Talanta 98 (2012) 211–219

Contents lists available at SciVerse ScienceDirect

Talanta

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

Accurate quantification of the mercapturic acids of acrylonitrile and its genotoxic metabolite cyanoethylene-epoxide in human urine by isotope-dilution LC-ESI/MS/MS

T. Schettgen *, J. Bertram, T. Kraus

Institute for Occupational and Social Medicine, Medical Faculty, RWTH Aachen University, Pauwelsstrasse 30, D-52074 Aachen, Germany

ARTICLE INFO

Article history:
Received 2 April 2012
Received in revised form
19 June 2012
Accepted 25 June 2012
Available online 2 July 2012

Keywords:
Human biomonitoring
LC/MS/MS
Metabolism
Occupational toxicology
Mercapturic acid
Carcinogenesis

ABSTRACT

Acrylonitrile is a highly important industrial chemical with a high production volume worldwide, especially in the plastics industry. It is classified as a possible human carcinogen by the International Agency for Research on Cancer (IARC group 2B). During metabolism of acrylonitrile, the genotoxic metabolite cyanoethylene-epoxide is formed. The urinary mercapturic acids of acrylonitrile, namely *N*-acetyl-*S*-(2-cyanoethyl)-L-cysteine (CEMA) and cyanoethylene-epoxide, namely *N*-acetyl-*S*-(1-cyano-2-hydroxyethyl)-L-cysteine (CHEMA) are specific biomarkers for the determination of individual internal exposure to acrylonitrile and its highly reactive metabolite.

We have developed and validated a sensitive method for the accurate determination of CEMA and CHEMA in human urine with a multidimensional LC/MS/MS-method using deuterium-labelled analogues for both analytes as internal standards. Analytes were stripped from urinary matrix by online extraction on a restricted access material, transferred to the analytical column and determined by tandem mass spectrometry. The limit of quantification (LOQ) for CEMA and CHEMA was 1 μg/L urine and allowed to quantify the background exposure of the (smoking) general population. Precision within and between series for CHEMA ranged from 2.6 to 8.0% at four concentrations ranging from 8.3 to 86 μg/L urine, mean accuracy was between 94 and 100%. For CEMA, precision within and between series ranged from 2.4 to 14.5% at four concentrations ranging from 15.1 to 196 μg/L urine, mean accuracy was between 91 and 104%. We applied the method to spot urine samples of 83 subjects of the general population with no known occupational exposure to acrylonitrile. Median levels (range) for CEMA and CHEMA in urine samples of non-smokers ($n=47$) were 1.9 μg/L (< 1–16.4 μg/L) and < 1 μg/L (< 1–3 μg/L), while in urine samples of smokers ($n=36$), median levels were 184 μg/L (2–907 μg/L) and 29.3 μg/L (< 1–147 μg/L), respectively. Smokers showed a significantly higher excretion of both acrylonitrile metabolites ($p<0.001$). Due to its automation and high sensitivity, our method is well suited for application in experimental studies on acrylonitrile metabolism or occupational studies.

1. Introduction

Acrylonitrile (CAS 107-13-1) is a highly reactive, colorless, volatile liquid that polymerises spontaneously. It is reasonably anticipated to be a human carcinogen based on sufficient evidence in animal experiments [1]. Consequently, IARC has rated acrylonitrile as a group 2B carcinogen, while the Deutsche Forschungsgemeinschaft (DFG) has classified it as a group 2 carcinogen [2,3]. An increased risk for lung cancer or prostate cancer has been linked with high occupational exposure to acrylonitrile of workers of the U.S. textile industry [1], but the results of several epidemiological studies are still inadequate to evaluate the relationship between human cancer and acrylonitrile exposure [1,4].

Acrylonitrile is one of the main basic chemicals in the production of synthetic fibres and plastics as well as rubber [1]. The production of acrylonitrile in the European Union was estimated to be 1.25 Mio t/year between 1994 and 1996 (USA: 1.5 Mio t in 1996) [1,5].

A main source of exposure to acrylonitrile for the general population is tobacco smoke, as it is a constituent of tobacco smoke with concentrations varying from 3 to 15 μg/cigarette [6]. Other exposure sources are negligible for the general population, since residual monomer concentrations of plastics (with potential contact to food) are regulated by law and ambient air concentrations are usually in the range of the LOD [5].

The metabolism of acrylonitrile has been intensively studied in rodents. Within metabolism of incorporated acrylonitrile, the

* Corresponding author. Tel.: +49 241 80 88285; fax: +492418082587.
E-mail address: tschettgen@ukaachen.de (T. Schettgen).

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http://dx.doi.org/10.1016/j.talanta.2012.06.074

highly reactive metabolite cyanoethylen-epoxide (CEO) is formed by liver cytochrome P 4502E1 [7]. The formation of CEO is considered to be responsible for the carcinogenic properties of acrylonitrile in animal experiments. CEO was shown to bind covalently to DNA at much higher rates than acrylonitrile itself and was shown to bind to the phosphate moiety of nucleosides, inducing DNA strand breaks [8,9]. Both acrylonitrile as well as the intermediate metabolite CEO might react with glutathione via enzymatic glutathione-S-tranferases (GSTs) as detoxification reaction. In case of direct reaction with glutathione, *N*-acetyl-*S*-cyanoethyl-cysteine (CEMA) is formed and excreted via urine [10,11]. Reaction of CEO with glutathione might lead to the formation of *N*-acetyl-*S*-(1-cyano)-2-hydroxethyl-cysteine (CHEMA) as well as to the formation of an intermediary cyanohydrin metabolite, which is unstable and finally leads to the excretion of *N*-acetyl-*S*-2-hydroxyethyl-cysteine (HEMA) after elimination of cyanide. The metabolism of acrylonitrile is illustrated in Fig. 1.

