Journal of Chromatography B, 877 (2009) 3743–3747

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Short communication

Direct determination of *N*-methyl-2-pyrrolidone metabolites in urine by HPLC-electrospray ionization-MS/MS using deuterium-labeled compounds as internal standard

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ARTICLE INFO

Article history:
Received 17 June 2009
Accepted 25 August 2009
Available online 28 August 2009

Keywords:
N-Methyl-2-pyrrolidone
5-Hydoxy-*N*-methyl-2-pyrrolidone
2-Hydroxy-*N*-methylsuccinimide
Electrospray ionization-MS/MS
Deuterium-labeled compound
Biological monitoring

ABSTRACT

N-Methyl-2-pyrrolidone (NMP) has been used in many industries and biological monitoring of NMP exposure is preferred to atmospheric monitoring in occupational health. We developed an analytical method that did not include solid phase extraction (SPE) but utilized deuterium-labeled compounds as internal standard for high-performance liquid chromatography-electrospray ionization-mass spectrometry using a C30 column. Urinary concentrations of NMP and its known metabolites 5-hydoxy-*N*-methyl-2-pyrrolidone (5-HNMP), *N*-methyl-succinimide (MSI), and 2-hydroxy-*N*-methylsuccinimide (2-HMSI) were determined in a single run. The method provided baseline separation of these compounds. Their limits of detection in 10-fold diluted urine were 0.0001, 0.006, 0.008, and 0.03 mg/L, respectively. Linear calibration covered a biological exposure index (BEI) for urinary concentration. The within-run and total precisions (CV, %) were 5.6% and 9.2% for NMP, 3.4% and 4.2% for 5-HNMP, 3.7% and 6.0% for MSI, and 6.5% and 6.9% for 2-HMSI. The method was evaluated using international external quality assessment samples, and urine samples from workers exposed to NMP in an occupational area.

1. Introduction

N-Methyl-2-pyrrolidone (NMP) has been used in many industries as an organic solvent for the dissolution of various resins [1,2]. Experiments show that NMP is absorbed by inhalation and ingestion [3,4]. Vaporous NMP is rapidly absorbed through the skin [5,6] and dermal absorption plays an important role [7,8]. Therefore, for exposure assessment, biological monitoring of individual workers is preferable to atmospheric monitoring in workplace environments.

In the human body, NMP is metabolized successively to 5-hydroxy-NMP (5-HNMP), *N*-methylsuccinimide (MSI), and 2-hydroxy-MSI (2-HMSI), and these compounds are excreted in urine. Recently, 2-pyrrolidone was also reported as a metabolite of NMP in human urine [9]. As major metabolites, 5-HNMP and 2-HMSI are present in urine in high concentrations [4]. In 2007, the American Conference of Governmental Industrial Hygienists (ACGIH) proposed 100 mg/L of 5-HNMP in urine as a biological exposure index (BEI) for NMP [10]. The Deutsche Forschungsgemeinschaft (DFG) proposed 150 mg/L of 5-HNMP in urine as a biological tolerance value (BAT; biologische arbeitsstoff-toleranzwerte) [11]. 2-HMSI is the second major metabolite present in the same magnitude of concentration as 5-HNMP, but has a longer biological half-life, which is favorable for its use as a biomarker for monitoring exposure [12].

Analytical methods for NMP and its metabolites have been developed using solid phase extraction (SPE) as an essential sample preparation step [3,13,14]. Because of its thermolability, 5-HNMP requires trimethylsilyl derivatization of its OH group before GC measurement [15,16]. We previously reported a GC method using SPE and a flame thermionic detector (SPE-GC/FTD) without the derivatization process, but excluded the determination of 5-HNMP because of its thermolability [17]. HPLC–MS/MS methods incorporating electrospray ionization (ESI) or atmospheric pressure ionization (APCI) have been developed for the analysis of 5-HNMP and 2-HMSI in urine and plasma, though these methods were combined with sample preparation using SPE [9,14].

However, from our experience with SPE using poly(divinylbenzene/hydroxymethacrylate), the recovery of an analyte of interest was severely limited by the variations of the operations, and all of these procedures were time-consuming [17]. Therefore,

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doi:10.1016/j.jchromb.2009.08.036

deuterium-labeled compounds have been used for urine sample preparation with SPE in order to correct for recovery variations. In addition, the metabolites of interest are not conjugated with glucoronic acid or sulfate [5], which encourages us to omit SPE use.

