Journal of Chromatography B, 1012 (2016) 89–96

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Quantification of naphthoquinone mercapturic acids in urine as biomarkers of naphthalene exposure

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ARTICLE INFO

Article history:
Received 30 September 2015
Received in revised form
21 December 2015
Accepted 23 December 2015
Available online 29 December 2015

Keywords:
Biomarker
Biomonitoring
Naphthalene
Naphthoquinone
Urine
Mercapturic acid

ABSTRACT

Naphthalene shows carcinogenic properties in animal experiments. As the substance is ubiquitary present in the environment and has a possibly high exposure at industrial workplaces, the determination of naphthalene metabolites in humans is of environmental–medical as well as occupational–medical importance. Here, biomarkers of 1,2- and 1,4-naphthoquinone, as possibly carcinogenic metabolites in the naphthalene metabolism, are of outstanding significance.

We developed and validated a liquid chromatography–tandem mass-spectrometric (LC–MS/MS) method for the simultaneous determination of the naphthoquinone mercapturic acids of 1,2- and 1,4-naphthoquinone in human urine samples as a sum of naphthoquinone- and dihydroxynaphthalene-mercapturic acid. Except for enzymatic hydrolysis and acidification, no further sample preparation is necessary. For sample clean-up, a column switching procedure is applied. The mercapturic acids are extracted from the urinary matrix on a restricted access material (RAM RP 18) and separated on a reversed phase column (Synergi Polar RP C18). The metabolites were quantified by tandem mass spectrometry using labelled D5-1,4-NQMA as internal standard. The limits of detection are 3 μg/l for 1,2-NQMA and 1 μg/l for 1,4-NQMA. Intraday- and interday precision for pooled urine (spiked with 10 μg/l and 30 μg/l of the analytes) ranges from 5.9 to 15.1% for 1,2-NQMA and from 2.0 to 10.8% for 1,4-NQMA. The developed method is suited for the sensitive and specific determination of the mercapturic acids of naphthoquinones in human urine. A good precision and low limits of detection were achieved. Application of those new biomarkers in biomonitoring studies may give deeper insights into the mechanisms of the human naphthalene metabolism.

1. Introduction

Naphthalene is generated, together with other polycyclic aromatic hydrocarbons (PAH), during all incomplete combustion processes. Thereby, it is quantitatively the main component in the PAH-mixture and shows a high volatility. In the environment, all individuals get into contact with naphthalene through different sources of exposure like polluted air, grilled or smoked food, tobacco smoke and even consumer products such as moth balls. This leads to a ubiquitary exposure for humans [1]. An occupational contact with naphthalene at different industrial workplaces, e.g. the chemical industry, the coking plants, the production of fireproof material etc., may lead to a significant increase of the naphthalene body burden beyond the background exposure [2,3].

Naphthalene causes haemolytic anaemia in humans [4]. In animal experiments, naphthalene shows clear carcinogenic effects in the respiratory tract [5]. International organizations like the International Agency for Research on Cancer (IARC) and the Commission for the Investigations of Health Hazards of Chemical Compounds in the Work Area of the German Research Foundation (DFG) evaluate naphthalene as "possibly carcinogenic for humans" in Category 2B (IARC) and Category 2 (DFG) for carcinogenic substances [6,7]. The Scientific Committee on Occupational Exposure Limits (SCOEL) classifies the substance as "non-genotoxic carcinogen" [8].

Abbreviations: 1,2-NQMA, 1,2-naphthoquinone-4-mercapturic acid; 1,4-NQMA, 1,4-naphthoquinone-2-mercapturic acid; D5-1,4-NQMA, D5-1,4-naphthoquinone-2-mercapturic acid; 1,2-DHMA, 1,2-dihydroxynaphthalene-4-mercapturic acid; 1,4-DHMA, 1,4-dihydroxynaphthalene-2-mercapturic acid; IS, internal standard.

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http://dx.doi.org/10.1016/j.jchromb.2015.12.052
1570-0232/

Naphthalene is of great importance in occupational and environmental medicine, due to its potentially carcinogenic properties and its occurrence at workplaces and in the environment. For an estimation of the health risk, it is essential to determine the internal burden by a biological monitoring using metabolites in human body fluids like blood and urine. The sole determination of naphthalene in the air (ambient monitoring) may underestimate the health risk, as percutaneously absorbed naphthalene is not considered.

So far, mainly 1- and 2-naphthol have been applied in the biological monitoring of the naphthalene body burden in humans [1,2,9–17].

However, they do not reflect the possibly carcinogenic properties of naphthalene. In the naphthalene metabolism, the generation of 1,2- and 1,4-naphthoquinone seems to be of fundamental significance for the carcinogenic properties. The high reactivity of those naphthoquinones complicates their direct analysis in biological material. However, their binding to glutathione and proteins was shown in-vitro, in animals and in humans [18–29]. Their precursors 1,2- and 1,4-dihydroxynaphthalene were already used as biomarkers in humans [30,31].

Our main interest was to establish biomarkers of 1,2- and 1,4-naphthoquinone in human urine reflecting the carcinogenic properties of naphthalene. A Michael-addition of glutathione to naphthoquinones could lead to naphthoquinone mercapturic acids (NQMA). The conjugation with endogenous glutathione and the following degradation to mercapturic acids is regarded as a detoxification mechanism of activated substances in the metabolism. Mercapturic acids, as biomarkers, have the advantage of longer half-life times than the highly reactive precursor compounds. Additionally, the structure of the original electrophilic metabolite is still identifiable.