As HEMA is also a metabolite of ethylene oxide as well as ethylene with varying urinary background levels [12,13], excretion of HEMA is no longer specific for exposure to acrylonitrile and cyanoethylene-epoxide, respectively. Animal experiments in rats showed that CEMA and HEMA account for 40 and 30% of the dose excreted via urine, while CHEMA makes up for another 13% of the excreted dose [7].

Thus, the excretion of CHEMA is the only specific urinary biomarker of internal exposure to CEO, the cancer initiating metabolite of acrylonitrile. As human biomonitoring should always be aimed to determine the internal dose of the most harmful agent within metabolism of hazardous substances, the quantification of CHEMA in urine would provide valuable data for an accurate risk assessment of human exposures to acrylonitrile. This is especially true as the formation as well as detoxification of CEO is influenced by a number of different (polymorphic) enzymes whose influence is yet unknown [14]. So far, CHEMA has only been identified in urine of highly exposed rats and mice by GC/MS and ^{13}C –NMR [7,15]. Most recently, CHEMA as well as CEMA was quantified in human urine [16]. However, a labelled internal standard for CHEMA was lacking in that study and human data on excretion of CHEMA and the fraction of oxidative metabolism in humans are still sparse.

Therefore, the aim of our present study was the development, validation and finally application of an accurate analytical method for the simultaneous determination of the mercapturic acids of acrylonitrile (CEMA) and cyanoethylene-epoxide (CHEMA) in human urine using a previously established column-switching technology [11].

2. Experimental

2.1. Reagents and standards

All solvents and chemicals used were HPLC or analytical reagent grade. CEMA (*N*-acetyl-*S*-(2-cyanoethyl)cysteine, chemical purity: 98%) and CHEMA (*N*-acetyl-*S*-(1-cyano-2-hydroxyethyl)cysteine dicyclohexylamine salt, chemical purity: 98%) were purchased from TRC (Toronto, Canada). The internal standards D_3-CEMA (*N*-acetyl-D_3-*S*-(2-cyanoethyl)cysteine, chemical purity: 98%, isotopic purity: > 98%) as well as D_3-CHEMA (*N*-acetyl-D_3-*S*-(1-cyano-2-hydroxyethyl)cysteine dicyclohexylamine salt, chemical purity: 98%, isotopic purity: > 98%) were also purchased from TRC (Toronto, Canada). Identity and purity of all standards was confirmed by ^1H –NMR spectroscopy and mass spectrometric analysis as stated by the supplier (www.trc-canada.com).

Formic acid (100%) was supplied by Merck (Darmstadt, Germany). Acetonitrile (HPLC grade) was purchased from J.T. Baker (Germany). Ammonium formate was supplied by Fluka (Buchs, Suisse). Standard solutions of both analytes as well as the labelled internal standards were prepared by dissolving 1 mg of CEMA, D_3-CEMA and CHEMA (complete amounts available) each in 1 ml of 0.1% aqueous formic acid, resulting in stock solutions of 1 g/L for CEMA and D_3-CEMA and 0.56 g/L for CHEMA (due to the molar ratio of the dicyclohexylamine salt to the free acid). In the case of D_3-CHEMA, 0.5 mg (complete amount available) was dissolved in 500 µl of 0.1% aqueous formic acid (0.56 g/L). A combined working solution of the internal standards was prepared by dilution with 0.1% aqueous formic acid (concentration 10 mg/L and 5.6 mg/L, respectively). All solutions were kept frozen at –20 °C in small dark brown screw top flasks.

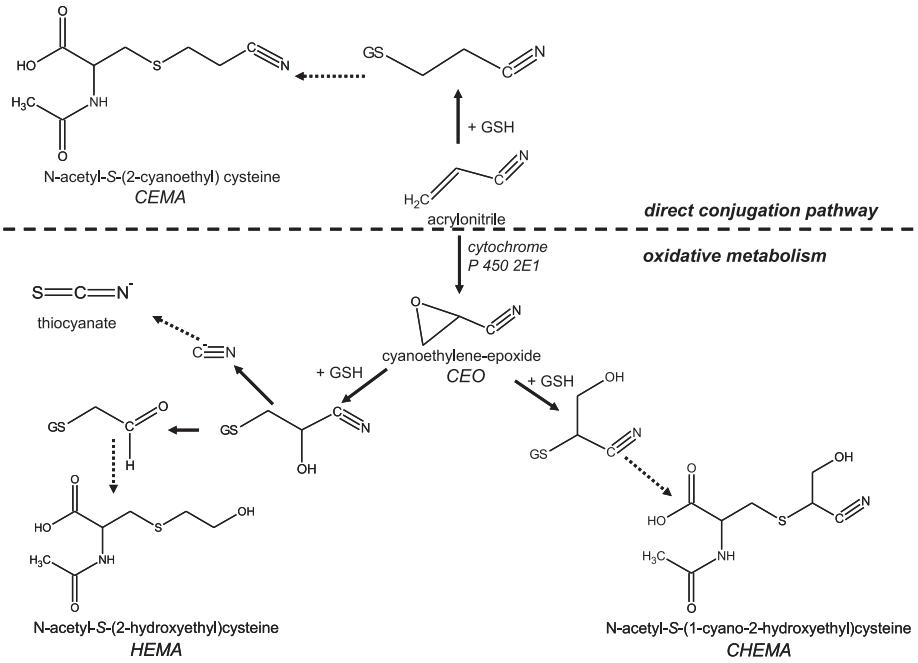

Fig. 1. Simplified metabolism of acrylonitrile (GSH = glutathione).