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Determination of 5-hydroxy-N-methylpyrrolidone and 2-hydroxy-N-methylsuccinimide in human urine

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Abstract

A method for simultaneous determination of 5-hydroxy-N-methylpyrrolidone and 2-hydroxy-N-methylsuccinimide in urine is described. These compounds are metabolites of N-methyl-2-pyrrolidone, a powerful and widely used organic solvent. 5-Hydroxy-N-methylpyrrolidone and 2-hydroxy-N-methylsuccinimide were purified from urine by adsorption to a C_8 solid-phase extraction column and then elution by ethyl acetate–methanol (80:20). After evaporation, the samples were derivatised at 100°C for 1 h by bis(trimethylsilyl)trifluoroacetamide. Ethyl acetate was then added and the samples were analysed by gas chromatography–mass spectrometry in the electron impact mode. The extraction recovery for 5-hydroxy-N-methylpyrrolidone was about 80% while that for 2-hydroxy-N-methylsuccinimide was about 30%. The intra-day precision for 5-hydroxy-N-methylpyrrolidone was 2–4% and the between-day precision 4–21% (4 and 60 μg/ml). The intra-day precision for 2-hydroxy-N-methylsuccinimide was 4–8% and the between-day precision 6–7% (2 and 20 μg/ml). The detection limit was 0.2 μg/ml urine for both compounds. The method is applicable for analysis of urine samples from workers exposed to N-methyl-2-pyrrolidone.

Keywords: 5-Hydroxy-N-methylpyrrolidone; 2-Hydroxy-N-methylsuccininide; N-Methyl-2-pyrrolidone

1. Introduction

N-Methyl-2-pyrrolidone (NMP; structural formula C_5H_9NO; CAS number 872-50-4; boiling point 202°C at 101.3 kPa), due to its strong and selective solvent power, is a widely used compound [1]. NMP dissolves most monomers and polymers and catalyses many polymerisation reactions. NMP is used increasingly as a substitute for other solvents of higher inherent toxicity in the occupational and environmental settings, e.g., dichloromethane in paint strippers. The use of NMP as a remover of graffiti has extremely increased [2]. Moreover, NMP is suggested as a skin penetration enhancer in transdermal therapy [1,3].

Experimental exposure in human volunteers shows an efficient absorption of NMP through the respiratory tract [4]. NMP is readily eliminated from the body, mainly by biotransformation to other compounds: only a minimal fraction is eliminated in urine as NMP. A metabolic pathway, where NMP is first hydroxylised to 5-hydroxy-N-methylpyrrolidone (5-HNMP) and then further oxidised to N-methylsuccinimde (MSI), which in turn is hydroxylised to 2-hydroxy-N-methylsuccinimide (2-HMSI), has been suggested [5].

Studies on reproductive toxicity show that NMP may cause developmental toxicity at doses causing

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no or mild maternal toxicity [6,7]. The large number of women exposed to NMP in the microelectronics fabrication industry makes it urgent to assess the exposure to NMP. Air monitoring methods have been described [8,9]. However, the extensive percutaneous absorption of NMP makes it necessary to develop methods for biological monitoring. A method aimed at the analysis of NMP itself in urine and plasma has recently been reported by our group [4]. However, the ready biotransformation of NMP to other compounds and the minor excretion of non-metabolized NMP in urine suggest that the metabolites of NMP, rather than NMP itself, can be the most suitable compounds for biological monitoring. No methods for the quantitative analysis of these metabolites have been described so far.

In the present study, we report a method for the determination of 5-HNMP and 2-HMSI in human urine.

2. Experimental

2.1. Apparatus

A Model 8065 gas chromatograph (Carlo-Erba, Milan, Italy) connected with a VG Trio 1000 quadrupole mass-spectrometer (Fisons, Manchester, UK) and fitted with an A200S auto-sampler (Carlo Erba) was used. The column (30 m×0.25 mm) with a DB-5 MS stationary phase and a film thickness of 0.25 μm was from J&W Scientific (Folsom, CA, USA). For the clean-up procedure, C_8 Isolute solid-phase extraction (SPE) columns (500 mg; IST, Hengoed, UK) were used. These disposable micro-columns were used with a VacElut SPS 24 (Varian, Palo Alto, CA, USA), which connected to an aspirating pump. A Model 3E-1 centrifuge (Sigma, Deisenhofen, Germany) was used to remove water from the Isolute columns after the washing step.

