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Development of a method for the determination of 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol in urine of nonsmokers and smokers using liquid chromatography/tandem mass spectrometry

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ABSTRACT

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) is an efficient biomarker of tobacco-specific carcinogen 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). The ability to monitor biomarker concentrations is very important in understanding potential cancer risk. An analytical method using molecularly imprinted polymer (MIP) column coupled with liquid chromatography/tandem mass spectrometry (LC–MS/MS) for the determination of total NNAL in human urine was developed and validated. The combination of MIP column extraction and LC–MS/MS can provide a high sensitive and relatively simple analytical method. The limit of detection (LOD) was 0.30 pg/ml and analysis time was 6 min. The method has been applied to urine samples of 36 nonsmokers and 207 smokers. NNAL was found to be significantly higher in the urine of smokers compared with nonsmokers. Compared with smokers with blended cigarettes, Chinese virginia cigarettes smokers had low urinary NNAL levels. There was a direct association between the 24-h mouth-level exposure of carcinogen NNK from cigarette smoking and the concentration of NNAL in the urine of smokers. However, there was not a positive correlation between urinary total NNAL levels in 24 h and tar. Total urinary NNAL is a valuable biomarker for monitoring exposure to carcinogenic NNK in smokers and in nonsmokers. A prediction model of cigarette smoke NNK and urinary average NNAL levels in 24 h was established ($y = 2.8987x - 245.38$, $r^2 = 0.9952$, $n = 204$).
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1. Introduction

Tobacco smoking is currently responsible for approximately 30% of cancer deaths in developed countries, and for an increasing proportion of the cancer deaths in developing countries. Furthermore, smoking causes more deaths from vascular, respiratory, and other diseases than it does from cancer [1]. 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a tobacco-specific nitrosamine found only in tobacco products and the ir smoke, causes lung cancer [2], adenocarcinoma [3], and liver cancer at the highest dose level ($P < 0.05$) in all laboratory animal species tested [4]. In humans, NNK is almost entirely reduced to 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), which is also carcinogenic and excreted in urine [3] (Fig. 1). Higher urine NNAL was also related to greater dyspnea, poorer physical health status, and more restricted activity [5]. The urine NNAL-to-creatinine ratio (per interquartile increment) was associated with greater chronic obstructive pulmonary disease severity. Study indicated there was a direct association between the NNK of 24-h mouth-level exposure from cigarette smoking and the NNAL concentration of its primary metabolite in the urine of smokers [6].

An important method to determine individual and collective risk from exposure to tobacco products is evaluation of tobacco carcinogen biomarkers [7,8]. Urinary metabolites have arose as highly practical biomarkers for determining uptake of specific carcinogens and toxicants in tobacco smoke and are likely to have more utility in predicting tobacco associated harm than machine measurements of smoke constituents. But the urine matrix was very complex, where the presence of many potentially interfering substances in concentrations far greater than those of the NNAL set demands for high selectivity and low limit of quantification (LOQ) for unequivocal identification and quantification. The analytical procedures for urinary NNAL are challenging indeed. Recently, liquid chromatography/tandem mass spectrometry (LC–MS/MS) have been developed for the analysis of urinary NNAL [9,10,11,12,13,14,15]. In order to get good analyte recoveries and improve method's accuracy and precision, complex sample cleanup is often required including liquid–liquid or solid-phase extraction (SPE). However, traditional liquid–liquid extraction need relatively large solvent

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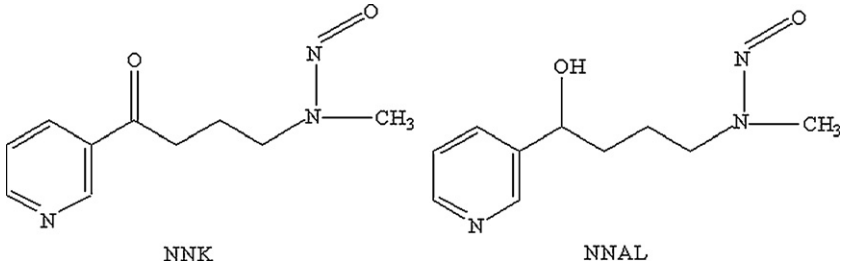

Fig. 1. Chemical structure of NNK and NNAL.

consumption, and the process is complex. SPE has many advantages compared to traditional liquid–liquid extraction methods. Bhat [13] described the use of LC–MS combined with a novel sample cleanup method using SPE on a WCX column developed specifically for extracting NNAL from urine samples. The method made it possible to analyze free NNAL in only 0.25 ml urine. But the pre-preparation was very complex, it involved derivatization, liquid–liquid extraction and SPE. Recently, molecularly imprinted polymer (MIP) column was applied to prepare for SPE of urinary NNAL. Xia [11] described the use of LC/APCI-MS/MS combined with a novel sample cleanup method using SPE for extracting NNAL from urine samples. However, the matrix suppression effect was strong. In 2009, Shah [9] improved the assay, by changing the liquid chromatography conditions, the response for this method was enhanced approximately 25-fold through avoidance of ionization suppression, and the lower LOQ for the assay was 20 pg/ml. The combination of MIP column extraction and LC/ESI-MS/MS can provide a sensitive and relatively simple analytical method.

