Talanta 83 (2011) 1562–1567

Contents lists available at ScienceDirect

Talanta

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

Screening of non-polar heterocyclic amines in urine by microextraction in packed sorbent-fluorimetric detection and confirmation by capillary liquid chromatography

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ARTICLE INFO

Article history:
Received 2 August 2010
Received in revised form 28 October 2010
Accepted 22 November 2010
Available online 30 November 2010

Keywords:
Screening
Microextraction in packed sorbent
Capillary liquid chromatography
Fluorescence detection
Heterocyclic aromatic amines
Urine

ABSTRACT

A rapid and simple procedure for the direct screening of urine samples is described. The method involves microextraction in a packed sorbent (MEPS) that is on-line coupled to a capillary liquid chromatograph with fluorimetric detection. The overall arrangement works as a screening/confirmatory system for monitoring non-polar heterocyclic aromatic amines (HAAs) in urine samples. This configuration allows the selective retention of HAAs from urine on a C_{18} MEPS cartridge integrated in the needle of a micro-well plate autosampler. Retained HAAs were eluted with methanol/water (90:10, v/v) and directly injected into the fluorimetric detector. This screening method provides a yes/no binary response that may require confirmation. The samples for which the concentration of HAAs was close to or above the established threshold limit (30 ng mL^{-1}) were subjected to capillary liquid chromatography (CLC) for confirmation purposes. A mobile phase of acetonitrile and triethylamine (25 mM) at pH 2.5, through a gradient of composition at a flow rate of 20 μL min^{-1}, resulted in good separations between the analytes in less than 11 min. This confirmation method allowed the determination of the analytes in the 10–100 ng mL^{-1} range for harmane and norharmane and from 20 to 200 ng mL^{-1} for 3-amino-1,4-dimethyl-5H-pyrido-[4,3-b] indole (Trp-P-1), 3-amino-1-methyl-5H-pyrido-[4,3-b] indole (Trp-P-2), 2-amino-9H-pyrido-[2,3-b] indole (AαC) and 2-amino-3-methyl-9H-pyrido-[2,3-b] indole (MeAαC), with relative standard deviation (RSD) values between 2.12% and 3.73%, and limits of detection between 1.6 and 5.6 ng mL^{-1} for all the HAAs.

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1. Introduction

Heterocyclic aromatic amines (HAAs) are chemicals that have high carcinogenic potential and are formed when amino acids and creatine react at high temperatures from the cooking of meats such as beef, pork, fowl and fish [1,2]. Free amino acid and sugar levels also influence the content of HAAs. The synthesis of these compounds depends on different cooking conditions such as temperature and time, the presence or absence of enhancers, pH, transport of water and water-soluble substances [3]. At temperatures above 150 °C, concentrations of HAAs become highly significant and the higher the temperature, the higher the level of apolar HAAs generated [1,4].

These compounds have proven to be carcinogenic in rodents and non-human primates [5–10]. Several studies (e.g., European Prospective Investigation into Cancer and Nutrition) have associated an increased risk of developing colorectal, pancreatic and breast cancer to high intakes of well-done, fried, or barbequed meat and fish, although from these studies there is insufficient scientific evidence to support the hypothesis that human cancer risk is specifically due to the greater uptake of heterocyclic aromatic amines [11,12]. However, the importance of other carcinogens, co-carcinogens and anticarcinogens in the diet – and genetic susceptibility to HAAs – should be taken into account. Consequently, HAAs may be considered as human carcinogens and The International Agency for Research

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doi:10.1016/j.talanta.2010.11.060

on Cancer (IARC) recommended a decrease in their consumption [13–15].

The quantification of HAAs in cooked foods and in human urine indicates the continual exposure of humans to low levels of these substances in the diet [16]. HAAs have been isolated and identified, not only in foods [17–21], alcoholic beverages [22] and cooking residues, such as cooking smoke and pan residue extracts [23,24], but also in biological matrices such as plasma, human tissues, hair, urine, bile, human milk [9,25–33] and even in environmental samples such as cigarette smoke [34], river and rain water [35,36] and air [37]. The trace quantities in which HAAs are present and the high level of interference in matrices require selective and sensitive methods to detect and identify these analytes. This requirement makes the extraction and clean-up steps for analyses extremely important. Liquid–liquid extraction, solid-phase extraction (SPE), solid-phase microextraction (SPME), focused microwave-assisted extraction and hollow fiber-supported liquid membrane extraction have been utilised to eliminate interference at the retention times of HAAs in real samples and, in general, large volumes of sample, solvent and time are employed [38–40].