Therefore, reference substances of the mercapturic acids were synthesised (see Fig. 1) and a LC–ESI–MS/MS-method for the determination of 1,2- and 1,4-NQMA in human urine was developed and validated.

2. Materials and methods

2.1. Chemicals and materials

The reference substances 1,2-naphthoquinone-4-mercapturic acid (1,2-NQMA), 1,4-naphthoquinone-2-mercapturic acid (1,4-NQMA), 1,2-dihydrxoynaphthalene-4-mercapturic acid (1,2-DHMA), 1,4-dihydroxynaphthalene-2-mercapturic acid (1,4-DHMA) as well as the internal standard D5-1,4-naphthoquinone-2-mercapturic acid (D5-1,4-NQMA) were custom-synthesised by KAdem Custom Chem, Göttingen (Germany). For structures of the reference substances, derived from 1,2- and 1,4-naphthoquinone, see Fig. 1. The identity and purity of the standards was verified with NMR spectroscopy and mass spectroscopy (see Supplemental material, Fig. S1). The isotopic purity of the labelled standard was tested by LC–MS/MS and contained no measurable unlabelled or partially labelled compound.

Supplementry material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2015.12.052.

Glacial acetic acid, ammonium acetate (p.a.), L-ascorbic acid, formic acid (>98%), acetonitrile (MeCN, gradient grade) and methanol (MeOH, LiChrosolv ≥99.9%) were obtained from Merck (Darmstadt, Germany). β-Glucuronidase/arylsulfatase (Helix pomatia, activity about 4.5–14 U/ml) was purchased from Roche Diagnostics (Mannheim, Germany).

2.2. Standard preparation

Stock solutions of 1,2-NQMA and 1,4-NQMA were prepared by dissolving the standard substances in MeOH, which was saturated with ascorbic acid to a concentration of 400 mg/l. This stock solution was stored on a deposit of solid ascorbic acid at −18 °C until further use. Spiking solutions I and II were freshly prepared for every analytical series by dilution of the stock solution with water (LiChrosolv) to final concentrations of 1 mg/l and 100 μg/l, respectively.

For the labelled internal standard (IS) D5-1,4-NQMA, an IS stock solution of 40 mg/l in MeOH, which was saturated with ascorbic acid, was prepared. The IS stock solution was diluted with MeOH (saturated with ascorbic acid) to an IS spiking solution (4 mg/l).

2.3. Calibration procedure

Calibration was carried out using six calibration levels prepared by spiking pooled urine with D5-1,4-NQMA as internal standard and different volumes of spiking solution I and II to achieve final concentrations of 1–100 μg/l of 1,2-NQMA and 1,4-NQMA. Additionally, identical pool urine was used as a blank sample and was included in each analytical series. The calibration standards were processed as described for the “Sample preparation”.

Linear calibration curves were obtained by plotting the quotients of the analyte’s peak areas and the peak area of the internal standard (D5-1,4-NQMA) as function of the concentration of the analytical standards.

2.4. Sample preparation

Aliquots of the urine samples were stored in polyethylene bottles at −18 °C. For analysis, the samples were thawed, equilibrated to room temperature and homogenised. To an aliquot of 2 ml urine, we added: 150 μl ascorbic acid solution (250 g/l), 25 μl of the internal standard’s spiking solution (D5-1,4-NQMA, 4 mg/l), 1 ml ammonium acetate buffer (0.1 M, pH 5.5) and 10 μl glucuronidase/arylsulfatase. The buffer is necessary to adjust the pH of the urine to the optimum of the enzymes. After vortex-mixing, the samples were hydrolysed for 3 h at 37 °C. Afterwards, the samples were acidified with 20 μl glacial acetic acid, vortex-mixed and centrifuged for 10 min at 3000 min^{-1}. The supernatant was transferred into glass-vials and analysed with LC–MS/MS.

2.5. Liquid chromatography–tandem mass spectrometry (LC–MS/MS)

Liquid chromatography was carried out on a Hewlett-Packard HP 1100 Series HPLC apparatus, equipped with auto sampler, quaternary pump and vacuum degasser (Agilent, Waldbronn, Germany), and an additional isocratic Merck–Hitachi L6000A pump (Merck, Darmstadt, Germany). The samples were analysed with two-dimensional HPLC (Fig. 2). Therefore, the isocratic pump was used to load an aliquot (300 μl) of the hydrolysed and acidified sample to a RAM (restricted access material) phase with a flow of 0.8 ml/min, using a mobile phase S1 of 1% aqueous solution of acetic acid and methanol (97:3, v/v). With the RAM phase, a LiChrosphere ADS RP-18 (RAM C18, 25 × 4 mm, 25 μm, Alkyl-Diol-modified silica gel from Merck, Darmstadt, Germany), online enrichment and clean-up of the sample is achieved.

After 8 min, the analytes were transferred to a reversed-phase HPLC-column Synergi 4u Polar-RP 80A (150 × 4.60 mm, 4u μm; precolumn C18 4 × 3.0 mm) (Phenomenex, Aschaffenburg, Germany) in backflush mode with a ten-port valve on the Sciex API 2000 MS/MS with software controlled 10 port valve (Applied Biosystems, Langen, Germany). Afterwards, the analytes were sep-