This work aimed to develop an HPLC-ESI-MS/MS method that excluded using SPE, but included the use of deuterium-labeled compounds to directly correct the remaining matrix effect for ESI detection. The method was evaluated with international external quality assessment samples for the determination of 2-HMSI and 5-HNMP in urine and tested using urine samples obtained from workers exposed to NMP in an occupational area.

2. Materials and methods

2.1. Chemicals

NMP was purchased from Wako Pure Chemicals (Osaka, Japan), 5-HNMP and 2-HMSI from Synthelec (Lund, Sweden), and MSI from Avocado Research Chemicals (Heysham, UK). Each deuterium-labeled compound (2-HMSI-d_3, 5-HNMP-d_4, and MSI-d_4,) was synthesized by Ramidus AB (Lund, Sweden). NMP-d_9 was obtained from Cambridge Isotope Laboratories (Andover, MA, USA). HPLC-grade acetonitrile and formic acid were purchased from Wako Pure Chemicals. Tap water was purified through a Milli-Q Element A10 (Millipore Japan, Tokyo, Japan) and used as pure water.

2.2. Sample preparation

Calibration standards were prepared in both water and 10-fold diluted urine (free from the compounds of interest) from 3 volunteers. These were spiked with NMP, 5-HNMP, MSI, and 2-HMSI at 0.1, 0.5, 1, 5, 10, and 20 mg/L, and also with NMP-d_9, 5-HNMP-d_4, MSI-d_4, and 2-HMSI-d_3 at 1 mg/L as internal standards. All analytical samples were prepared by mixing 100 μL of urine, 100 μL of a 10 mg/L mixture of NMP-d_9, 5-HNMP-d_4, MSI-d_4, and 2-HMSI-d_3, and 800 μL of pure water as 1 mL of final volume in a vial. These solutions were stable during storage at −20 °C for 3 months.

2.3. HPLC-ESI-MS/MS measurement

A highly hydrophobic C30 stationary column (Develosil C30-UG-5; 250 mm long × 2.0 mm i.d., 5 μm particle size; Nomura Chemical, Seto, Japan) and a guard column (10 mm × 1.5 mm i.d.) were used. The mobile phase was a linear gradient between solvent A (water containing 0.1% formic acid) and solvent B (acetonitrile containing 0.1% formic acid), and programmed for solvent B as follows: 0–9 min, 0 → 30%; 9–9.5 min, 30 → 100%; 9.5–10.5 min, held at 100%; 10.5–11 min, 100 → 0%; and 11–25 min, 0% for the equilibration. Thus, a measurement cycle took 25 min. The flow rate was 0.2 mL/min, column temperature was 40 °C, and sample injection volume was set to 5 μL. An HPLC system (Alliance 2695; Waters, MA, USA) was used. Detection used a Quattro micro API tandem quadrupole mass spectrometer (Waters) with ESI-positive ion mode by setting the capillary voltage to 5.0 kV, ion source temperature to 120 °C, desolvation nitrogen gas temperature to 450 °C, desolvation gas flow to 700 L/h, and cone gas flow to 50 L/h. The divert valve was set to introduce only the HPLC effluent to the mass analyzer from 5 to 13 min. Collision-induced dissociation (CID) was performed with argon gas. Multiple reaction monitoring (MRM) optimized by the infusion of each aqueous standard solution was applied to a fragment combination of each compound, as listed in Table 1.

3. Results and discussion

3.1. Chromatographic conditions and their influence on ESI efficiency

Co-eluting compounds very readily reduce the ionization efficiency of ESI and result in a reduced sensitivity, known as *Ion Suppression* [18]. Therefore, an appropriate sample preparation and/or an improved chromatographic separation are required [18–20]. The inorganic ions of urine constituents and highly hydrophilic organic compounds in urine emerge in the solvent front of reversed-phase (RP) chromatography. Among the metabolites of interest, 2-HMSI, which elutes earlier than other compounds, is significantly influenced by these interferences. Thus, in this work, a pre-cut method with valve switching was incorporated in order to waste the column effluent before the emergence of 2-HMSI.