Microextraction in a packed sorbent (MEPS) represents a new technique for miniaturized SPE. In MEPS, about 1 mg of solid packing material is inserted into a syringe (100–250 μL) or integrated into autosampler robotics, i.e. not into a separate column, and this allows an online connection to a GC or LC. MEPS is based on multiple extractions in which the sample flows through a bed of solid extractant, the size (and particle size) of which has to be as small as possible to speed up the transfer of analytes from the sample to the solid phase. The transfer is also facilitated by the close contact between these phases. The packed sorbent can also be reused, more than 100 times for plasma or urine samples and more than 400 times for water samples, whereas a conventional SPE column can only be used once. MEPS can handle volumes from 10 to 1000 μL and is more robust when compared with SPME. This new technique can be used for complex matrices (such as plasma, urine, blood and organic solvents), which is not always the case with SPME, and it is fairly sensitive to the nature of the sample matrix. Moreover, high extraction recoveries can be achieved (>60%) [41,42]. The MEPS technique has been used to extract a wide range of analytes from different matrices (urine, plasma, blood). Hence, several drugs such as local anaesthetics and their metabolites [43–45], neurotransmitters such as dopamine, serotonin [46], methadone [41] and cotinine [47] have been extracted from biological samples such as blood, plasma or urine using the MEPS technique. References regarding the use of MEPS for the extraction of HAAs have not been published to date.

HPLC, GC and capillary electrophoresis (CE) coupled to mass spectrometric (MS), photometric, fluorimetric (FLD), evaporative light scattering (ELSD) and electrochemical detectors (ECD) have been used for the identification and quantification of HAAs in real food and biological samples [38,48,49]. Daily intake and exposure to HAAs can be estimated by their determination in urine samples, which is useful to correlate the uptake of these compounds with cancer risk. In this sense, qualitative analysis provides binary yes/no responses with the least possible delay in order that timely decisions can be made. However, this technique does not provide quantitative data. To date it has been common to identify an analyte or a group of analytes or, in the case of qualitative analysis devoted to sample classification, to make a rapid and reliable classification of samples on the basis of previously established criteria, for example a cut-off concentration fixed by the legislation or client. Consequently, screening methods can reduce purchase and maintenance costs of equipment, diminishing the use of capillary liquid chromatography (CLC) to process only the positive samples tested with the screening system.

The objective of the work described here was to develop a method for the extraction and determination of HAAs in urine samples through the use of a screening system to obtain rapid responses for the presence or absence of these analytes. The use of CLC to identify and quantify the HAAs present in urine samples was markedly reduced because only those samples that gave positive responses in the screening system were analysed. The interferences present in the samples were also reduced by the use of MEPS integrated in the micro-well plate autosampler, thus avoiding time-consuming procedures for sample preparation. Good repeatability of the responses and high sensitivity were achieved. This method was applied to determine six HAAs in urine samples with less reagent consumption and low cost instrumentation.

2. Experimental

2.1. Chemicals, materials and samples

The heterocyclic aromatic amines, 9H-pyrido-[3,4-b] indole (norharman, NH) and 1-methyl-9H-pyrido-[3,4-b] indole (harman, H) standards were purchased from Sigma–Aldrich (St. Louis, MO, USA). 2-Amino-9H-pyrido-[2,3-b] indole (AαC), 3-amino-1-methyl-5H-pyrido-[4,3-b] indole (Trp-P-2), 2-amino-3-methyl-9H-pyrido-[2,3-b] indole (MeAαC), and 3-amino-1,4-dimethyl-5H-pyrido-[4,3-b] indole (Trp-P-1) standards were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada) (Fig. 1). Acetonitrile (HPLC grade) and phosphoric acid were supplied by Panreac (Barcelona, Spain). Methanol (HPLC grade) and triethylamine (TEA) used to prepare the mobile phase were purchased from Sigma–Aldrich. Water was purified with a Milli-Q filtration system from Millipore (Bedford, MA, USA). All solutions prepared for capillary liquid chromatography were passed through a 0.45 μm nylon filter before use. Microextraction packed sorbents (MEPS) and syringes were purchased from SGE Analytical Science (Ringwood, Australia), C_{18} solid phase extraction cartridges were obtained from Varian (Palo Alto, CA, USA) and Análisis Vínicos (Tomelloso, Spain). Strata-X cartridges were purchased from Phenomenex (Bellefonte, PA, USA) and Sep-Pak solid phase extraction cartridges were obtained from Waters (Milford, MA, USA). Urine samples were donated by volunteers.

2.2. Equipment

Chromatographic analysis of the HAAs was carried out on an Agilent 1200 capillary liquid chromatograph equipped with a binary pump, a degasser, a micro-well plate autosampler and a column heater-cooler. The chromatograph was coupled with an Argos 250B fluorescence detection system from Flux Instruments AG (Basel, Switzerland), equipped with a fluorescence flow-cell directly connected to the excitation and emission light guides. The emitted light was guided along the capillary by total reflection and focused on the emission light guide using a patented light cone. This configuration allowed direct detection at the flow cell. The detection light path was defined by the inner diameter of the capillary. The detector was also equipped with a xenon–mercury (Xe–Hg) lamp as the irradiation source (power 75 W) with a radiation range lying between 185 and 2000 nm, and included a photomultiplier tube (PMT) detection unit working in the 185–700 nm range. The chromatograms were recorded using a program developed in-house and controlled by Labview software (Labview, National Instruments, USA) running on a computer fitted with a multipurpose interface card (NI USB-9161). The analytical column was a capillary polar encapped C_{18} reverse phase Luna® column (250 mm × 500 μm i.d., 5 μm) from Phenomenex.

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