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Determination of N-methylsuccinimide and 2-hydroxy-N-methylsuccinimide in human urine and plasma

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Abstract

A method for determination of N-methylsuccinimide (MSI) and 2-hydroxy-N-methylsuccinimide (2-HMSI) in human urine and of MSI in human plasma was developed. MSI and 2-HMSI are metabolites of the widely used organic solvent N-methyl-2-pyrrolidone (NMP). MSI and 2-HMSI were purified from urine and plasma by C_8 solid-phase extraction and analysed by gas chromatography–mass spectrometry in the negative-ion chemical ionisation mode. The intra-day precisions in urine were 2–6% for MSI (50 and 400 ng/ml) and 3–5% for 2-HMSI (1000 and 8000 ng/ml). For MSI in plasma it was 2% (60 and 1200 ng/ml). The between-day precisions in urine were 3–4% for MSI (100 and 1000 ng/ml) and 2–4% for 2-HMSI (10 000 and 18 000 ng/ml) and 3–4% for MSI in plasma (100 and 900 ng/ml). The recoveries from urine were 109–117% for MSI (50 and 400 ng/ml) and 81–89% for 2-HMSI (1000 and 8000 ng/ml). The recovery of MSI from plasma was 91–101% (50 and 500 ng/ml). The detection limits for MSI were 3 ng/ml in urine and 1 ng/ml in plasma and that of 2-HMSI in urine was 200 ng/ml. The method is applicable for analysis of urine and plasma samples from workers exposed to NMP.

Keywords: N-Methylsuccinimide; 2-Hydroxy-N-methylsuccinimide; N-Methyl-2-pyrrolidone

1. Introduction

N-Methyl-2-pyrrolidone (NMP) is an organic solvent with an extensive use in the chemical industry [1]. Its use can be expected to increase in the next few years since NMP has been suggested as a substitute of solvents of higher inherent toxicity. NMP has also been suggested as a skin penetration enhancer in transdermal therapy [2,3].

Animal studies indicate that NMP may be a reproductive toxic compound [1,4–8]. Moreover, a stillbirth after occupational exposure to NMP has recently been described in a case report [8]. Thus, the large number of women exposed to NMP in industry makes it urgent to develop methods for assessment of exposure to NMP. Methods for analysis of NMP in air have been described [9–11]. However, methods for biological monitoring of the exposure to NMP may have several advantages over air monitoring. For example, these methods may reflect percutaneous absorption which can be expected to be a major source of exposure to NMP. Biological monitoring methods may also compensate for individual respiratory rates during various work

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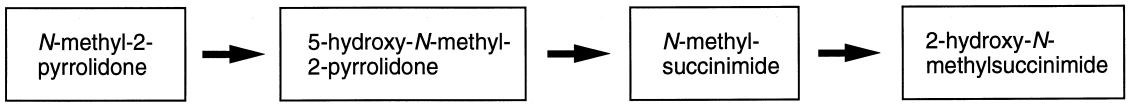

Fig. 1. Scheme of the major metabolic pathway for NMP.

loads. Moreover, biological monitoring makes it possible to estimate exposure even when protective devices are used. In addition, individual differences in metabolism may be of relevance for biological monitoring of risk.

NMP is readily absorbed through the skin in the rat [12]. In an in vitro study it was shown that NMP also possess a high permeability through human skin [13]. In man, it has been shown that NMP is efficiently absorbed through the respiratory tract [11] and eliminated from the body, mainly by biotransformation to other compounds [14]. A metabolic pathway, where NMP is first hydroxylised to 5-hydroxy-N-methyl-2-pyrrolidone (5-HNMP) and then further oxidized to N-methylsuccinimde (MSI) which in turn is hydroxylised to 2-hydroxy-N-methylsuccinimide (2-HMSI), has been suggested (Fig. 1). Only a minimal fraction was found to be eliminated in urine as NMP [11,14].

The extensive biotransformation of NMP to other compounds and the minor excretion of unmetabolized NMP in urine suggests that the metabolites of NMP, rather than NMP itself, may be the most suitable compounds for biological monitoring of the exposure. Moreover, the use of metabolites as biomarkers eliminates the risk of contamination of the samples. Methods for the determination of 5-HNMP and 2-HMSI in urine [15] and plasma [16] have recently been suggested. However, there is no validated analysis method reported for MSI. Here we describe a simple and sensitive method for the determination of MSI in human urine and plasma. In addition, the method is applicable for the determination of 2-HMSI in urine.

