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Quantification of *N*-methylmalonamic acid in urine as metabolite of the biocides methylisothiazolinone and chloromethylisothiazolinone using gas chromatography-tandem mass spectrometry

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ABSTRACT

Methylisothiazolinone and the mixture of chloromethylisothiazolinone/methylisothiazolinone (MCI/MI, 3:1) are widespread biocides used in cosmetic and household products. Due to their skin permeability, they might be taken up by the general population via use of products containing these biocides. As both compounds are known skin sensitizers, the use of these products is under discussion by regulatory agencies.

In order to evaluate the possible uptake of MI and/or MCI/MI by human biomonitoring, we have developed and validated a highly sensitive and specific GC/MS/MS-method for the quantification of *N*-methylmalonamic acid (NMMA), a known metabolite of MI and MCI in urine of rats. After freeze-drying of urine, the analyte is derivatised with pentafluorobenzyl bromide in anhydrous solution and the PFB-derivative is extracted into n-hexane. After concentration, the derivative is finally quantified by GC/MS/MS in EI-mode using $^{13}C_3$-NMMA as internal standard. The limit of quantification for NMMA was 0.5 ng mL^{-1} urine. Precision within and between-series was determined to range between 3.7–10.9% using native and spiked quality control samples. Accuracy ranged between 89 and 114%.

In a pilot study we applied this method to spot urine samples of 63 persons not knowingly exposed to MI and/or MCI/MI. NMMA was quantifiable in every urine sample analysed, with no significant difference in urinary levels between male and female participants. The median (95th percentile) levels for urinary NMMA were 3.6 (7.4) ng mg^{-1} creatinine and 2.9 (9.1) ng mg^{-1} creatinine for males (n = 32) and females (n = 31), respectively. In a volunteer experiment, a relation of exposure to MI and/or MCI/MI and subsequent NMMA-excretion was shown. Our method is the first to report human urinary background levels of NMMA. However, the possibility of formation and urinary excretion of NMMA within physiological processes cannot be ruled out.

1. Introduction

Methylisothiazolinone (MI) and the reaction mixture of chloromethylisothiazolinone/methylisothiazolinone (3:1, MCI/MI, trade name for example: Kathon CG®, ACTICIDE® SPX) are important biocides that are used in cosmetic products as well as in household and industrial products (e.g. wet wipes, cleaners, paints, etc.) to prevent growth of mold or bacteria in these products [1,2]. The maximum permitted concentration of the mixture MCI/MI in cosmetic products (only "rinse off"-products) in the European Union is 15 ppm, while for MI alone, the maximum concentration is 100 ppm [3]. Concerning household and industrial products, there are no maximum amounts for MI or MCI/MI defined. Recent surveys in Belgium constrain the widespread use of these biocides in consumer products with rather high concentrations found (e.g. up to 163 ppm of MI in a baby shampoo) [4]. MI and MCI/MI are readily taken up via skin contact [5]. However, due to the semivolatile character of both compounds, they can also be determined in indoor air after application of paints or cleaning agents containing MI or MCI/MI as recently shown [6,7]. Possible occupationally relevant applications are the use of MI and MCI/MI as preservative in cutting fluids and cooling systems as well as in the production of paper and cellulose [5].

While the acute and chronic toxicity of both substances in rats, mice and dogs was shown to be low in animal experiments, both substances are well known for their skin sensitizing potential [1,2]. In the last years, dermatologists worldwide have observed an

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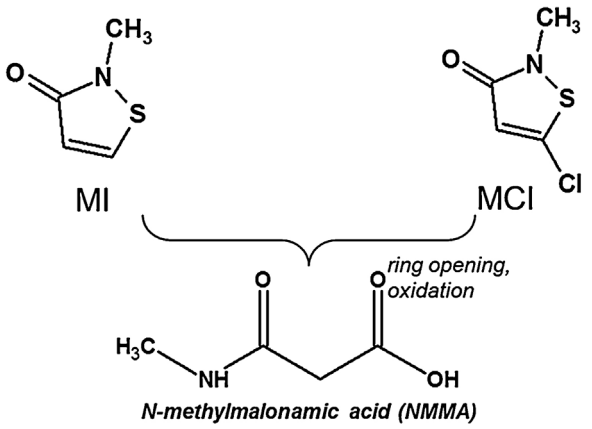

Fig. 1. Simplified metabolism scheme of methylisothiazolinone (MI) and chloromethylisothiazolinone (MCI).

alarming increase in skin sensitizations against both substances in the general population using patch tests [2,8]. As MI and MCI/MI have in recent times often replaced the possibly endocrine disrupting parabens as biocides in cosmetic products, this growing number of observed sensitizations is probably connected with an increasing exposure of the general population to MI and MCI/MI. For the quantification of this exposure human biomonitoring is the method of choice, which means the quantification of chemicals or their metabolites in human body fluids [9]. Therefore, a biomarker of exposure to MI and/or MCI/MI is highly required.

MI and MCI were shown to be rapidly metabolized and excreted in urine in animal experiments in rats [10]. Within metabolism, the nitrogen-sulfur-bond of the thiazolinone ring undergoes nucleophilic attack as first step and the sulfur atom (and chlorine atom in case of MCI) is eliminated. Following oxidation, N-methylmalonamic acid (NMMA) is formed and was shown to be the main urinary metabolite of MI (and MCI) in Sprague-Dawley-rats after oral dosage of 5 and 50 mg ^{14}C-labelled MI, representing 21–23% of the dose applied. In this study, the radiolabelled metabolites were further identified using LC/MS, and NMR [10]. A simplified metabolism scheme of MI and MCI together with the structures of the chemicals of interest is shown in Fig. 1.