Fig. 1(A) shows baseline separations of a urine sample from German External Quality Assessment Scheme (G-EQUAS), which was diluted 10-fold and spiked with the deuterium-labeled compounds at 1 mg/L. The separation in Fig. 1(B) was performed with a C18 column (Genesis C18; 50 mm × 2.1 mm i.d., 4 μm particle size; Grace Davison Discovery Sciences, IL, USA) following Carnerup et al. [14]. There is a peak adjacent to 2-HMSI and 2-HMSI-d_3 in the C18 column separation, which is not seen in the C30 column separation. The common noisy baselines for NMP are due to the expansion at very low concentrations.

The C30 column stationary phase used in this work, 5 times longer than the C18 column, had a highly hydrophobic stationary phase [21], which retarded the elution of hydrophobic urinary constituents. Without the pre-cut, the total UV detector trace (not shown) reported large peaks starting at 3 min, which is close to the void volume (3 min = 0.6 mL of column void volume/0.2 mL/min of flow rate), as is common for urine samples using RP separations.

Next, the urine samples from 10 individuals were examined using the present method. The analytical sample was prepared from 100 μL blank urine samples obtained from 10 volunteers who were not exposed to NMP and were confirmed not to have 2-HMSI, 5-HNMP, MSI, and NMP in their urine. These were mixed with 100 μL of a 10 mg/L aqueous mixture of the standards, 100 μL of the deuterium-labeled compounds at the same concentrations, and 700 μL of pure water. Thus, a sample solution contained (as final concentrations) 1 mg/L of each analyte and its internal standard, along with a 10-fold dilution of the urine sample. The ion suppression, which is expressed as peak area ratio, similar to the recovery (i.e., between spiked urine sample and aqueous standard solution),

Table 1
Parameters of multiple reaction monitoring (MRM).

Compounds	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)	Cone voltage (V)	Collision energy (eV)	Acquisition period (min)
2-HMSI	130.0	57.8	20	20	5.5–7.5
2-HMSI-d_3	133.0	60.8	20	22	5.5–7.5
5-HNMP	116.0	84.8	20	14	6.5–8.5
5-HNMP-d_4	120.0	88.9	20	14	6.5–8.5
MSI	114.0	85.8	25	14	9.0–11.0
MSI-d_4	118.0	89.9	25	14	9.0–11.0
NMP	99.9	57.8	35	23	10.5–12.5
NMP-d_9	109.0	61.8	35	26	10.5–12.5

