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Quantification of Four Major Metabolites of Embryotoxic *N*-Methyl- and *N*-Ethyl-2-pyrrolidone in Human Urine by Cooled-Injection Gas Chromatography and Isotope Dilution Mass Spectrometry

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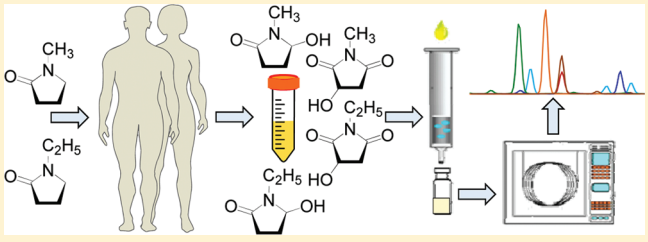
Supporting Information

ABSTRACT: *N*-Methyl- and *N*-ethyl-2-pyrollidone (NMP and NEP) are frequently used industrial solvents and were shown to be embryotoxic in animal experiments. We developed a sensitive, specific, and robust analytical method based on cooled-injection (CIS) gas chromatography and isotope dilution mass spectrometry to analyze 5-hydroxy-*N*-ethyl-2-pyrrolidone (5-HNEP) and 2-hydroxy-*N*-ethylsuccinimide (2-HESI), two newly identified presumed metabolites of NEP, and their corresponding methyl counterparts (5-HNMP, 2-HMSI) in human urine. The urine was spiked with deuterium-labeled analogues of these metabolites. The analytes were separated from urinary matrix by solid-phase extraction and silylated prior to quantification. Validation of this method was carried out by using both, spiked pooled urine samples and urine samples from 56 individuals of the general population with no known occupational exposure to NMP and NEP. Interday and intraday imprecision was better than 8% for all metabolites, while the limits of detection were between 5 and 20 μg/L depending on the analyte. The high sensitivity of the method enables us to quantify NMP and NEP metabolites at current environmental exposures by human biomonitoring.



INTRODUCTION

N-Alkyl-2-pyrrolidones are organic solvents, miscible with water and capable of dissolving nonpolar, polar, and even inorganic chemicals. For this reason, they are essential industrial solvents used worldwide in the production of polymers, coating materials, paints, pesticide formulations, cosmetics, and drugs.[1,2] The two most important compounds of this class are *N*-methyl-2-pyrrolidone (NMP) and *N*-ethyl-2-pyrrolidone (NEP). The majority of data, however, is available for NMP,[3] whereas studies related to NEP are scarce.

NMP is a high-production volume chemical and its annual world production in 2003 was estimated to be up to 150 000 t.[2] The acute toxicity of NMP is low.[4,5] However, studies in rats have shown dose-related decreases in fetal body weights after oral and inhalation exposure and at exposure levels below maternal toxicity. In addition, fetal malformations were found at maternal toxic doses.[6,7] For these reasons, NMP has recently been classified in category 1b for developmental toxicity (may cause harm to the unborn child) by the European Union thus resulting in restrictions and limitations in its use.[8] All industrial formulations and consumer products containing ≥5% NMP from now on must be labeled as embryotoxic worldwide according to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS).

Besides ingestion and inhalation, uptake of NMP can also occur by penetration through the skin.[9−11] In particular, dermal absorption is an important route of uptake at the workplace,[12,13] whereas ingestion has to be considered an additional route of uptake in the general population. Human biomonitoring can integrate exposure via all three routes and is therefore an important tool for risk assessment.[12,13] The determination of specific metabolites in urine and plasma is a well-established technique for exposure assessment of NMP at workplaces.[14−16]

NMP is successively oxidized to 5-hydroxy-*N*-methyl-2-pyrrolidone (5-HNMP), *N*-methylsuccinimide (MSI), and 2-hydroxy-*N*-methylsuccinimide (2-HMSI) in humans (Figure 1). 5-HNMP and 2-HMSI are major metabolites of NMP in urine, accounting for 44% and 20% of the administered dose.[17] Conjugation of metabolites with glutathione or sulfation was not observed.

Analytical methods for the determination of 5-HNMP or 2-HMSI in urine have been previously reported with use of gas chromatography and mass spectrometry (GC-MS),[17−20] liquid chromatography and tandem mass spectrometry (LC-MS/MS),[21,22] and gas chromatography in combination with flame

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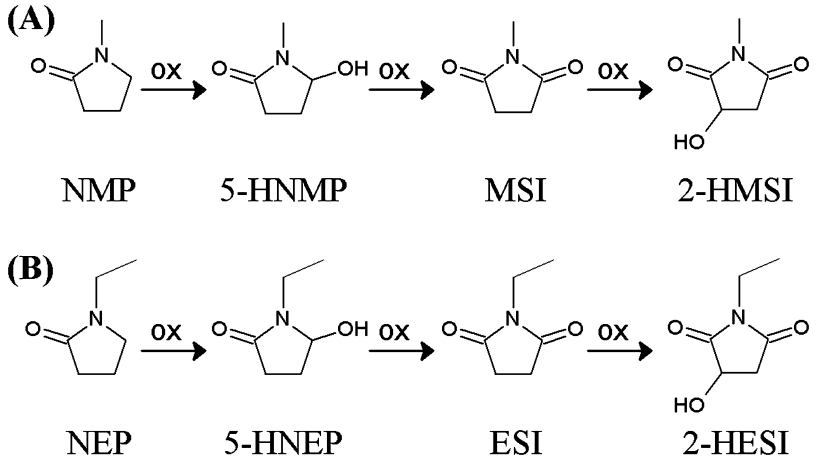

Figure 1. Oxidative metabolism of NMP according to Åkesson et al.[17] (A) and proposed metabolism of NEP based on the known metabolites of NMP (B) (ox = oxidation).

ionization detection (GC-FID).[23] Up to now, these methods have been exclusively used to study work-related exposures[14,16] or experimental exposures of volunteers in inhalation chambers.[24−26] Internal standardization and quantification were carried out by using commercially available 5-HNMP and 2-HMSI standards and their deuterium labeled analogues—5-hydroxy-*N*-methyl-2-pyrrolidone-d_4 (5-HNMP-d_4) and 2-hydroxy-*N*-methylsuccinimide-d_3 (2-HMSI-d_3)—in the case of analytical methods based on mass spectrometry. The limits of detection (LODs) were between 6 and 1000 μg/L for 5-HNMP and 30 and 1000 μg/L for 2-HMSI and therefore suitable for the quantification of the internal dose after occupational exposure to NMP.