Consequently, NMMA as probable human metabolite was chosen as a candidate biomarker of human exposure to MI and MCI/MI within the cooperation project between the German Federal Ministry for the Environment (BMUB) and the German Chemical Industry Association (VCI). This 10-year research project is aimed at the development and validation of suitable human biomonitoring methods for the quantitative exposure assessment of chemicals of concern (such as MI and MCI/MI) in the general population.

So far, there has been no previous analytical method for the determination of urinary NMMA with the exception of the identification as (radiolabelled) metabolite in animal experiments using LC/MS and NMR [10]. Therefore, our study was aimed at the development and validation of a highly sensitive and specific method for the quantification of NMMA in human urine as metabolite of the biocides MI and/or MCI. In order to achieve a maximum in both specificity and sensitivity, we have applied gas chromatography coupled to tandem mass spectrometry which is highly superior to other detection techniques as previously shown for pesticides [11]. In a pilot study we have applied this method to spot urine samples of a group of 63 persons with no known exposure to MI and/or MCI/MI. To our knowledge, this study is the first method that covers that topic and reports background levels of NMMA in urine samples taken from the general population

2. Experimental section

2.1. Chemicals

N-methylmalonamic acid (3-(methylamino)-3-oxopropanoic acid, 100%) was obtained from ChemBridge (San Diego, CA, USA). ^{13}C-N-methylmalonamic acid (> 98%, isotopic purity: 99.8%) was custom synthesized in the laboratory of Dr. Belov (Göttingen, Germany) and characterized by ^{1}H NMR, mass spectrometry and HPLC. *N*-acetyl-cysteine (> 99%) was purchased from Merck (Darmstadt, Germany) and 2,3,4,5,6-pentafluorobenzylbromide (PFBBr, 99%) was obtained from Aldrich (Steinheim, Germany). Potassium carbonate (p.a.) and dried acetonitrile (SeccoSolv, max. 0.005% water) was also obtained from Merck (Darmstadt, Germany). Toluene and *n*-hexane were of the highest analytical grade available and supplied by Merck (Darmstadt, Germany). Synthetic urine (95% water, according to DIN EN 1616) was purchased by Synthetic urine e.K. (Eberdingen-Nußdorf, Germany, www.synthetic-urine.de).

To prepare a reagent solution for derivatization of NMMA, 5 g of pentafluorobenzylbromide were weighed in a 20-ml beaker and dissolved in 6 ml of dry acetonitrile (SeccoSolv). This solution was transferred to a 10 ml brown-glass vial with Teflon-lined screw top (close thightly!) and is ready for use in sample preparation. This solution was kept at + 4 °C in a fridge and is stable for about 3 months.

Note: pentafluorobenzylbromide is a strong lachrymator! Care has to be taken when handling this substance and their solutions. All work has to be done in a fume hood and used pipette tips should be disposed in basic methanol in a fume hood!

2.2. Preparation of standard solutions

A stock solution of NMMA was prepared by dissolving 10 mg of NMMA with acetonitrile in a 10-ml glass volumetric flask (1 mg mL^{-1}). From this stock solution, four spiking solutions with concentrations of 50 μg mL^{-1}, 5 μg mL^{-1}, 500 ng mL^{-1} and finally 5 ng mL^{-1} were prepared and used for the preparation of standards and quality controls.

The stock solution of $^{13}C_3$-NMMA was prepared by dissolving 10 mg of $^{13}C_3$-NMMA with acetonitrile in a 10-ml glass volumetric flask and filling to the mark (1 mg mL^{-1}). To prepare the working solution of the internal standard, 100 μl of this stock solution of $^{13}C_3$-NMMA (1 mg mL^{-1} in acetonitrile) were placed in a 20-ml glass volumetric flask and diluted to the mark with water (c = 5 μg mL^{-1}). This working solution of the internal standards is ready for use in sample preparation.

2.3. Sample preparation

An aliquot of 100 μl of the urine sample was transferred to a 5 ml glass vial with screw top and spiked with 10 μl of the working solution of the labelled internal standard ($^{13}C_3$NMMA, 5 μg mL^{-1}). The urine sample was then subjected to freeze drying in a pre-cooled freeze-dryer (Model P3, Piatkowski Forschungsgeräte, Munich, Germany) at −20 °C and a vacuum pressure below 0.4 mbar for app. 8 h.

The freeze-dried urine samples were then redissolved in 1 ml of dry acetonitrile (SeccoSolv) by short vortexing and subsequent placing in an ultrasonic bath for 2 min. 100 μl of the derivatization solution of PFBBr (5 g in 6 ml acetonitrile) were added and after that, app. 10–20 mg of potassium carbonate were added to the sample. The vials were sealed tightly and placed in an oven at 60 °C overnight for 16 h. After cooling down to room temperature, 1 ml of n-hexane and 3 ml of a solution of N-acetylcysteine in water (10 g L^{-1}) were added and the solution was vortexed for 60 s. Following centrifugation at 800*g* for 5 min, the upper n-hexane