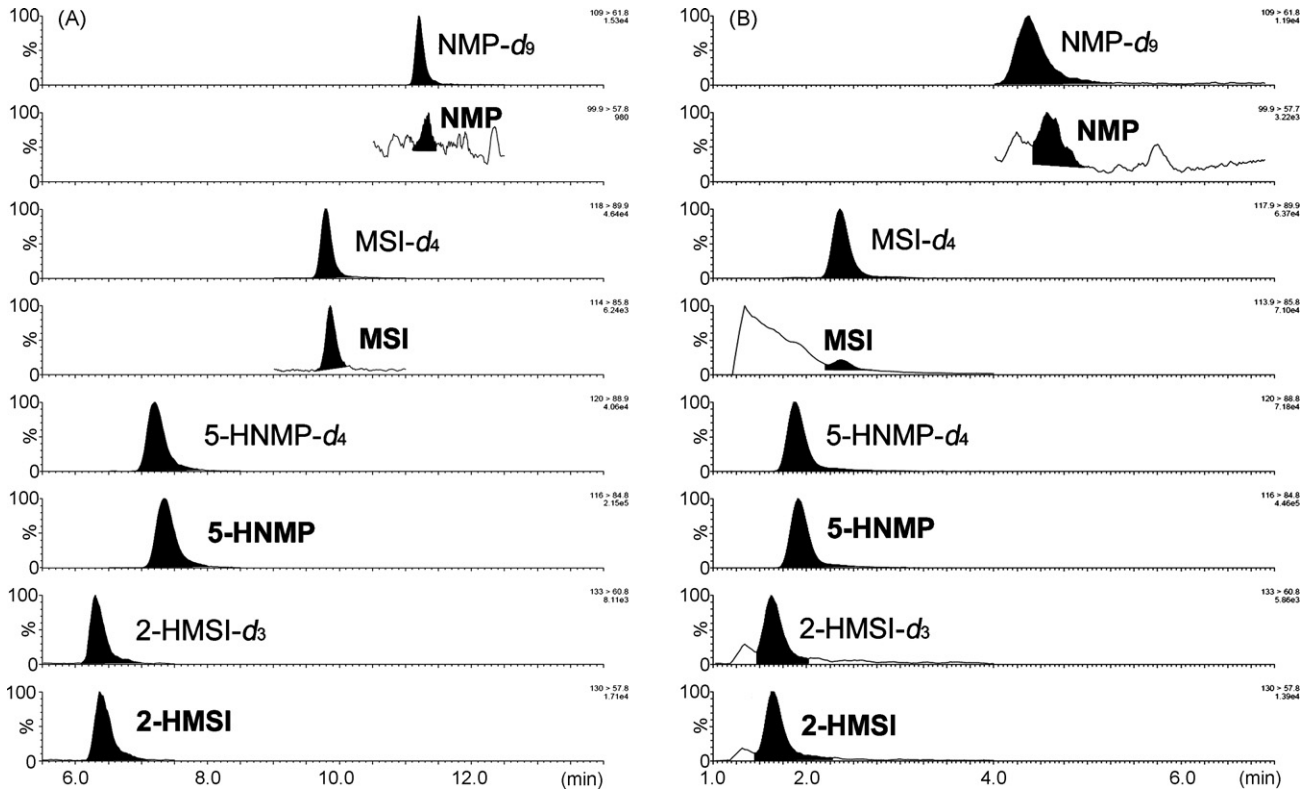

Fig. 1. MRM chromatograms of 2-HMSI, 5-HNMP, MSI, and NMP in 10-fold diluted urine sample of G-EQUAS 41st-3A, to which 2-HMSI-d_3, 5-HNMP-d_4, MSI-d_4, and NMP-d_9 were spiked each as 1 mg/L, and separated with a C30 column (A) and a C18 column (B).

found 65% recovery for 2-HMSI and higher for others. However, the deuterium-labeled compounds used to correct for the ionization gave 95.8 ± 1.0% for 2-HMSI, 107.4 ± 1.7% for 5-HNMP, 108.0 ± 3.5% for MSI, and 108.7 ± 2.4% for NMP. These results suggested that the C30 column separation and the use of each deuterium-labeled internal standard made this method workable when allowing for a 10% variation range, similar to the variation using SPE-GC/FTD [17].

3.2. Calibration curves, limits of detection, and precision

Linear calibrations were examined by preparing aqueous solutions and 10-fold diluted blank urine samples (from 3 volunteers). These were spiked with NMP and its metabolites at 0.1, 0.5, 1, 5, 10, and 20 mg/L, and also with deuterium-labeled compounds at 1 mg/L. A calibration curve was prepared by plotting the peak area relative to each internal standard (y) against the known concentration of each compound (x). Slopes (a) and intercepts (b) were calculated by linear regression, $y = ax + b$, after weighting the ordinate values by $1/x^2$.

The results (summarized in Table 2) indicate that the calibration equations obtained with 10-fold diluted spiked urine samples were, irrespective of the urine sources, very similar to each other as well as to those obtained with aqueous solutions (i.e., similar slopes, small intercepts, and high correlation coefficients). Linear concentration responses ranged up to 20 mg/L for 2-HMSI, 5-HNMP, and MSI, but the range was only up to 5 mg/L for NMP. These limits were

Table 2
Calibration equations for 2-HMSI, 5-HNMP, MSI, and NMP in aqueous solution and 10-fold diluted urine samples spiked with standards.

Compounds	Matrix	Concentration range (mg/L)	Slope (L/mg)	Intercept	Correlation coefficient
2-HMSI	Water	0.1–20	1.58	0.010	0.992
	1/10 Urine A[a]	0.1–20	1.59	−0.007	0.997
	1/10 Urine B[a]	0.1–20	1.67	−0.002	0.980
	1/10 Urine C[a]	0.1–20	1.59	−0.010	0.994
5-HNMP	Water	0.1–20	2.46	0.036	0.996
	1/10 Urine A[a]	0.1–20	2.36	0.019	0.995
	1/10 Urine B[a]	0.1–20	2.46	0.023	0.996
	1/10 Urine C[a]	0.1–20	2.41	0.014	0.996
MSI	Water	0.1–20	1.50	−0.012	0.995
	1/10 Urine A[a]	0.1–20	1.49	−0.111	0.989
	1/10 Urine B[a]	0.1–20	1.58	−0.147	0.998
	1/10 Urine C[a]	0.1–20	1.56	−0.010	0.996
NMP	Water	0.1–5	19.7	0.333	0.994
	1/10 Urine A[a]	0.1–5	18.1	0.094	0.999
	1/10 Urine B[a]	0.1–5	20.9	0.290	0.987
	1/10 Urine C[a]	0.1–5	19.9	0.372	0.992

[a] Urine samples A, B, and C were from NMP non-exposed individuals.