2.2. Chemicals and regents

2-HMSI was from Aldrich (Gillingham, UK), methanol and ethyl acetate were from LabScan (Dublin, Ireland), and bis(trimethylsilyl)trifluoroacetamid (BSTFA) with 1% trimethylchlorosilane was from Sigma (St Louis, MO, USA). 5-HNMP, tetra-deuterium labeled 5-HNMP ($[^2H_4]$-5-HNMP) and tri-deuterium labeled 2-HMSI ($[^2H_3]$-2-HMSI) were synthesised by Synthelec (Lund, Sweden).

Synthetic urine was prepared as described by Hesse et al. [10] except that the trypticase soy broth was excluded. The following chemicals were added to water: $CaCl_2 \cdot 2H_2O$ (0.65 g/l), $MgCl_2 \cdot 6H_2O$ (0.65 g/l), NaCl (4.60 g/l), Na_2SO_4 (2.30 g/l), Na_2 oxalate (0.02 g/l), KH_2PO_4 (2.80 g/l), KCl (1.60 g/l), NH_4Cl (1.00 g/l), urea (25.0 g/l), Na_3 citrate (0.65 g/l) and creatinine (1.10 g/l). The synthetic urine was diluted ten times before use.

2.3. Storage of urine samples

Urine samples were stored without any pretreatment in 10 ml polyethylene test tubes at −20°C until analysis.

2.4. Preparation of standards

Standard solutions of 5-HNMP and 2-HMSI were prepared by addition of 25 mg of each compound to 25 ml of water. Solutions in desired concentrations (range: 0.2–60 μg 5-HNMP/ml and 0.2–20 μg 2-HMSI/ml) were prepared from this point by further dilution in water. Urine standards containing 5-HNMP and 2-HMSI were prepared by adding 50 μl aliquots of these standard solutions to a mixture of 0.2 ml blank urine and 0.8 ml 10% synthetic urine.

For the determination of the recovery, standard solutions of 5-HNMP and 2-HMSI were prepared in ethyl acetate at the desired concentrations.

2.5. Work-up and derivatisation procedure

The 5-HNMP and 2-HMSI were extracted by C_8 columns previously conditioned with 5 ml of methanol followed by 10 ml of water. Internal standards, 15 μg $[^2H_4]$-5-HNMP and 4 μg $[^2H_3]$-2-HMSI, in 50 μl aliquots of water were added to a mixture of 0.2 ml urine sample and 0.8 ml 10% synthetic urine. The samples were applied to the columns and followed by 1 ml of water for washing. The columns were then dried by air suction for 5 min and centrifuged at 3000 *g* for 10 min and thereafter eluted with 2 ml of a mixture containing 80% ethyl acetate and 20% methanol. The organic phase was

evaporated under a nitrogen stream and 50 μl BSTFA was added. The samples were heated at 100°C for 1 h, 1 ml of ethyl acetate added, and transferred to the auto-sampler injection vials. The derivatized samples were stored at 4°C until analysis.

2.6. Analysis

Aliquots (2 μl) were injected with a splitless technique at 250°C. The split exit valve was kept closed for 0.5 min after the injection. The column carrier gas was helium at a pressure of 70 kPa. The initial column temperature was 70°C for 1 min. The temperature was then increased by 15°C/min to 250°C. The MS interface was at 250°C, the ion source at 200°C and the MS was in the electron impact (EI) mode at 70 eV. Selected ion monitoring (SIM) for the trimethyl silyl derivative of 5-HNMP (TMS-5-HNMP) was performed at m/z 98 and 186 while m/z 102 and 190 were chosen for the internal standard. SIM for the trimethyl silyl derivative of 2-HMSI (TMS-2-HMSI) was performed at m/z 144 and 186 while only m/z 189 was used for [$^2H_3$]-labeled TMS-2-HMSI. The peak area ratios were used for the determinations.