In contrast to NMP, information on NEP is limited. The acute oral toxicity of NEP has been reported to be approximately 4-fold higher than that of NMP.[5] After oral administration, NEP has been shown to display a similar spectrum of developmental toxic and teratogenic effects in rodents, as was reported previously for NMP. The onset of the observed effects, however, was less pronounced.[27] The use of NEP is expected to gain importance due to the classification and labeling requirements of NMP in the EU. In many industrial formulations and consumer products NMP is already substituted by the nonclassified NEP due to their very similar physicochemical properties.[28]

Owing to the toxicity of NMP and NEP and their broad application ranges, it is necessary to study both workplace and low-level environmental exposures. Low levels of NMP have been detected in the environment such as municipal and industrial wastewaters and in US drinking water supplies.[1,29,30] Exposure of the general population to low environmental levels of NMP and NEP has not been studied yet mostly due to the lack of sensitive and specific analytical methods. Only the previously published method by Suzuki et al.[22] is considered sensitive enough to assess low-level exposures to NMP (LODs of 6 and 30 μg/L for 5-HNMP and 2-HMSI). This method, however, has not been used to assess exposure of NMP in the general population. In addition, no analytical method at all for the determination of NEP metabolites is available so far.

Based on the well-known metabolism of NMP in humans, we postulated the formation of 5-hydroxy-*N*-ethyl-2-pyrrolidone (5-HNEP) and 2-hydroxy-*N*-ethylsuccinimide (2-HESI) as major metabolites of NEP in humans (Figure 1). To prove this hypothesis, we first synthesized and characterized 5-HNEP and 2-HESI metabolites and their deuterated analogues (5-HNEP-d_5, 2-HESI-d_5). Then we developed and validated a sensitive and specific analytical method based on solid phase extraction of 5-HNMP, 2-HMSI, 5-HNEP, and 2-HESI from urine samples, followed by silylation of the analytes. The quantification was carried out by cooled-injection gas chromatography and isotope dilution mass spectrometry (GC-MS). Finally, we assessed environmental exposures to NMP and NEP in persons of the general population, and thus proved the practical suitability of our method in a field study.

EXPERIMENTAL SECTION

Reagents and Materials. Acetonitrile (ACN), ethyl acetate (EtOAc) and methanol (MeOH) for GC, ethanol (EtOH), and acetic acid (100%) of highest available analytical grade were purchased from VWR International (Darmstadt, Germany). *N*-(*tert*-Butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA) and pyridine were purchased from Sigma-Aldrich (Seelze, Germany). SPE cartridges containing hydroxylated polystyrene−divinylbenzene copolymer (Isolute ENV$^+$, 1 mL, 100 mg) were obtained from Biotage AB (Uppsala, Sweden). Deionized water was prepared by a Milli-Q Plus system (Millipore, Eschborn, Germany). Nitrogen for solvent evaporation during sample preparation and helium as carrier gas for GC was of 4.5 and 6.0 quality. 5-HNMP, 2-HMSI, and their deuterated analogues 5-HNMP-d_4 and 2-HMSI-d_3 were purchased from Ramidus AB (Lund, Sweden) and were ≥95% isotopic purity. Since analytical standards for 5-HNEP and 2-HESI were not commercially available, they were synthesized and characterized along with their deuterated analogues 5-HNEP-d_5 and 2-HESI-d_5. For this purpose, *N*-ethylsuccinimide (TCI Deutschland GmbH Eschborn, Germany), (±)-malic acid, and a 70% aqueous solution of ethyl amine (Sigma-Aldrich, Seelze, Germany), acetonitrile-d_3 (99.8%, Deutero GmbH, Kastellaun, Germany), and $LiAlD_4$ (99%, Fluka Analytical, Buchs, Germany) were used as starting materials.

Instrumentation for Standard Characterization. All synthesized standards were characterized by ^{1}H and ^{13}C NMR, MS, HPLC, and IR. ^{1}H NMR was performed on a Varian MERCURY-400-MR spectrometer at 400 MHz. Chemical shifts (δ) are given in ppm relative to TMS. ^{13}C NMR was performed at 100 MHz on a Varian 400-MR spectrometer, using attached proton test for multiplet detection. Multiplicities of signals are described as singlet (s), doublet (d), triplet (t),

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quartet (q), and multiplet (m). Mass spectra of the synthesized standards were obtained with electro spray ionization (ESI), using LCQ and ESI-TOF mass spectrometers (MICROTOF (focus), Bruker, Bremen, Germany). A Knauer smartline gradient HPLC system (Knauer, Berlin, Germany) including two pumps (1000), UV detector (2500), column thermostat (25 °C), and a 20 μL injection loop was used during standard preparation. A Eurospher-100 C_{18} column (5 μm, 250 × 4 mm, Knauer, Berlin, Germany) was used as the analytical column at a flow rate of 1.1 mL/min. HPLC grade water containing 0.1% trifluoroacetic acid (TFA) was used as solvent A and acetonitrile containing 0.1% TFA as solvent B. The detection of the analytes was carried out at 220 nm. Purity of the standards was controlled by thin-layer chromatography on Macherey Nagel ready-to-use plates with silica gel (G/UV254), using 10% molybdenum−phosphoric acid in ethanol (Sigma-Aldrich, Seelze, Germany) as a developer. Column chromatography was carried out on MERCK silica gel (grade 60, 0.040−0.063 mm). IR spectra (KBr) were recorded on Spectrum BXII (Perkin-Elmer, Waltham, USA).

