Journal of Chromatography A, 1322 (2013) 69–73

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Simultaneous quantification of ethylpurine adducts in human urine by stable isotope dilution nanoflow liquid chromatography nanospray ionization tandem mass spectrometry

Hauh-Jyun Candy Chen*, Chao-Ray Lin

Department of Chemistry and Biochemistry, National Chung Cheng University, 168 University Road, Ming-Hsiung, Chia-Yi 62142, Taiwan

ARTICLE INFO

Article history:
Received 3 September 2013
Received in revised form 29 October 2013
Accepted 29 October 2013
Available online 6 November 2013

Keywords:
DNA adduct
3-Ethyladenine
7-Ethylguanine
Nanoflow LC
Mass spectrometry
Urine

ABSTRACT

Ethylating agents contained in cigarette smoke can damage DNA producing ethylated DNA adducts, including N^3-ethyladenine (3-EtAde) and N^7-ethylguanine (7-EtGua). In this study, a highly specific and sensitive assay based on stable isotope dilution nanoflow liquid chromatography nanospray ionization tandem mass spectrometry (nanoLC-NSI/MS/MS) was used to measure 3-EtAde and 7-EtGua in human urine. These urinary adducts were enriched by a polymeric reversed phase solid-phase extraction column before the nanoLC-NSI/MS/MS analysis. The on-column detection limits ($S/N \geq 3$) of 3-EtAde and 7-EtGua were 15 fg (92 amol) and 10 fg (56 amol), respectively, while the lower quantification limits of 3-EtAde and 7-EtGua were 930 and 840 amol, respectively. Urinary concentrations of 3-EtAde and 7-EtGua in 21 smokers were 68.6 ± 29.4 and 18.7 ± 13.8 pg/mL, respectively. In 20 nonsmokers, concentrations of 3-EtAde and 7-EtGua were 3.5 ± 3.8 and 2.4 ± 3.0 pg/mL, respectively. The urinary concentrations of 3-EtAde and 7-EtGua were statistically significantly higher in smokers than in nonsmokers ($p < 0.0001$). Moreover, 3-EtAde and 7-EtGua concentrations are significantly correlated with the number of cigarettes smoked per day and with the smoking index. This highly specific and sensitive assay based on stable isotope dilution nanoLC-NSI/MS/MS assay should be clinically valuable in assessing the possibility of measuring urinary ethylpurines as noninvasive biomarkers for smoking-related cancers in humans.

1. Introduction

Cigarette smoke contains thousands of chemicals and more than seventy of them are carcinogens [1]. The fact that more than 80% of lung cancer patients are tobacco smokers or ex-smokers renders smoking as a primary cause of lung cancer [2]. Because tobacco intake is a major risk factor in lung and oral cancers, DNA ethylation might be associated with carcinogenesis in these cancers. Furthermore, the finding of cigarette smoking increased levels of ethylated DNA adducts in tissues and urine strongly suggests that ethylating agents are contained in the smoke [3–9]. The recent finding of 7-EtGua in calf thymus DNA treated with extracts of areca nut suggests that ethylating agents are also present in areca nut extracts [10].

Treatment of rats with the mutagenic *N*-ethyl-*N*-nitrosourea or *N*-nitrosodiethylamine causes tumor formation in brain and liver, along with formation of ethylated DNA adducts [11,12]. Ethylated DNA bases identified in humans include N^3-ethyladenine (3-EtAde), O^6-ethylguanine, N^7-ethylguanine (7-EtGua) and O^2-, O^4-, and N^3-ethylthymine [3–5,8,9,13–16]. In human tissues, 7-EtGua was quantified in liver and leukocyte DNA [14,17,18], while 3-EtAde was analyzed in leukocyte DNA [17]. Although results from animal studies indicated association between DNA ethylation and cancer [19,20], studies in humans are limited. The only connection was the reports in lung cancer patients that higher levels of O^4-ethylthymidine in DNA from normal lung tissue adjacent to tumors in smokers than in nonsmokers [7,13].

Urinary DNA adducts have been used as biomarkers for carcinogen exposure [21]. The glycosidic linkages of 3-EtAde and 7-EtGua are labile, with the respective half-lives of ca. 20–30 and 100–150 h at 37 °C under neutral pH [22,23]. Thus, 3-EtAde and 7-EtGua can undergo spontaneous depurination. At the same time, they can be removed and repaired by DNA repair enzymes. As a result, these ethylpurine adducts have been detected in smokers' urine [3–5,8,16]. Furthermore, higher levels of urinary 3-EtAde and 7-EtGua in smokers than in nonsmokers have been reported [3–5,8]. Therefore, 3-EtAde and 7-EtGua should be potential biomarkers for smoking-induced DNA damage.