In this present study, a method for the determination of total NNAL in human urine by extraction on a MIP column and LC/ESI-MS/MS was developed and validated. Higher sensitivity and good recovery were achieved. The limit of detection (LOD) was 0.30 pg/ml and analysis time was 6 min. The validated method was applied to quantify urinary NNAL levels of 207 smokers and 36 nonsmokers, then the relationship between the urinary concentrations of total NNAL and Chinese virginia cigarette smoke analytes of NNK and tar were assessed.

2. Experimental

2.1. Chemical

Ammonium acetate, acetic acid, methanol and acetonitrile were obtained from TEDIA Company Inc. (Fairfield, OH, USA). Dichloromethane and hexane were purchased from J.T. Baker Chemical Products Co. Ltd. (Philipsburg, MO, USA). All solvents were high performance liquid chromatography (HPLC)-grade. β-Glucuronidase (type IX-A, *Escherichia coli*) was obtained from Sigma–Aldrich (Taufkirchen, Germany). Potassium dihydrogen phosphate and heptane were purchased from CNW Technologies GmbH (Dusseldorf, Germany). NNK, NNAL, NNAL-d_3 and NNK-d_4 were purchased from Toronto Research Chemical (North York, Ontario, Canada).

2.2. Urine sample preparation

The sample preparation was processed and optimized with a TSNA MIP cartridge (50 mg, 10 ml, Supelco, Taufkirchen, Germany) according to a previous published method [9] after slight modification. 5 ml of 50 mM phosphate buffer (pH 6.3; 50 mM) was added to 5 ml of urine. Adjusting pH to 6.3, then 20 μl methanol containing 100 ng/ml of NNAL-d_3 and 100 μl β-glucuronidase in phosphate buffer (pH 7.2, 100 units/μl) were added to mixture. The mixture was incubated overnight (24 h) at 37 °C in the dark. The cartridge was preconditioned with 1 ml dichloromethane, 1 ml methanol and 1 ml water, and then washed with 2 ml water, 1 ml heptane, 1 ml hexane and eluted with 3 ml dichloromethane/methanol (9:1). The extract was reduced to dryness in a SpeedVac evaporator (ThermoFisher, Dreieich, Germany) and redissolved in 100 μl 0.1% acetic acid in water.

2.3. Instrument and analytical conditions

All samples were analyzed using Agilent 1200 rapid resolution liquid chromatograph from Agilent Technologies (Wilmington, NC, USA) coupled with an API 5500 triple quadruple mass spectrometer equipped with a TurboIonSprayTM source from Applied Biosystems (Foster City, CA, USA). An Agilent Zorbax Eclipse XDB-C18 column 2.1 mm × 150 mm, 3.5 μm from Agilent Technologies (Wilmington, NC, USA) was used for LC separation. LC conditions were as follows: column temperature, 50 °C; mobile phase, solvent A (0.1% acetic acid in water) and 50% solvent B (0.1% acetic acid in methanol); flow-rate, 0.2 ml/min; the injected volume, 5 μl. A linear gradient condition was used as follows (time, solvent A:solvent B): 0–3 min, 50:50–10:90; 4–5 min, 0:100; 5.5–6 min, 50:50. ESI-MS conditions were as follows: nebulizer gas, N_2 (50 psi); ionspray voltage, 5000 V; the turbo ion spray temperature, 450 °C; declustering potential, 35 V; entrance potential, 8 V; collision energy and collision cell exit potential, 10 V; the dwell time, 100 ms; ionization mode, positive ion. NNAL and NNAL-d_3 were assayed by quantifying the multiple reaction monitoring (MRM) transition of $[M+H]^+$ ion of NNAL at m/z 210.2 → 180.2 and NNAL-d_3 at 213.0 → 183.0.

2.4. Calibration

The method was calibrated by spiking a nonsmoker pooled urine with 3, 20, 100, 200, 400, 1000 pg/ml NNAL. Method validation was performed with an injection volume of 5 μl. The background peak area ratio in unspiked urine was zero for NNAL. Each calibrator was analyzed twice. The means of the analyte/internal standard ratio was used for calculation of the regression function. The regression curves were forced through the origin.

2.5. Method validation

The method was validated according to the U.S. Food and Drug Administration (FDA) guidelines for bioanalytical methods [16]. Recovery rates were determined by comparing the analyte concentrations at the low, middle and high level, measured when nonsmoker urine extract was spiked after sample work-up and before the LC–MS/MS measurements (reference, 100%), and when the nonsmoker urine was spiked at the beginning of the sample work-up procedure. As a criterion, the accuracy at a average concentration level tested in these six matrices should be in the range of 85–115%. Intra-day precision was determined by evaluating three analyte concentration levels in authentic human urine samples,

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