Synthesis of Standards. *(±)-5-Hydroxy-N-ethyl-2-pyrrolidone (5-HNEP).* *N*-Ethylsuccinimide (500 mg, 3.93 mmol) was dissolved in 26 mL of anhydrous ethanol. The solution was cooled in an ice bath, and 30 mg of $NaBH_4$ was added in small portions at regular intervals (10−15 min), followed by the addition of 2 drops of 4 M HCl in dioxane after each portion of $NaBH_4$. The course of the reaction was monitored by HPLC (solvent A/B = 90/10, retention times (t_R) of the starting compound 7.6 min, product 4.0 min, side product 3.9 min). The reaction was complete in about 2 h, when 300 mg (7.93 mmol) of $NaBH_4$ and 20 drops (~1 mL) of HCl were added. The solvent was evaporated in vacuo (50−60 mbar) at 0 °C into a trap cooled with an acetone−dry ice bath, and the residue was extracted with 100 mL of cold CH_2Cl_2. The suspension was filtered through a glass filter, and the filter cake was washed with 2 × 25 mL of cold CH_2Cl_2. The combined solutions were evaporated in vacuo at 0 °C. The residue was taken up in CH_2Cl_2, transferred to a column with 75 g of SiO_2, and eluted with a CH_2Cl_2/MeOH mixture (gradient 20:1 → 10:1) to obtain 5-HNEP as a colorless oil (300 mg, yield 59%, R_f = 0.4 (CH_2Cl_2/MeOH, 15:1)). Characterization: HPLC: 0−3 min, 99% A; 3−13 min, 99−95% A; 13−20 min, 95−60% A; t_R = 12.9 min, area: 97%. ^{1}H NMR (Figure S-1, Supporting Information, CD_3OD): δ 1.17 (t, J = 7 Hz, 3 H, CH_3), 1.82 (m, 1 H, $C\underline{H}^aH^bCH(OH)$), 2.29 (m, 2 H, CH_2CO), 2.49 (m, 1 H, $CH^a\underline{H}^bCH(OH)$), 3.20 (A-part of ABX_3 system, J_{AB} = 14.4 Hz, J_{AX} = 7.2 Hz, 2 H, $C\underline{H}^aH^bN$), 3.43 ($CH^a\underline{H}^bN$), 5.22 (dd, J = 6.4 and 2.51, 1 H, CHOH) ppm. ^{13}C NMR (Figure S-2, Supporting Information, CD_3OD): δ 12.8 (CH_3), 28.6 (CH_2), 29.7 (CH_2), 35.5 (CH_2N), 83.8 (CHO), 176.5 (C═O) ppm. EI-MS (Figure S-3, Supporting Information): m/z (%) 129 (100%) $[M]^{\bullet+}$, 112 (45%) $[M - OH]^+$, 96 (30%) $[M - H_2O - CH_3]^+$, 85 (30) $[C_4H_7NO]^{\bullet+}$. ESI-MS, positive mode: m/z (%) 281 (100) $[2M + Na]^+$, 152 (58) $[M + Na]^+$; negative mode: m/z (%) 257 (100) $[2M - H]^-$, 128 (69) $[M - H]^-$. IR (KBr): ν 3337 (OH), 2978/2939 (CH), 1670 (CO). Purity was determined as >97%.

2-Hydroxy-N-ethylsuccinimide (2-HESI). (±)-Malic acid (5.36 g, 40.0 mmol) was added at 0 °C to the stirred solution of 4.5 mL of 70% aqueous ethylamine (d = 0.8, 2.5 g of $EtNH_2$, 56 mmol) in 50 mL of ethanol and the reaction mixture was stirred at RT overnight. Volatile materials were evaporated in vacuo, and the residue was coevaporated with 2 × 25 mL of toluene. Toluene (500 mL) was added and the mixture was refluxed for 24 h with a Dean−Stark trap for the azeotropic removal of water. After cooling to RT, the reaction mixture was washed with 80 mL of water and 50 mL of brine, then dried over Na_2SO_4. The solvent was evaporated in vacuo, and the residue was recrystallized from a hexane−ether mixture (5/1, v/v) as a colorless solid (4.67 g, yield 72%) and with a mp of 81.5−82.4 °C (lit.[31] mp 81−82 °C). Characterization: HPLC: 0−3 min, 99% A, 3−13 min, 99−95% A, t_R = 9.6 min, area: 100%. ^{1}H NMR (Figure S-4, Supporting Information, CD_3OD): δ 1.12 (t, J = 7.2 Hz, 3 H, CH_3), 2.44 (dd, A-part of ABX-system, J_{AB} = 18.0 Hz, J_{AX} = 4.4 Hz, 1 H, $C\underline{H}^aH^bCH_xOH$), 3.01 (B-part of ABX-system, J_{AB} = 17.9 Hz, J_{BX} = 8.3 Hz, 1 H, $CH^a\underline{H}^bCH^xOH$), 3.50 (q, J = 7.2 Hz, 2 H, CH_2N), 4.53 (dd, J_{AX} = 4.4 Hz, J_{BX} = 8.3 Hz,1 H, CH^xOH) ppm. ^{13}C NMR (Figures S-5 and S-6, Supporting Information, CD_3OD): δ 13.4 (CH_3), 34.7 (CH_2N), 39.1 (CH_2), 68.2 (CHO), 176.8 (C═O), 180.0 (C═O) ppm. EI-MS (Figure S-7, Supporting Information): m/z (%) 143 (42%) $[M]^{\bullet+}$, 115 (76%) $[M - C_2H_4]^{\bullet+}$, 72 (100) $[C_3H_6NO]^+$. ESI-MS positive mode: m/z (%) 166 (100) $[M + Na]^+$; negative mode: m/z (%) 285 (92) $[2M - H]^-$, 142 (100) $[M - H]^-$. IR (KBr): ν 3373 (OH), 2992/2977/2961/2940 (CH), 1685 (CO). Purity was determined as >99%.