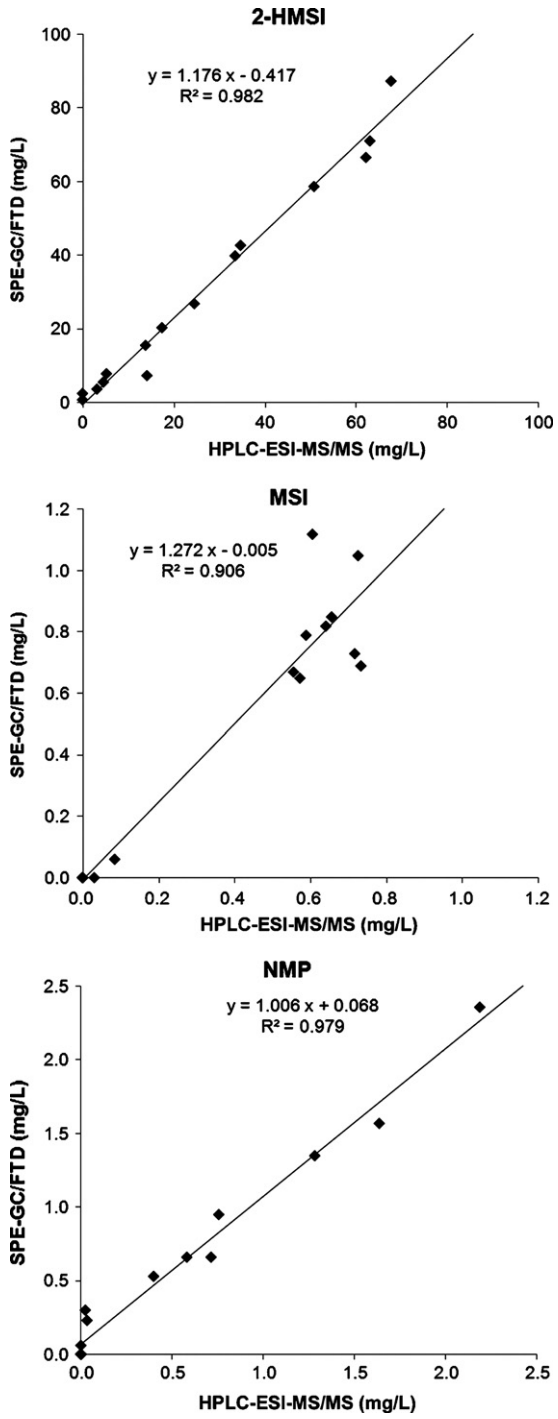

Fig. 2. Correlations of measurements between HPLC-ESI-MS/MS and SPE-GC/FTD for urine samples from workers exposed to NMP in an occupational environment.

due to the specific ionization characteristics of the compounds (i.e., 2-HMSI at the lowest detection and NMP at the highest, as found in the infusion experiments with aqueous solutions). In practice, the urinary concentration of NMP is one-digit lesser than the others, as seen in Fig. 2, and the upper concentration linearity limits match the ACGIH BEI value of 100 mg/L [10] and the DFG BAT value of 150 mg/L [11] for 5-HNMP in raw urine.

The limits of detection (LOD) for NMP and its metabolites were calculated according to the definition given by Gibbons [22]. The samples used for LOD determinations were aqueous solutions of standards and the 10-fold diluted urine samples from 3 volunteers, but spiked at concentrations of 0.2 mg/L 2-HMSI, 0.02 mg/L 5-HNMP, 0.04 mg/L MSI, and 0.01 mg/L NMP, which gave chromatographic peaks with S/N ratios of 10–16 (peak-to-peak) for each peak. The calculated LODs were 0.02 mg/L for 2-HMSI, 0.005 mg/L for 5-HNMP, 0.01 mg/L for MSI, and 0.0003 mg/L for NMP in aqueous solutions, and 0.03 mg/L for 2-HMSI, 0.006 mg/L for 5-HNMP, 0.008 mg/L for MSI, and 0.0001 mg/L for NMP in 10-fold diluted spiked urine samples. There was little difference in LODs between aqueous solutions and the 10-fold diluted spiked urine samples.