There is a practical need to establish analytical methods for DNA adducts as biomarkers in surrogate tissues or biological fluids to assess DNA damage in the body because DNA adducts are often present in trace amounts in human tissues which are inaccessible.

* Corresponding author. Tel.: +886 5 242 8176; fax: +886 5 272 1040.
E-mail address: chehjc@ccu.edu.tw (H.-J.C. Chen).

0021-9673/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.chroma.2013.10.092

The development of analytical methods allowed characterization and quantification of trace levles of DNA adducts in biological tissues and fluids [24]. Among these methods, liquid chromatography tandem mass spectrometry is the state of the art choice [25,26]. In this study, a highly accurate, specific and sensitive method based on stable isotope dilution nanoflow liquid chromatography nanospray ionization tandem mass spectrometry (nanoLC-NSI/MS/MS) is used for simultaneous quantification of 3-EtAde and 7-EtGua in human urine.

2. Materials and methods

2.1. Chemicals and reagents

[$^{13}C_1$,$^{15}N_2$]Adenine and [$^{15}N_5$]2′-deoxyguanosine were purchased from Cambridge Isotope Laboratories (Andover, MA). Standard 3-EtAde, 7-EtGua, [$^{13}C_1$,$^{15}N_2$]3-EtAde, and [$^{15}N_5$]7-EtGua were synthesized and characterized as previously reported [17]. All reagents are of reagent grade or above.

2.2. Adduct enrichment by SPE

An aliquot of 24-h urine stored at −80 °C was defrosted and centrifuged at 23,000 × *g* for 20 min at 4 °C. To the supernatant (100 μL) was added 100 pg each of [$^{13}C_1$,$^{15}N_2$]3-EtAde and [$^{15}N_5$]7-EtGua and the solution (140 μL) was loaded on a polymeric reversed phase SPE column [Strata-X, 100 mg, 1 mL, Phenomenex (Torrance, CA)] after conditioning with 5 mL of methanol, followed by 5 mL of water. After sample loading, the SPE column was washed with 1 mL of 15% aqueous MeOH, and the adducts were eluted with 1 mL of 40% aqueous MeOH. The fraction containing these adducts was evaporated, redissolved in 10 μL of 0.01% formic acid (pH 3.7), and passed through a 0.22 μm Nylon syringe filter. A volume of 2 μL of the processed sample was subjected to the nanoLC-NSI/MS/MS analysis described below.

2.3. nanoLC-NSI/MS/MS analysis

A 2 μL injection loop was connected to a six-port switching valve injector into an LC system consisting of an UltiMate 3000 Nano LC system (Dionex, Amsterdam, The Netherlands) and a reversed phase column packed in-house (180 μm × 11 cm, Magic C18AQ, 5 μm, 200 Å, Michrom BioResource, Album, CA). The pump output (30 μL/min) was split before the injection port to a flow rate of 700 nL/min. Mobile phase A was 0.01% formic acid (pH 3.7), and mobile phase B was 0.1% formic acid in acetonitrile. The elution started with a linear gradient of 10% mobile phase B to 100% mobile phase B from 0 to 17 min, and maintained at 100% B in the next 13 min before conditioning with 100% mobile phase A.