Ethylamine-d_5 ($C_2D_5NH_2$). Ethylamine-d_5 ($C_2D_5NH_2$) was prepared from acetonitrile-d_3 and $LiAlD_4$ according to Meese[32] with the following modification. After quenching the reaction with water, volatile amine, ether, and water were slowly distilled under gradually diminishing pressure (p = 500−50 mbar) into a receiving flask, cooled with a mixture of dry ice and acetone. In contrast to the original report, however, the flask did not contain HCl. Cold ethanol was carefully added to the distillate, until it became homogeneous (at 0 °C). The yield was considered to be 80%, and the intermediate was not isolated. Half of this solution was used for the preparation of *N*-ethylsuccinimide-d_5 and the other half for the synthesis of 2-HESI-d_5.

(±)-N-Ethylsuccinimide-d_5. Succinic acid anhydride (4.0 g, 40 mmol) was added at 0 °C to a stirred solution of 2.8 g (56 mmol) of ethylamine-d_5 in an ether/ethanol/water mixture (10/2/1, v/v/v), and the reaction mixture was stirred at RT overnight. Volatile materials were evaporated in vacuo and the residue was coevaporated with 2 × 25 mL of toluene. Then, 200 mL of toluene was added and the mixture was refluxed for 24 h with a Dean−Stark trap for azeotropic removal of water. After cooling to RT, the reaction mixture was washed with 50 mL of water and 25 mL of brine and dried over Na_2SO_4. The solvent was evaporated in vacuo, and the residue was filtered through 25 g of SiO_2 with a hexane/ethyl acetate mixture (1:1, v/v) as an eluent. Evaporation of the solvents in vacuo yielded *N*-ethylsuccinimide-d_5 as oil (3.9 g, yield 74%). Characterization: HPLC: solvents A/B = 90/10, t_R = 7.6 min, ^{1}H NMR ($CDCl_3$): δ 2.57 (s, CH_2).

(±)-5-Hydroxy-N-ethyl-2-pyrrolidone-d_5 (5-HNEP-d_5). 5-HNEP-d_5 was prepared from *N*-ethylsuccinimide-d_5 and $NaBH_4$ in anhydrous ethanol with 4 M HCl, as described above for 5-HNEP. Characterization: HPLC: solvents A/B = 90/10, t_R = 4.0 min, area 100%. ^{1}H NMR (Figure S-8, Supporting Information, CD_3OD): δ 1.82 (m, 1 H, $C\underline{H}^aH^bCH$-(OH)), 2.3 (mc, 2 H, CH_2CO), 2.49 (m, 1 H, $CH^a\underline{H}^bCH$-(OH)), 5.22 (dd, J = 2.6 and 6.4 Hz 1 H, CHOH) ppm. ^{13}C NMR (Figure S-9, Supporting Information, CD_3OD): δ 29.0 (CH_2), 30.1 (CH_2), 84.2 (CHO), 176.9 (C═O) ppm. EI-MS

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(Figure S-3, Supporting Information): m/z (%) 117 (100%) $[M - OH]^+$, 85 (17) $[C_4H_7NO]^{\bullet+}$. ESI-MS: positive mode: m/z (%) 291 (100) $[2M + Na]^+$, 157 (65) $[M + Na]^+$; negative mode: m/z (%) 267 (100) $[2M - H]^-$, 133 (79) $[M - H]^-$. IR (KBr): ν 3337 (OH), 2952 (CH), 2233 (CD), 1666 (CO). Purity was determined >98%.

(±)-2-Hydroxy-N-ethylsuccinimide-d_5 (2-HESI-d_5). 2-HESI-d_5 was prepared from 5.36 g (40.0 mmol) of (±)-malic acid and the solution of 2.8 g (56 mmol) of ethylamine-d_5 in an ether/ethanol/water mixture according to the method described above for 2-HESI (mp 82.3−83.4 °C). Characterization: HPLC: 0−3 min, 99% A, 3−13 min, 99−95% A, t_R = 9.6 min, area: 100%. ^{1}H NMR (Figure S-10, Supporting Information, CD_3OD): δ 2.46 (dd, A-part of ABX-system, J_{AB} = 18.0 Hz, J_{AX} = 4.4 Hz, 1 H, $CH^aH^bCH_xOH$), 3.01 (dd, B-part of ABX-system, J_{AB} = 17.9 Hz, J_{BX} = 8.3 Hz, 1 H, $CH^aH^bCH^xOH$), 4.53 (dd, J_{AX} = 4.4 Hz, J_{BX} = 8.3 Hz,1 H, CH^xOH) ppm. ^{13}C NMR (Figure S-11, Supporting Information, CD_3OD): δ 39.1 (CH_2), 68.3 (CHO), 176.9 (C═O), 180.0 (C═O) ppm. EI-MS (Figure S-7, Supporting Information): m/z (%) 148 (48%) $[M]^{\bullet+}$, 120 (76%) $[M - C_2H_4]^{\bullet+}$, 77 (100) $[C3D5HNO]^+$. ESI-MS positive mode: m/z (%) 171 (100) $[M + Na]^+$; negative mode: m/z (%) 295 (89) $[2M - H]^-$, 147 (100) $[M - H]^-$. IR (KBr): ν 3373 (OH), 2664/2361/2237 (CD), 1685 (CO). Purity was determined as >99%.