3. Results and discussion

3.1. Standards

The identity of the synthesised 5-HNMP was confirmed by proton NMR. In this a multiplet was found at 5.1 ppm (one ring proton next to a hydroxyl group), a doublet at 4.6 ppm (one hydroxyl proton), a singlet at 2.8 ppm (three methyl protons) and three multiplets at 1.9, 2.3 and 2.5 ppm, respectively (four ring protons). The proton purity was found to be better than 90%. No attempts were performed to check the purity by GC since 5-HNMP is a thermolabile compound which can not even be analysed quantitatively as such using on-column injections. The identity of 5-HNMP and 2-HMSI in urine from NMP exposed subjects was confirmed by collection of EI mass spectra of the TMS derivatives (see Section 3.5).

3.2. Stability

5-HNMP and 2-HMSI proved to be stable in water or ethyl acetate for several months at 4°C. Urine samples containing between 1 and 60 μg 5-HNMP/ml lost 5% of the 5-HNMP during a 5 month storage at −20°C. Urine samples containing between 0.2 and 18 μg 2-HMSI/ml lost 9% of the 2-HMSI during a 8 month storage at −20°C. The TMS derivatives, especially the 5-HNMP derivative, proved unstable upon storage, even at −20°C. Thus, we recommend that the samples should be analysed within 3 days of derivatisation.

3.3. Work-up procedure

It has already been reported from NMP exposed subjects that there is no conjugation with glucuronic acid or sulfate for either 5-HNMP or 2-HMSI [5]. Thus, no hydrolysis was performed prior to urine extraction.

In preliminary tests, liquid–liquid extraction of 5-HNMP and 2-HMSI from urine with ethyl acetate gave low extraction recoveries (10–30%). Thus, SPE columns were chosen. Several different disposable SPE columns were tested for the extraction of 5-HNMP from urine. Isolute C_8 columns gave recoveries of about 80%. This column proved also to be the best column for 2-HMSI extractions (recovery about 30%) and was chosen for urine work-up. Later it was found that a new extraction column, Isolute ENU+, gave similar recoveries but it was not tested further. Isolute C_{18} and Bond Elut C_8 (Varian) gave extraction recoveries between 60 and 70%, Bond Elute C_{18}, Bond Elut C_2 and end-capped Isolute C_{18} gave worse recoveries. All columns had 500 mg sorbent (except the Isolute ENU+ which had 200 mg). The extraction recovery of 2-HMSI was lower than that of 5-HNMP and it was found that most of the 2-HMSI was lost during the washing step with water, but it was found that the washing was necessary for the derivatisation with BSTFA. As far as elution is concerned, after testing different ethyl acetate to methanol ratios, it was found that 80:20 gave optimal recovery, i.e., about 80% for 5-HNMP and about 30% for 2-HMSI.

The absolute recovery of urine extraction using the Isolute C_8 columns was further studied. Ten different

Table 1
Average SPE recovery of 5-HNMP and 2-HMSI from urine using Isolute C_8

Compound	Concentration (μg/ml)	Recovery (%)	Precision[a] (%)
5-HNMP	4	83	5.4
5-HNMP	60	80	2.5
2-HMSI	2	31	6.4
2-HMSI	20	29	4.8

Ten different urine samples were used at each concentration.
[a] Given as coefficients of variation.

urine samples spiked with 4 μg/ml or 60 μg/ml of 5-HNMP and with 2 μg/ml or 20 μg/ml of 2-HMSI but without internal standard added underwent this extraction procedure. The internal standards was added in ethyl acetate after the extraction but before the derivatisation and subsequently the samples were analysed. Besides, the ten urine samples were extracted without the addition of 5-HNMP, 2-HMSI, or internal standards before extraction, and these compounds were added only before the derivatisation procedures. Thus, the extraction recovery was calculated as the fraction of area ratio in the extracted samples versus the area ratio in the non-extracted samples. The extraction recoveries and relative precisions are shown in Table 1.

