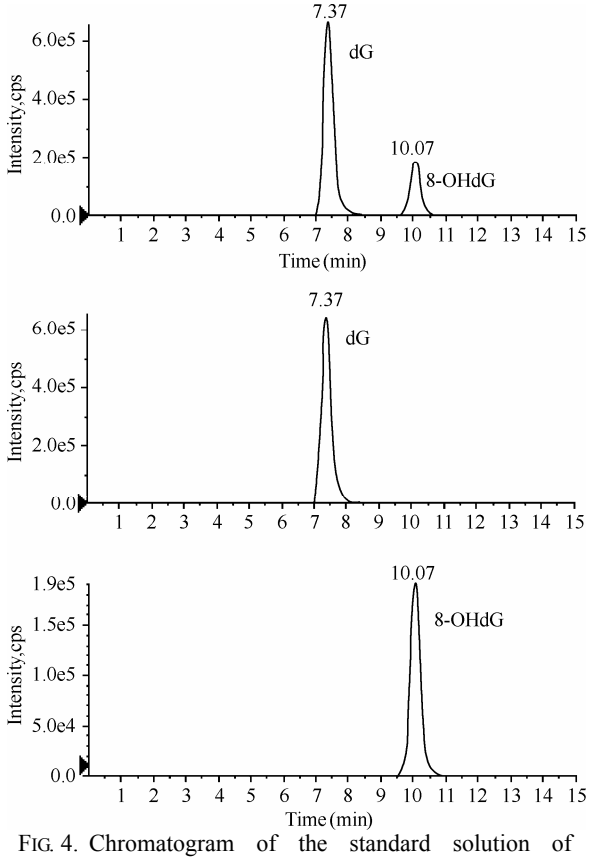

FIG. 4. Chromatogram of the standard solution of 8-OHdG (150 mg/L) and dG (50 mg/L) in the MRM mode using HPLC-MS/MS. Under the HPLC conditions used, 8-OHdG and dG were eluted at 10.02 and 7.37 min, respectively. 8-OHdG and dG were monitored by four pairs of transitions (284.3/168.4, 284.3/140.2, 306.1/190.2, and 306.1/262.1) and three pairs of transitions (268.5/152.2, 268.5/135.3, and 268.5/117.3), respectively.

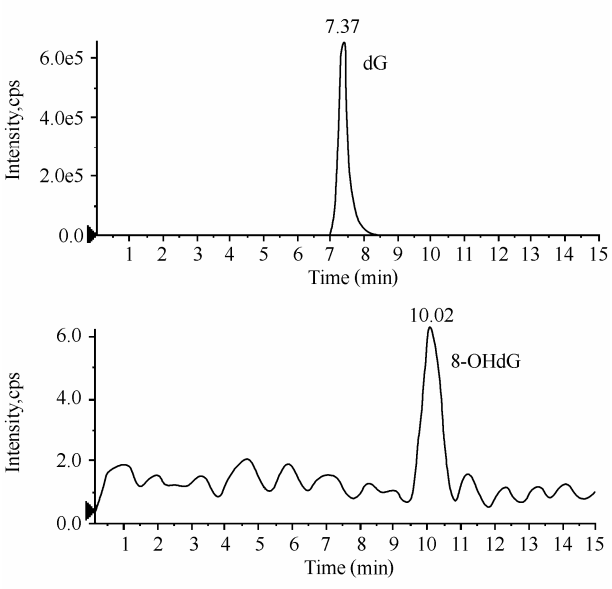

FIG. 5. Representative HPLC-MS/MS chromatogram for a control in MRM mode (The transitions monitored for 8-OHdG and dG see Fig. 4).

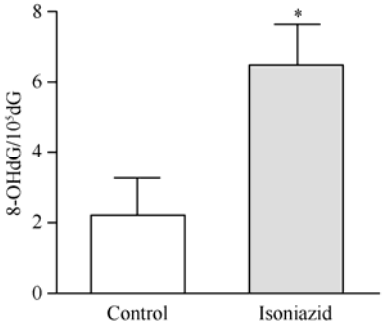

FIG. 6. Induction of 8-OHdG levels by isoniazid teatment in precision-cut rat liver sections. n=3. $\bar{x} \pm$ s. *P<0.05 *vs* control.

instrument[18]. The MRM mode is a more sensitive detection method for 8-OHdG than selected-ion monitoring (SIM) mode since the background can be dramatically reduced in MRM mode. The results indicate that HPLC-MS/MS analysis is a fast and reliable measurement to quantify the low level of oxidative DNA damage in various biological samples due to its sensitivity and specificity.

The ratio of 8-OHdG to dG is used to describe the level of 8-OHdG in precision-cut liver slices, which is 2.2±1.8/10^5 dG in the control slices. It is notable that the level of 8-OHdG range widely from 0.1 to 100/10^5 dG in previous studies using cellular DNA[19]. The discrepancies may be due to different analytical methods, biological samples used and others. In precision-cut liver slices, the level of 8-OHdG is expected to be relative to the viability of the slices. Preincubation can effectively reduce the impact of mechanical injury and maintain the viability of the slices during incubation, as indicated by the stable ALT and Na^+, K^+-ATPase activities. The improvement in the viability would increase the sensitivity and reliability of the slices to xenobiotic induced oxidative stress, and improve the potential of using slices in long-term experiments. Moreover, INH treatment can markedly induce the levels of 8-OHdG in rat liver slices, which supports our previous deduction that ROS generated from hydrazine, a toxic metabolite of INH, plays a principal role in INH-induced hepatotoxicity mediated by CYP2E1[12].

In conclusion, HPLC-MS/MS coupled with precision-cut tissue slices is a sensitive and reliable analytical technique to evaluate oxidative DNA damage of the target tissues caused by xenobiotics. Precision-cut tissue slices technique is a suitable *in vitro* model for pharmacological and toxicological investigations as the drug-metabolizing enzyme systems and the intercellular communication are maintained. Therefore, this can be a convenient model for the investigation of the toxicological mechanisms of procarcinogens and cytotoxins, and for the screening of possible protective compounds by HPLC-MS/MS coupled with precision-cut tissue slices.

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