Preparation of Standards and Quality Control Material. Individual stock solutions (1 g/L) of 5-HNMP, 2-HMSI, 5-HNEP, and 2-HESI were prepared by weighing 10 mg of each compound in a 10-mL-volumetric flask and dissolving with acetonitrile. One milliliter of each stock solution was combined in a 10-mL-volumetric flask and diluted with water to the mark yielding a working solution 1 of 100 mg/L for each standard. Working solution 1 was diluted with water to a concentration of 50 mg/L (working solution 2) and 5 mg/L (working solution 3). Six calibration standards (0.05 to 10 mg/L) were prepared by diluting the working solutions described earlier in pooled urine from multiple donors. The pooled urine was frozen, thawed, and filtrated before use. An unspiked urine sample from the same pooled urine was used as the urine blank sample. The calibration standards and the urine blank sample were aliquoted in 1 mL portions that could be stored at −20 °C up to 1 year. Within each analytical run a full set of calibration standards including a urine blank sample and a reagent blank (water) was analyzed.

Individual stock solutions (1 g/L) of the deuterium-labeled standards (5-HNMP-d_4, 2-HMSI-d_3, 5-HNEP-d_5, and 2-HESI-d_5, Figure 2) were prepared in acetonitrile as described above for the stock solutions of the nonlabeled standards. An internal standard IS working solution (5 mg/L of each standard) was prepared in water by combining 50 μL of each stock solution in a 10-mL-volumetric flask and diluting with water to the 10 mL mark.

Certified quality control material is not available for metabolites of NMP and NEP. Therefore we prepared in house quality control (QC) material at two concentrations (0.2 and 2.0 mg/L). For this purpose, 0.2 and 2.0 mL of working solution 1 (100 mg/L) each were diluted in a 100-mL-volumetric flask, using pooled urine from multiple donors. Again, the pooled urine was frozen, thawed, and filtrated prior use. QC material of both concentrations was analyzed within each analytical run. All solutions were stored at −20 °C in the dark, making them stable up to 1 year.

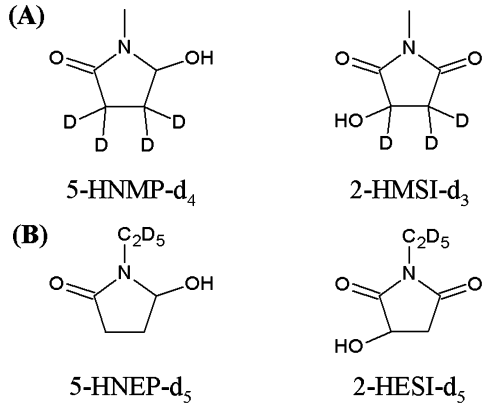

Figure 2. Deuterated internal standards of 5-HNMP, 2-HMSI, 5-HNEP, and 2-HESI used for sample preparation and isotope dilution mass spectrometry.

Sample Preparation. After equilibration to RT the urine samples were homogenized and an aliquot of 600 μL was transferred to a 2-mL vial. Then, 200 μL of the IS working solution and 200 μL of 1% HAc in water were added. The solution was homogenized on a vortex mixer. The ENV$^+$ solid phase extraction cartridges were placed onto a Vacmaster SPE station (Biotage AB, Uppsala) and preconditioned with 2 × 500 μL of EtOAc/EtOH (4/1, v/v), 2 × 500 μL of MeOH, and 4 × 500 μL of 1% HAc in water. The preconditioned ENV$^+$ cartridges were loaded with 1 mL of the diluted urine sample. The cartridges were washed with 250 μL of 1% HAc in water and 750 μL of water. Afterward the extraction cartridges were dried in vacuo. The analytes were eluted with 1.75 mL of EtOAc/EtOH (4:1, v/v) into 2-mL vials. The eluate was evaporated to dryness in a stream of nitrogen. The residue was reconditioned in 200 μL of acetonitrile and again evaporated to dryness in order to remove trace residues of water prior to derivatization. The residue was then dissolved in 30 μL of pyridine and 30 μL of MTBSTFA was added. Derivatization was performed at 110 °C within 60 min. After being cooled to RT the sample was diluted with 50 μL of EtOAc and transferred to a microinsert. Cooled injection GC-MS analysis was performed by injection of 2 μL of each sample. If necessary, the final extracts could be stored up to 2 weeks at −20 °C until analysis.

Cooled Injection Gas Chromatography/Isotope Dilution Mass Spectrometry. Cooled injection GC-MS analysis was performed on an Agilent 7890 GC system with a CTC CombiPAL autosampler (Agilent Technologies, Waldbronn, Germany) and a KAS 4 cold injection system (Gerstel GmbH & CoKG, Mühlheim a.d. Ruhr, Germany), whereas mass spectrometric detection was performed on an Agilent 5975 mass spectrometer. A DB-35MS capillary column (60 m, 0.25 mm i.d., 0.25 μm film thickness, Agilent Technologies, Waldbronn, Germany) was used for chromatographic separation. Helium 6.0 (1 mL/min) was used as the carrier gas. The KAS injector was operated in solvent vent mode (split 1:50) for 0.5 min after injection and splitless between 0.5 and 2.3 min. The injector was programmed from 40 °C (0.5 min) to 240 °C (120 deg/min; 0 min) and to 260 °C (600 deg/min; 10 min). The column temperature was 50 °C (4 min), raised to 90 °C (25 deg/min; 1 min), then to 190 °C (10 deg/min; 3 min) and to a final temperature of 280 °C (30 deg/min; 10 min). The injector was cooled to the initial temperature with air at the end of the oven program. The temperature of the transfer line was

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280 °C, the ion source was set to 230 °C, and the quadrupole was set to 150 °C. Electron ionization was carried out at 70 eV. Selective and sensitive mass traces were used for quantitation (quantifier) and confirmation (qualifier) of the target analytes in single ion monitoring mode (Table 1). The ratio of quantifier and qualifier in the samples had to be consistent with the ratio determined from standard solutions. Variations of ≤30% of the ratio quantifier to qualifier were accepted.