3.4. Derivatisation procedure

Pentafluorobenzoyl chloride was initially tested for derivatisation but the derivatisation recoveries were poor with this regent. Because of the wide use of different TMS reagents for the derivatisation of hydroxyl compound, we tested BSTFA and this reagent was found to give good results. No attempts were made to investigate the recovery of the derivatisation procedure since the TMS compounds were rather unstable. However, it was found that the highest recoveries were obtained when the BSTFA was kept in the injection solutions during analysis.

3.5. Mass spectrometry and chromatography

The EI mass spectra of the TMS-5-HNMP and tetra-deuterium labeled TMS-5-HNMP are shown in Fig. 1. The molecular ion (M^+) for the unlabeled compound at 187 was a major fragment and so were the fragments at 186 (M^+-H), 172 (M^+-CH_3) and the base peak at m/z 98 ($M^+-O-TMS$). The corresponding fragments were found in the spectrum of the internal standard. In Fig. 2 the EI spectra of TMS-2-HMSI and tri-deuterium labeled TMS-2-HMSI are shown. No M^+ for TMS-2-HMSI was obtained at m/z 201. The base peak, derived from the loss of a methyl group from the M^+, was at 186. Other major fragments were at m/z 158 (M^+-

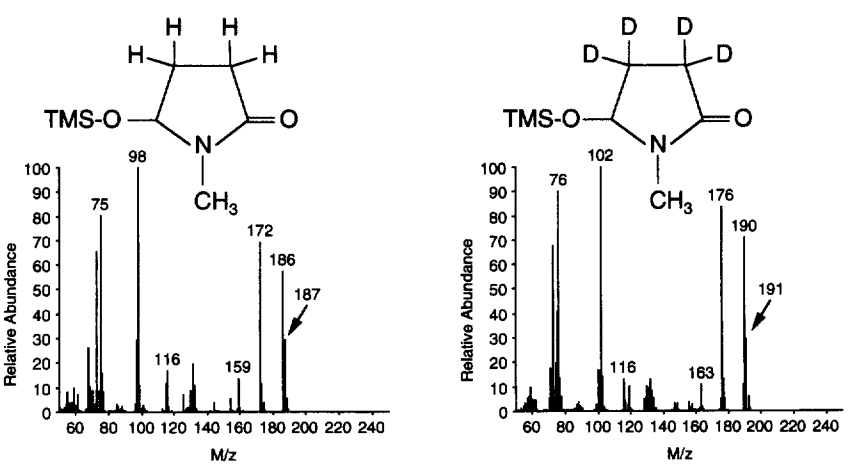

Fig. 1. Electron impact mass spectra of the trimethyl silyl derivatives of 5-hydroxy-N-methylpyrrolidone (TMS-5-HNMP) and tetra-deuterium labeled TMS-5-HNMP.

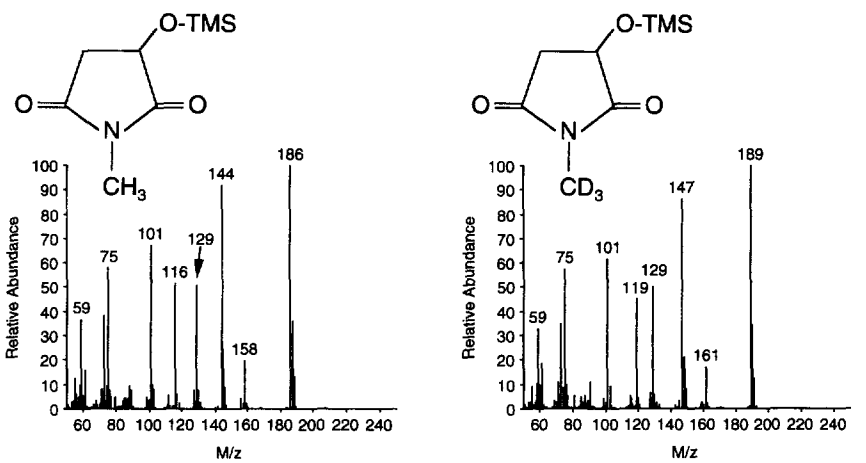

Fig. 2. Electron impact mass spectra of the trimethyl silyl derivatives of 2-hydroxy-N-methylsuccinimide (TMS-2-HMSI) and tri-deuterium labeled TMS-2-HMSI.