The precision of the HPLC–MS/MS measurements was determined using 9 diluted urine samples from 3 volunteers, spiked at urinary concentrations of 1, 10, and 100 mg/L each of 2-HMSI, 5-HNMP, MSI, and NMP. These were subjected to 10 replicate measurements for the within-run precision, or 5 replicates in 4 different runs for the total precision. Within-run precision (CV, %) was 6.5% for 2-HMSI, 3.4% for 5-HNMP, 3.7% for MSI, and 5.6% for NMP. Total precision (CV, %) was 6.9% for 2-HMSI, 4.2% for 5-HNMP, 6.0% for MSI, and 9.2% for NMP. These results seem to be reasonable by comparison with the reported within-day and between-day precisions obtained with SPE-HPLC–MS/MS [9,14].

3.3. Method evaluation with test urine samples

First, the present method was applied to urine samples of G-EQUAS 41st and 42nd intercomparison programs held in 2008 for 2-HMSI and 5-HNMP [23]. These urine samples were diluted 10-fold with pure water, the deuterium-labeled internal standards were added as concentrations of 1 mg/L, then measured ($n = 5$). All of the measured values were found in the tolerance range, as seen in Table 3. Thus, the accuracy of this method was reasonable.

Next, to compare the present method with the SPE-GC/FTD method [17], urine samples from 5 workers exposed to NMP, which had been stored frozen at −20 °C, were subjected to the present HPLC-ESI-MS/MS measurements. The relationships of measured values between HPLC-ESI-MS/MS and SPE-GC/FTD are shown in Fig. 2. Correlations between the two methods are significant: $r^2 = 0.982$ for 2-HMSI, $r^2 = 0.979$ for NMP and $r^2 = 0.906$ for MSI with several scattered data points.

Comparable data by SPE-GC/FTD were not available for 5-HNMP because of its thermal instability for GC measurements, as previously mentioned. However, the concentration of 5-HNMP found in these 5 workers' urine samples (collected at the end of a shift) was 37.5 ± 17.2 mg/L (mean ± SD). These workers were exposed to 3.2 ± 1.1 ppm NMP vapor in the occupational atmosphere for 8 h

Table 3
Measured values, reference values, and tolerance ranges for 2-HMSI and 5-HNMP in G-EQUAS urine samples.

G-EQUAS urine samples	2-HMSI (mg/L)			5-HNMP (mg/L)		
	Measured value	Reference value	Tolerance range	Measured value	Reference value	Tolerance range
41st-3A	17.7 ± 0.3	21.2	16.3–26.1	30.1 ± 0.4	30.4	24.4–36.3
41st-3B	36.6 ± 0.9	42.4	36.0–49.8	101.7 ± 1.5	110.4	94.5–126.3
42nd-3A	20.9 ± 0.5	25.1	19.1–31.2	95.9 ± 2.4	96.7	82.7–110.6
42nd -3B	42.6 ± 2.1	43.2	34.7–51.6	150.4 ± 4.6	151.7	131.2–172.1

The values are expressed as means ± SD ($n = 5$).

[17]. This urinary concentration of 5-HNMP seems to be reasonable based on the proportionality of NMP exposure level and the concentration of 5-HNMP excreted in urine [12]. It is also in line with the values reported by Workplace Environmental Exposure Level [24] of 10 ppm (40 mg/m^3) NMP and its excretion of 100 mg/L 5-HNMP (BEI) [10], and of 19 ppm (80 mg/m^3) NMP for Maximum Concentrations at the Workplace (MAK) and its excretion of 150 mg/L 5-HNMP [11].

4. Conclusion

The present analytical method using HPLC-ESI-MS/MS, which comprises highly hydrophobic chromatographic separation and the use of deuterium-labeled compounds as internal standards, allows the direct determination of NMP and its urinary metabolites by simple aqueous dilution of urine samples and without laborious SPE preparations. This method is useful for high-throughput biological monitoring of NMP exposure in the workplace.

Acknowledgments

This study was a part of the Research and Development Project on the 13 Fields of Occupational Injuries and Illness of the Japanese Labour Health and Welfare Organization.

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