Table 1. Retention Times, Quantifiers and Qualifiers, and Their Ratios of the Four Metabolites of NMP and NEP (t_R, retention time)

analyte	t_R [min]	quantifier [m/z]	qualifier [m/z]	ratio
5-HNMP	21.45	172	98	53
5-HNMP-d_4	21.41	176	102	
2-HMSI	21.27	186	144	39
2-HMSI-d_3	21.23	189	147	
5-HNEP	21.69	186	112	61
5-HNEP-d_5	21.65	191		
2-HESI	21.40	200	158	31
2-HESI-d_5	21.34	205	163	

Method Validation. Method validation involved the determination of the limit of detection (LOD), recovery, intraday and interday imprecision, and matrix effects. The LOD was defined as a signal-to-noise ratio of 3. For calibration, the ratio of the peak area of each standard vs internal standard was plotted against the respective concentration. The slope of the calibration function was used for quantification because no urine without traces of NMP and NEP metabolites was available for calibration. Water was used as reagent blank during method validation. Intraday and interday imprecision was determined in pooled urine samples at two concentrations (0.2 and 2.0 mg/L). Relative standard deviations (RSD) were determined for intraday and interday variations, based on a set of 8 and 12 measurements. Recovery also was tested at 0.2 and 2.0 mg/L. For this purpose, eight different urine samples with a wide range of creatinine concentrations between 0.26 and 3.11 g/L were selected to consider varying urine compositions when determining the recovery of the analytes rather than using pooled urine samples with a particular creatinine level.

The eight urine samples were analyzed unspiked to determine the background level of NMP and NEP metabolites and spiked with 0.2 and 2.0 mg/L. The analyte concentrations in the spiked and unspiked urine samples were calculated from the ratio of the area of the analyte and internal standard based on an external calibration curve. Finally, the relative recovery was calculated by subtraction of the base levels derived in the unspiked samples from the corresponding concentrations determined in the spiked samples.

Biological Samples. Spot urine samples from 56 individuals (31 females, 25 males) with a median age of 40 years (range 18−64) not known to be exposed to NMP or NEP were collected to study the urinary concentrations of 5-HNMP, 2-HMSI, 5-HNEP, and 2-HESI in the general population and to prove that our method was sensitive and specific enough to determine background levels of NMP and NEP metabolites in the general population. All samples were stored at −20 °C until analysis was carried out. An approval of the ethics committee of the Ruhr Universität Bochum (No. 3867-10) is available for the collection of the urine samples.

RESULTS AND DISCUSSION

No data are currently available on the metabolism of NEP, making it difficult to identify specific metabolites of NEP that can be used for method development. Thus, we postulated the formation of 5-HNEP and 2-HESI based on the previously reported formation of 5-HNMP and 2-HMSI in humans after exposure to NMP.[17] 5-HNEP and 2-HESI, however, were not commercially available. Therefore, in-house synthesis of 5-HNEP and 2-HESI along with their deuterated analogues was necessary.

Standard Synthesis. Synthesis of 5-HNEP and other *N*-alkyl derivatives has been previously reported in the course of a multistep sequence via a spontaneous cyclization of the theanine Strecker aldehyde and starting from *cis*-4-octen-1,8-dioic acid.[33,34] The most straightforward synthetic approach to 5-HNEP, however, is based on the transformation of succinic acid anhydride into *N*-ethylsuccinimide in the presence of ethylamine, followed by a partial reduction with $NaBH_4$. The reduction of cyclic imides with $NaBH_4$ in the presence of HCl was shown to provide 5-hydroxy-*N*-alkyl-2-pyrrolidones.[35] The exact amount of HCl, however, was not specified and this reaction has not been described for *N*-ethylsuccinimide and $NaBH_4$ so far. We found that the outcome of this reaction strongly depends on the amount of added HCl as well as on the temperature during the reduction and the workup procedures. The best results were obtained when 2 mol of $NaBH_4$ and 1 mol of HCl were added sequentially in small portions to 1 mol of imide in ethanol at 0 °C. An excess of HCl caused "overreduction" or led to instability of the product. The removal of the solvent and extraction of 5-HNEP with dichloromethane also had to be carried out at 0 °C. The synthesis of 2-HESI was achieved by the cyclization of malic acid with ethylamine.