CH_3-CO), 144, 129, 116 and 101 (all with complex fragmentations which include migration of hydrogens). Corresponding ions for the internal standard were found at m/z 189, 161, 147, 129, 119 and 101. The overall chromatographic behaviour of the TMS compounds was good. The procedure using synthetic urine for the dilutions was employed because this seemed to give a slightly better peak shape compared to dilution with pure water. Chromatograms from a urine containing 3.9 μg 5-HNMP/ml and 1.2 μg 2-HMSI/ml and a blank urine sample are shown in Fig. 3. All fragments used for the determination of

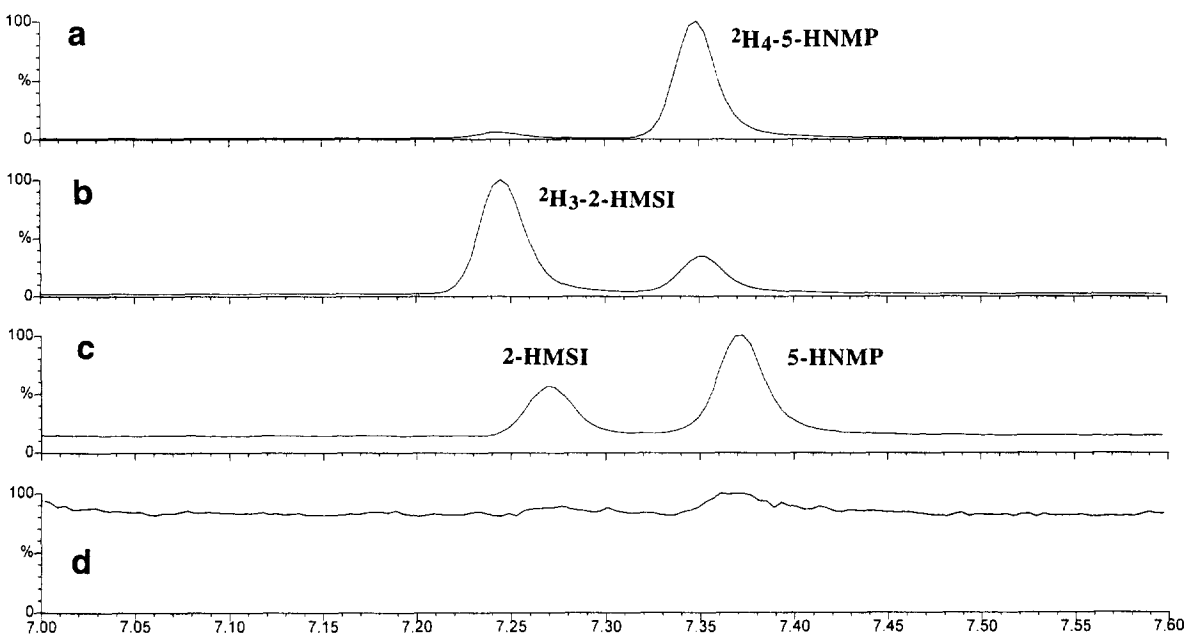

Fig. 3. Chromatograms from (c) a urine sample containing 3.9 μg 5-HNMP/ml and 1.2 μg 2-HMSI/ml using m/z 186 and (d) a blank urine sample using m/z 186. The chromatograms in (a) and (b) are from the internal standards using the fragments at m/z 190 and 189, respectively.

5-HNMP and 2-HMSI in urine samples were influenced by signals from the matrix. For quantitative analysis of 5-HNMP the best fragments seemed to be *m/z* 98. *m/z* 186 was also tested for the determinations but a fairly high imprecision was obtained in the lower concentration range (see Section 3.6.3). For analysis of 2-HMSI, both fragments at 144 and 186 could be used for the determinations. Thousands of injections were made without any observed break down of the column but the injection glass liner had to be cleaned after some hundreds of injections.