The effluent was subjected to analysis by a triple quadrupole mass spectrometer, TSQ Quantum Ultra EMR mass spectrometer (Thermo Electron Corp., San Jose, CA), equipped with a nanospray ionization (NSI) interface under the positive-ion mode for the NSI-MS/MS. The column effluent enters the spray chamber through a tapered emitter constructed from a 180-μm i.d. fused-silica capillary and is directly electrosprayed into the mass spectrometer in the positive ion mode. The spray was monitored by a built-in CCD camera. The spray voltage was 1.5 kV, and the source temperature was at 270 °C. In MS/MS experiments, argon was used as the collision gas and the pressure of the collision cell was 1.5 mTorr. The adduct-enriched sample was analyzed by nanoLC-NSI/MS/MS using the transition from the precursor ion $[M+H]^+$ focused in quadrupole 1 (Q1) and dissociated in a collision cell (Q2), yielding the product ion, which was analyzed in quadrupole 3 (Q3). Under the highly selected reaction monitoring (H-SRM) mode, the mass width of Q1 and Q3 was 0.2 and 0.7 *m*/*z*, respectively, and the dwell time was 0.1 s. Two H-SRM transitions were simultaneously employed: method 1 monitored 3-EtAde in Q1 for the precursor ion $[M+H]^+$ at *m*/*z* 164.2 and in Q3 for the product ion $[M+H-C_2H_4]^+$ at *m*/*z* 136.0. For [$^{13}C_1$,$^{15}N_2$]3-EtAde, Q1 and Q3 were at *m*/*z* 167.2 and *m*/*z* 139.0, respectively. For 7-EtGua, Q1 and Q3 were for the precursor ion $[M+H]^+$ at *m*/*z* 180.1 and for the product ion $[M+H-C_2H_4]^+$ at *m*/*z* 152.0, respectively, while those for [$^{15}N_5$]7-EtGua were at *m*/*z* 185.1 (Q1) and *m*/*z* 157.0 (Q3). The collision energy was 20 eV for these four H-SRM transitions. The H-SRM method 2 monitored Q1 for the precursor ion $[M+H]^+$ at *m*/*z* 164.2 and Q3 for the product ion $[M+H-C_2H_4-NH_3]^+$ at *m*/*z* 119.0 for 3-EtAde and from *m*/*z* 167.2 to *m*/*z* 122.0 for [$^{13}C_1$,$^{15}N_2$]3-EtAde, with a collision energy of 30 eV for both transitions. For 7-EtGua, Q1 and Q3 was for the precursor ion $[M+H]^+$ at *m*/*z* 180.1 and for the product ion $[M+H-NH_3]^+$ at *m*/*z* 163.0, respectively, while that for [$^{15}N_5$]7-EtGua was at *m*/*z* 185.1 (Q1) and *m*/*z* 167.0 (Q3), with a collision energy of 20 eV for both transitions.

2.4. Method validation

The detection limits was defined by injecting the smallest amount of standard 3-EtAde and 7-EtGua on-column with $S/N \geq 3$. The calibration curves were obtained by adding various amount of the standards (0, 0.01, 0.15, 2, 6, 15, and 20 pg) in the presence of 100 pg each of [$^{13}C_1$,$^{15}N_2$]3-EtAde and [$^{15}N_5$]7-EtGua to a urine sample with non-detectable amounts of 3-EtAde and 7-EtGua. Each solution went through the SPE column described in Section 2.2. The fraction containing these adducts was evaporated and reconstituted in 10 μL of 0.01% formic acid (pH 3.7), and 2 μL of the aliquot was subjected to the nanoLC-NSI/MS/MS analysis described above. The lowest amount of analyte showing linearity with $S/N \geq 10$ was defined as the lower limit of quantification (LLOQ).

The precision of the assay was assessed by the relative standard deviation (RSD) in analyzing 3-EtAde and 7-EtGua in a urine sample in triplicates, and the experiments were performed on three different days using the optimized assay conditions.

The accuracy of this assay was evaluated by adding known amount of 3-EtAde and 7-EtGua (6, 12, 18 pg each) to 0.1 mL of a urine sample and measured the total amount. The y-intercept obtained by linear regression was compared with the adduct level in this sample without addition of the standards.

2.5. Study-subjects

This study is approved by the Institutional Review Board of the National Chung Cheng University (IRB No. 100112902). The study-subjects were healthy individuals recruited from employees and students of the National Chung Cheng University, including 21 male smokers and 20 nonsmokers (10 male and 10 female). The mean (±standard deviation (SD)) age was 37 ± 16 for smokers and 36 ± 11 for nonsmokers. The mean (±SD) smoking index (number of cigarette per day × years smoked) of the study-subjects was 203 ± 201 in a range of 4–690. The subjects received a written warranty stating that the information was for research purposes only and that their personal information would be kept confidential.

2.6. Statistical analysis

GraphPad InStat version 3.00 for Windows 95, GraphPad Software (San Diego, CA, www.graphpad. com) was used for statistical analysis. The nonparametric Mann–Whitney test was used to analyze levels of 3-EtAde and 7-EtGua in urine between the smokers and nonsmokers. The correlation between each adduct and the number of cigarettes smoked per day or the smoking index as well