In addition, the synthesis of stable isotope-labeled standards of 5-HNEP and 2-HESI was necessary for internal standardization by isotope dilution. Commercially available standards of 5-HNMP-d_4 and 2-HMSI-d_3 are labeled in the ring. Therefore, we first tried to introduce the deuterium labels into the pyrrole ring to obtain the corresponding ethyl derivatives 5-HNEP-d_4 and 2-HESI-d_3. For this purpose, succinic anhydride-d_4 and malic acid-d_3 were used as starting materials. Heating these compounds with ethylamine in toluene indeed provided the required imides. The deuterium−hydrogen exchange that was observed under these conditions, however, made this approach inappropriate. The $CD_2CONC_2H_5$-groups are prone to protonation at the oxygen atom (with participation of the COOH-residue), and the protonated tertiary amide residue may easily lose a deuterium atom at the α-position to the carbonyl group. Therefore, we have chosen to introduce the deuterium labels into the ethyl moiety of 5-HNEP and 2-HESI. Synthesis of 5-HNEP-d_5 and 2-HESI-d_5 followed the same route as described above for the nonlabeled compounds except that ethylamine-d_5 was not commercially available. For this reason, ethylamine-d_5 was prepared from acetonitrile-d_3 and $LiAlD_4$ and immediately used to synthesize 2-HESI-d_5 and *N*-ethylsuccinimide-d_5. *N*-Ethylsuccinimide-d_5 was smoothly reduced to the required 5-HNEP-d_5 at high yields. No loss of deuterium labels was observed under these conditions. Purity of all standard substances (5-HNEP, 2-HESI, 5-HNEP-d_5, and 5-HESI-d_5) was >97%.

Method Development. Once 5-HNEP, 2-HESI, and their deuterium-labeled analogues were synthesized, purified, and

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characterized, we were able to develop a highly sensitive and specific analytical method. We included 5-HNMP and 2-HMSI and their deuterated internal standards to quantify exposure to NMP and NEP in one analytical run. Methods of Jönsson et al.[18] and Bader et al.[20] were taken into account during method development. We used GC-MS since enhanced separation efficiency and the detection of specific mass fragments were required due to the structural similarities of the metabolites of NMP and NEP.

The high polarity of NMP and NEP metabolites and the complexity of the urinary matrix presented a challenge for method development. Efficient separation of the metabolites from urine was achieved by solid-phase extraction. The SPE material (ENV+) chosen, a cross-linked styrene−divinylbenzene copolymer, combines polar and nonpolar retention mechanisms and was previously shown to be highly suitable for extraction of polar metabolites from a urinary matrix.[36] To ensure a protonation of the hydroxyl-functions of the metabolites, we slightly acidified the urine samples prior to SPE. The best results for the elution step were achieved by using a mixture of ethyl acetate and ethanol. Ethanol, however, has to be completely removed prior to the derivatization step since even trace amounts will significantly reduce the derivatization yield. Derivatization of the hydroxyl-groups was performed by silylation because the extracts can be directly injected into the GC-MS system without any further cleanup steps thus speeding up sample preparation and minimizing losses during sample cleanup. Derivatization with MTBSTFA was superior to that with BSTFA (*N*,*O*-bis(trimethylsilyl)-trifluoroacetamide) in view of better chromatographic separation of the structurally very similar metabolites. In addition, fragmentation was more specific for MTBSTFA derivates compared to those after BSTFA derivatization, since the loss of the methyl moiety of the BSTFA derivates was identical to the mass difference between NMP and NEP metabolites. Pyridine serves as a solvent of the residue that remained after evaporation of the sample to dryness and as a catalyst in the silylation reaction. The use of pyridine resulted in reduced amounts of MTBSTFA, and therefore decreased contamination of the analytical system, reducing instrument maintenance when running a large number of samples.

Cold-injection was chosen to increase the sample volume for GC analysis in order to improve LODs. At the same time solvent vent mode was used to compensate for the increased amounts of the injected solvent and MTBSTFA, in order to protect the analytical column. Suitable mass traces for quantitation and confirmation of the analytes were identified in full scan mode (m/z 50−300) by injection of the derivatized standards in ethyl acetate during method development. The fragments with the highest abundance were the dimethylsilyl ether fragments of the pyrrolidones and succinimides, which were chosen for quantitation (quantifiers) later on. The same fragments were detected for the deuterium-labeled internal standards (Table 1). All analytes were well separated from one another despite their structural similarity (Figure 3). The stereoisomers of the analytes were not separated from each other.

Method Validation. Excellent LODs of 20 (5-HNMP), 5 (2-HMSI), 15 (5-HNEP), and 5 μg/L (2-HESI) were achieved. Three-fold LODs were considered as limits of quantification. Previously published methods efficiently analyze NMP metabolites alone and reported 10−100-fold higher LODs[18,20−23,37] with the exception of the method by Suzuki et al.[22] with LODs in a similar range for 5-HNMP and 2-HMSI. The calibration curve was linear from 0.05 to 10 mg/L with coefficients of correlation of 0.998 or higher for all analytes, thus covering a broad range of potential environmental and occupational exposures. The slopes of the calibration curves in water and urine samples were similar, indicating that there is no significant matrix effect. Nevertheless, calibration was carried out in urine rather than water to increase long-term stability of the calibration standards during storage, based on the buffering capacity of urine. The formation of artifacts from the use of deuterium-labeled internal standard solutions during sample preparation and measurement was excluded since none of the reagent blanks spiked with internal standards contained any traces of unlabeled NMP and NEP metabolites. The reproducibility of the method presented as relative standard deviations of the measured concentrations in spiked urine samples was excellent and <8% for all analytes and at all spiked levels (Table 2).