3.6. Quantitative analysis

3.6.1. Calibration

Equations describing the calibration graphs of 5-HNMP and 2-HMSI in urine in the concentration range between 0.2 and 60 μg 5-HNMP/ml ($n=6$) and 0.2 and 25 μg 2-HMSI/ml ($n=6$) are shown in Table 2. Urine samples containing more than 60 μg 5-HNMP/ml or more than 25 μg 2-HMSI were diluted ten times with water before analysis. All calibration graphs were linear over the whole range of concentrations.

3.6.2. Detection limit

Urine samples were collected from ten volunteers who were presumed to be unexposed to NMP, 5-HNMP, MSI or 2-HMSI. Internal standards were added and the samples were derivatised and analysed according to the method described in Section 2.5 Section 2.6. The detection limit was calculated as reported by Miller and Miller [11], as the concentration corresponding to the peak area ratios with the same retention time as trimethylsilylated 5-HNMP or 2-HMSI plus three times the standard deviation of these. The detection limits for 5-HNMP and 2-HMSI in urine are shown in Table 3.

Table 3
Detection limits for 5-HNMP and 2-HMSI in urine

Compound	Fragment *m/z*	Detection limit (μg/ml)
5-HNMP	98	0.2
5-HNMP	186	0.3
2-HMSI	144	0.2
2-HMSI	186	0.2

3.6.3. Precision

The intra-day precision of the overall method was determined by analysis of ten different urine samples spiked with 4 μg/ml or 60 μg/ml of 5-HNMP and 2 μg/ml or 20 μg/ml of 2-HMSI, respectively. The coefficients of variation (C.V.) were determined for the ratio between the area for 5-HNMP and 2-HMSI and their respective internal standards. The results are shown in Table 4.

The between-day precision was studied by analysing one urine sample from a NMP exposed volunteer containing 4 μg 5-HNMP/ml and 20 μg 2-HMSI/ml and one urine sample from a volunteer containing 60 μg 5-HNMP/ml and 2 μg 2-HMSI/ml seven times during a period of five months. The C.V.s for the determinations are shown in Table 4. A fairly high imprecision (21%) was obtained at 4 μg 5-HNMP/ml for the ion at *m/z* 186. Thus, the mass fragment at *m/z* 98 looks preferable.

3.7. Application

A urine sample from a subject experimentally exposed to 25 mg NMP/m^3 for 8 h was collected

Table 2
Calibration equations for 5-HNMP and 2-HMSI in urine

Compound	*m/z*	Concentration range (μg/ml)	Slope (ml/μg)	Intercept	Correlation coefficient
5-HNMP	98	0.2–60	0.012	0.0013	0.999
5-HNMP	186	0.2–60	0.015	0.0029	0.999
2-HMSI	144	0.2–25	0.039	0.0003	0.999
2-HMSI	186	0.2–25	0.059	0.0005	0.999

Table 4
Precision of the analysis of urine samples with different concentrations of 5-HNMP and 2-HMSI

Compound	Fragment *m/z*	Concentration (μg/ml)	Intra-day precision[a] (%)	Between-day precision[b] (%)
5-HNMP	98	4	4	7
5-HNMP	98	60	3	5
5-HNMP	186	4	2	21
5-HNMP	186	60	3	4
2-HMSI	144	2	8	7
2-HMSI	144	20	4	7
2-HMSI	186	2	7	6
2-HMSI	186	20	4	7

[a] Given as coefficients of variation ($n=10$).
[b] Given as coefficients of variation ($n=7$).

immediately after the end of exposure and analysed by the present method. The levels in urine were 94 μg 5-HNMP/ml and 6.7 μg 2-HMSI/ml. The Swedish occupational exposure limit is 200 mg NMP/m^3.

4. Conclusions

The proposed method is simple and rapid and shows high precision for both 5-HNMP and 2-HMSI. The detection limits are sufficiently low for determinations of 5-HNMP and 2-HMSI in urine from workers exposed to NMP within the Swedish occupational exposure limit. Thus, the method is proposed for the biological monitoring of the exposure to NMP.

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