Clinica Chimica Acta 436 (2014) 290–297

Contents lists available at ScienceDirect

Clinica Chimica Acta

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

A high-throughput robotic sample preparation system and HPLC-MS/MS for measuring urinary anatabine, anabasine, nicotine and major nicotine metabolites ☆

Binnian Wei *, June Feng *, Imran J. Rehmani, Sharyn Miller, James E. McGuffey, Benjamin C. Blount, Lanqing Wang

Tobacco and Volatiles Branch, Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, GA, United States

ARTICLE INFO

Article history:
Received 11 April 2014
Received in revised form 3 June 2014
Accepted 8 June 2014
Available online 23 June 2014

Keywords:
Robotic
High-throughput
Biomonitoring
Biomarker
Tobacco exposure
Urinary metabolites

ABSTRACT

Background: Most sample preparation methods characteristically involve intensive and repetitive labor, which is inefficient when preparing large numbers of samples from population-scale studies.
Methods: This study presents a robotic system designed to meet the sampling requirements for large population-scale studies. Using this robotic system, we developed and validated a method to simultaneously measure urinary anatabine, anabasine, nicotine and seven major nicotine metabolites: 4-Hydroxy-4-(3-pyridyl)butanoic acid, cotinine-N-oxide, nicotine-N-oxide, trans-3′-hydroxycotinine, norcotinine, cotinine and nornicotine. We analyzed robotically prepared samples using high-performance liquid chromatography (HPLC) coupled with triple quadrupole mass spectrometry in positive electrospray ionization mode using scheduled multiple reaction monitoring (sMRM) with a total runtime of 8.5 min.
Results: The optimized procedure was able to deliver linear analyte responses over a broad range of concentrations. Responses of urine-based calibrators delivered coefficients of determination (R^2) of >0.995. Sample preparation recovery was generally higher than 80%. The robotic system was able to prepare four 96-well plate (384 urine samples) per day, and the overall method afforded an accuracy range of 92–115%, and an imprecision of <15.0% on average.
Conclusions: The validation results demonstrate that the method is accurate, precise, sensitive, robust, and most significantly labor-saving for sample preparation, making it efficient and practical for routine measurements in large population-scale studies such as the National Health and Nutrition Examination Survey (NHANES) and the Population Assessment of Tobacco and Health (PATH) study.

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1. Introduction

Humans are exposed to complex chemicals from both natural and anthropogenic sources that exist in both ambient surroundings and indoor microenvironments through daily inhalation, ingestion and dermal absorption. Tobacco smoke, for example, contains more than 8000 chemicals [1], many of which are associated with adverse health outcomes, such as cancer, respiratory and cardiovascular diseases, in both smokers and nonsmokers [2–5].

Assessing the exposure to and risk from chemicals caused by tobacco use by external measurements, e.g. their indoor air concentrations and surface loadings, is always challenging. Biomonitoring, measuring trace levels of suspected chemicals in biological matrices (i.e., blood, urine, and tissues), is able to provide the actual absorbed levels of these chemicals in human fluids and tissues, providing the "gold standard" for assessing exposure to chemicals [6].

However, biological samples are usually complicated matrices, containing thousands of chemicals from external exposure, their metabolites, and a number of other endogenous constituents, such as proteins, phospholipids and salts, which could potentially interfere with the analysis. As a result, biological samples require specific pre-treatment procedures to minimize or eliminate potential interferences and matrix effects and thus to improve the sensitivity and specificity for measuring the target analytes [7,8]. Typical pre-treatment techniques include liquid–liquid extraction (LLE) [9,10], solid phase extraction (SPE) [11–13], soxhlet extraction [14], supercritical fluid extraction (SFE) [15], microwave-assisted extraction (MAE) and pressurized solvent extraction (PSE) [16]. The robotic sample preparation procedure

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* Corresponding authors at: Centers for Disease Control and Prevention, 4770 Buford Hwy, NE Rd, Mail stop: F44, Atlanta, GA 30041, United States. Tel.: +1 770 488 7980; fax: +1 770 488 7518.
E-mail addresses: bwei@cdc.gov (B. Wei), czf2@cdc.gov (J. Feng).

http://dx.doi.org/10.1016/j.cca.2014.06.012
0009-8981/Published by Elsevier B.V.

described here uses a validated acetone precipitation step to deplete endogenous proteins, salts and phospholipids and to remove the exogenous enzyme that is added to the urine samples to hydrolyze conjugates [17].

Target analytes in pretreated samples are often chromatographically resolved using gas chromatography (GC) or high-performance liquid chromatography (HPLC), which is often coupled with detectors varying from the ultraviolet (UV)/visible spectroscopy and single quadrupole mass spectrometry (MS) [10,18] to tandem MS/MS [11,19–21]. MS/MS provides a more sensitive and selective means for simultaneously measuring multiple analytes.

Most sample preparation methods characteristically involve intensive and repetitive labor, which is inefficient when preparing large numbers of samples from population-scale studies. These types of studies include the National Health and Nutrition Examination Survey (NHANES) designed to assess the health and nutritional status of adults and children in the United States [22], and the Population Assessment of Tobacco and Health (PATH) study aimed to assess the behavioral, social, and health impact of tobacco use [23]. The need for high through-put sample analysis motivated the development of a robotic procedure for pretreatment of biological urine samples, specifically for measurement of anatabine (ANAT), anabasine (ANAB), nicotine (NIC) and seven major nicotine metabolites in the present study.