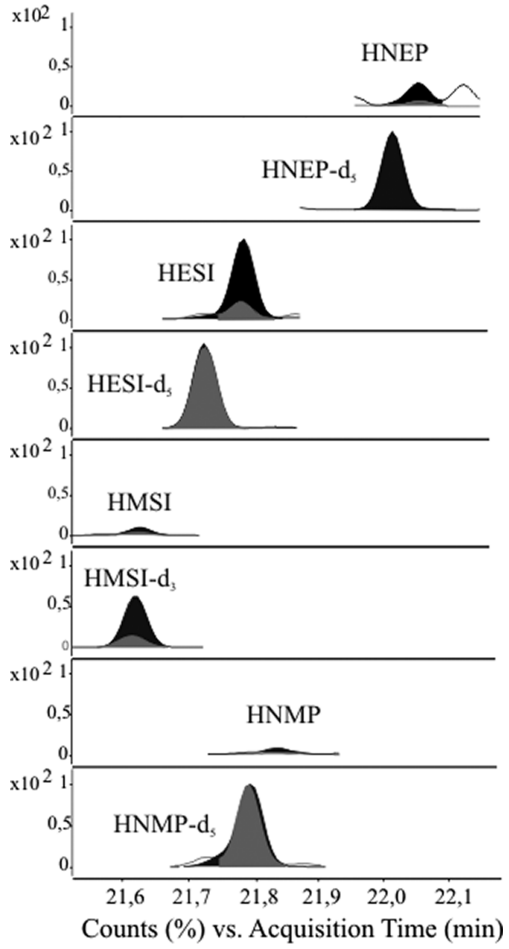

Figure 3. Chromatogram of a native urine sample from a person representing the general population not occupationally exposed to NMP and NEP (quantifier and qualifier are in black and gray). The concentrations of 5-HNMP, 2-HMSI, 5-HNEP, and 2-HESI are 129, 87, 143, and 235 μg/L, respectively.

Intraday imprecision (n = 8) at 0.2 and 2.0 mg/L was 2.1−4.9% and 1.5−4.1% depending on the analyte. Interday imprecision (n = 12) at the same concentrations was similar and determined to be 4.4−7.6% and 2.3−6.4%.

Since urine is an extremely varying and complex matrix, it is necessary to determine the robustness of the method in

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Table 2. Validation Data for the Determination of NMP and NEP Metabolites[a]

		mean rel recovery (n = 8)		range rel recovery (%)		intraday imprecision (%) (n = 8)		interday imprecision (%) (n = 12)	
analyte	LOD (μg/L)	0.2 μg/L	2.0 μg/L	0.2 μg/L	2.0 μg/L	0.2 μg/L	2.0 μg/L	0.2 μg/L	2.0 μg/L
5-HNMP	20.0	91	97	83−112	92−107	4.9	4.1	6.1	6.4
2-HMSI	5.0	100	99	97−101	95−101	2.3	1.9	7.6	4.9
5-HNEP	15.0	96	98	92−106	95−101	2.1	1.5	6.2	5.1
2-HESI	5.0	104	104	98−113	100−109	2.8	2.0	4.4	2.3

[a]Intraday and interday imprecision is presented as relative standard deviation of the spiked concentrations.

different samples. Urinary creatinine can be used as an indicator for dilution and different matrix load of urine samples. For this purpose, eight different urine samples of nonexposed persons with creatinine concentrations from 0.26 to 3.10 g/L were selected to cover the complete range of common urines as recommended by the World Health Organization.[38] The urine samples were analyzed unspiked and spiked at two concentration levels (0.2 and 2.0 mg/L) to determine the relative recovery of the method. Metabolite levels of 44−277 μg/L (5-HNMP), 12−92 μg/L (2-HMSI), <LOD−198 μg/L (5-HNEP), and <LOD−288 μg/L (2-HESI) were quantified in the unspiked urine samples of the recovery experiments. The mean relative recoveries for all analytes were between 91% and 104% (range: 83−112%) at 0.2 mg/L and 97−104% (range: 92−109%) at 2.0 mg/L, thus proving the high robustness of the analytical method even for extremely varying urine samples.

Method Application. We used our newly developed method to verify whether or not 5-HNEP and 2-HESI are human metabolites of NEP and finally assessed environmental exposures to both NMP and NEP in persons of the general population with no known occupational exposure. We were able to show that 5-HNEP and 2-HESI are human metabolites of NEP thus proving our initial hypothesis that NEP is metabolized in analogy to the NMP in humans. In addition, we show for the first time that parts of the general population are exposed to both NMP and NEP (Figure 3). NMP metabolites were quantified in >96% of urine samples of the general population, whereas NEP metabolites were found in >32% of the samples (Table 3). The high incidence of NMP positive

Table 3. Results of the Biomonitoring Study in 56 Persons from the General Population with No Known Occupational Exposure

analyte	>LOD (%)	median [μg/L]	95 percentile [μg/L]	maximum [μg/L]
5-HNMP	96.4	69.5	337.0	620.0
2-HMSI	98.2	63.5	200.4	256.2
5-HNEP	32.1	<15.0	642.6	769.3
2-HESI	46.4	<5.0	238.4	310.8

samples in the general population reflects the ubiquitousness of NMP in our environment. The lower prevalence of NEP metabolites in urine may reflect the currently less frequent use of NEP. Nevertheless, our results already indicate an increased use of NEP as a substitute of NMP. This interpretation may also be supported by the fact that higher median levels of NMP metabolites (5-HNMP: 70 μg/L; 2-HMSI: 64 μg/L) were found in urine compared to NEP metabolites (5-HNEP and 2-HESI: <LOD), although up to now, no information is available on kinetics and the quantitative fractions of 5-HNEP and 2-HESI in human NEP metabolism. Nevertheless, the observed maximum levels and 95th percentiles of NEP metabolites suggest that individuals within the general population are presently exposed to NEP at levels comparable to NMP.

CONCLUSION

Our newly developed method provides for the first time a tool for the simultaneous and specific analysis of major metabolites of the two most frequently used *N*-alkyl-2-pyrrolidones, NMP and NEP. This method was demonstrated to be highly robust and selective, and therefore applicable for routine analysis. In addition, the high sensitivity allows for the quantification of NMP metabolites and presumed NEP metabolites in the low microgram per liter range, making the method suitable for environmental exposure assessment with a biomonitoring approach.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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