Nicotine, a primary tobacco-specific alkaloid in tobacco and tobacco smoke, does not directly cause most diseases associated with tobacco use. However, due to its addictiveness, users often choose to continuously/repeatedly use tobacco products, exposing themselves to the carcinogens and bioactive compounds in tobacco [24]. Thus, measuring nicotine and its major metabolites can determine tobacco exposure, and will play an important role in assessing tobacco exposure and making control regulations. Nicotine is absorbed by smokers mainly through direct inhalation of the mainstream smoke [24]. Non-smokers also can be involuntarily exposed to second-hand smoke (SHS), or even third-hand smoke through inhalation, dermal absorption, and dust ingestion [25,26]. Nicotine in mammals is metabolized predominantly into cotinine (COT) (Fig. S1), accounting for approximately 70–80% of NIC dose. COT is further converted through various metabolism pathways, (e.g., cytochrome P450), to form other metabolites, including trans-3′-hydroxycotinine (3HC, accounting for 40–49% of NIC dose), cotinine-N-oxide (CNO, 2–5%) and norcotinine (NorCOT, 1–2%), leaving approximately 22–32% total COT in urine. Another three major NIC metabolites include 1-(3-Pyridyl)-1-butanol-4-carboxylic acid (HPBA, 7–9%), nicotine-N-oxide (NNO, 4–7%), and nornicotine (NorNIC, 0.4–0.8%) [24].

Tobacco plants synthesize nicotine as well as minor tobacco alkaloids such as ANAT and ANAB. This pattern of alkaloids is characteristic of tobacco leaf and tobacco products. Tobacco use leads to concurrent exposure to NIC, ANAT, and ANAB, as well as the presence of measurable levels of biomarkers of exposure to these alkaloids [27]. Not surprisingly, ANAT and ANAB are variably glucuronidated [17] and excreted in the urine with elimination half-lives similar to those for nicotine and its metabolites. Thus the presence of biomarkers of exposure to NIC, ANAT, and ANAB has been validated as proving tobacco product use [27]. Conversely, nicotine replacement therapy uses pharmaceutical-grade nicotine that is essentially free of minor tobacco alkaloids; therefore subjects abstaining from tobacco use and undergoing nicotine replacement therapy have urinary ANAT and ANAB levels <2 ng/ml.[27] Based on these findings ANAT and ANAB are validated biomarkers for identifying non-compliance for participants using NIC replacement therapy (NRT) [17,27]. Our specific aim in this study was to develop and validate a robotic sample preparation method and a HPLC-MS/MS analytical method to simultaneously determine the urinary concentrations of NIC and seven major NIC metabolites in samples from NHANES and PATH studies including both “free" (non-conjugated) and “conjugated" (mostly glucuronide) forms. Our automated method was able to prepare four 96-well plates (384 samples) per day simultaneously with a sample preparation recovery higher than 80%. The method was optimized to provide a wide linear dynamic range for analyte concentrations. Urine calibrators produced coefficients of determination (R^2) of greater than 0.995. The accuracy, precision, and robustness make this method efficient and practical for large population-scale studies.

2. Materials and methods

2.1. Reagents and standards

Acetone, ammonium hydroxide, ammonium acetate and ß-glucuronidase (type H-1, *Helix pomatia* and *Escherichia coli*, type IX-A) were from Sigma-Aldrich. Acetonitrile and methanol were from Honeywell; hydrochloric acid was purchased from Fisher Scientific and HPLC water was from J.T. Baker. Ten native analytes, including NIC, HPBA, CNO, NNO, 3HC, NorCOT, COT, NorNIC, ANAT and ANAB and their corresponding isotopically labeled standards were from Toronto Research Chemicals. Details for product sources are listed in Tables S1 and S2.

2.2. Biological samples

Urine samples from smokers (n = 40) used to develop study methods were collected with no identifiable information by Tennessee Blood Services. Two hundred urine samples from non-smokers were collected from anonymous donors with Institutional Review Board (IRB) approval. Since the smoker urine samples were purchased from commercial sources, the analysis of these samples did not meet the definition of human subjects as specified in 45 CFR 46.102 (f) [28].

2.3. Blank urine pool preparation and Quality control (QC) materials

Blank urine used as matrix material for calibration standards and quality control (QC) was prepared using the following procedures: First, urine samples collected from non-users (n = 200) were screened to eliminate those samples with detectable levels of the analytes. Then the samples containing non-detectable levels of the target analytes were pooled to form a blank urine pool and held at 4 °C overnight to ensure thorough mixing.

High and low QC pools made from the collected smoker urine samples (n = 40) were prepared according to the following procedures: First, we screened smoker urine samples to determine the analyte concentrations in each sample; then, we combined different samples and diluted them using pooled blank urine to obtain a desired concentration for each analyte. It was necessary to spike some pools with native stock solutions to obtain the desired concentrations of some analytes in the pools when their concentrations did not yield high enough concentration levels. Aliquoted QC pools were analyzed daily for two months to obtain analyte means and standard deviations.

2.4. Standard preparation

Individual stock solutions were gravimetrically prepared for both native and labeled standards using certified materials in acidified HPLC water (0.1% hydrochloric acid in HPLC water). We prepared 12 calibration standard solutions by diluting the native stock solutions with pooled blank urine. We prepared internal standard spiking solution by mixing isotope-labeled stock solutions and diluting them with HPLC water. Details about the calibration ranges and isotope-labeled spiking internal standards are provided in Table 1.

2.5. HPLC mobile phase

Fresh mobile buffer “A” was prepared based on the volume needed for the total batch samples. For a total volume of 1.0 l buffer, we added 10 ml of 650 mmol/l of stock ammonium acetate solution to 990 ml of HPLC-grade water, yielding a running buffer of 6.5 mmol/l