2. Experimental

2.1. Apparatus

A Model 8065 gas chromatograph (Carlo-Erba, Milan, Italy) connected to a VG Trio 1000 quadrupole mass spectrometer (Fisons, Manchester, UK) and fitted with an A200S autosampler (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; International Sorbent Technology, Hengoed, UK) were used. The columns were connected to a VacElut SPS 24 (Varian, Palo Alto, CA, USA) which connected to an aspirating pump. A Model 3E-1 centrifuge (Sigma, Harz, Germany) was used for sedimentation of the magnesium sulfate and to remove liquid from the Isolute columns.

2.2. Chemicals

MSI and 2-HMSI were from Aldrich (Gillingham, UK), methanol and ethyl acetate were from LabScan (Dublin, Ireland), and anhydrous magnesium sulfate was from Merck (Darmstadt, Germany). Deuterium labeled MSI (labeled at the four ring positions; [$^2H_4$]MSI) and deuterium labeled 2-HMSI (labeled at the three methyl positions; [$^2H_3$]-2-HMSI) were synthesised by Synthelec (Lund, Sweden).

2.3. Sampling and storage

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

Blood samples were collected by venepuncture in 10 ml evacuated tubes (Venoject, Terumo Europe, Leuven, Belgium) containing sodium heparin. After sampling, the blood was allowed to cool to room temperature and then centrifuged at 1500 *g* for 10 min. The plasma was stored in 10 ml polyethylene test tubes at −20°C until analysis.

2.4. Preparation of standards

Standard solutions of MSI and 2-HMSI were prepared by addition of 25 mg MSI or 2-HMSI to 25 ml water. Solutions in desired concentrations were

prepared from these solutions by further dilution in water. The internal standards were prepared in the same way. Urine or plasma standards of MSI and 2-HMSI were prepared by adding 50 μl aliquots of these standard solutions to blank urine or plasma.

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

2.5. Work-up procedure

The MSI and 2-HMSI were extracted from the urine and plasma by Isolute C_8 columns previously conditioned by 5 ml of methanol followed by 10 ml of water. The internal standards, 110 ng [$^2H_4$]MSI and 4000 ng [$^2H_3$]-2-HMSI, were added in 50 μl aliquots in water to 1.0 ml urine or plasma. The samples were then applied to the columns. The columns were dried by air suction for 5 min and then centrifuged at 1500 *g* for 10 min to remove any remaining liquid before the elution of the MSI and 2-HMSI by 2 ml of ethyl acetate. The samples were dried by magnesium sulfate, centrifuged for sedimentation, and the supernatant was transferred to autosampler injection vials and stored at 4°C until analysis.

2.6. Analysis

Aliquots of 2 μl were injected with a splitless technique at 300°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 thereafter increased by 20°C/min to 170°C and then by 40°C/min to 250°C where cooling immediately started. The mass spectrometer interface was at 300°C, the ion source at 200°C and the mass spectrometry (MS) system was in the negative-ion chemical ionisation (NICI) mode with ammonia as moderating gas. Selected ion monitoring (SIM) for MSI and 2-HMSI were both performed at *m/z* 112 while *m/z* 115 was chosen for the two internal standards. Peak area ratio measurements were used for the determinations.

3. Results and discussion

3.1. Stability

MSI and 2-HMSI were stable in water or ethyl acetate for several months at 4°C. No degradation of MSI was found in urine samples containing between 5 and 300 ng MSI/ml after an 8 months storage at −20°C. It has previously been described that 9% 2-HMSI was lost from urine samples containing between 200 and 18 000 ng/ml during an 8 months storage at −20°C [15]. No degradation was found in five plasma samples containing 50 ng MSI/ml after a 2.5 months storage at −20°C.

Analysis of urine samples containing between 5 and 400 ng MSI/ml and between 500 and 20 000 ng 2-HMSI/ml directly after work-up and after a week in room temperature showed the same results. Also, analysis of plasma samples containing between 2 and 200 ng MSI/ml directly after work-up and after a week in room temperature showed the same results. Thus, it seems that both worked-up urine and plasma samples can be stored for some time before analysis.

3.2. Work-up procedure

No conjugation with glucorinic acid or sulfate of 2-HMSI in urine from NMP exposed subjects has been found [14] and thus, no hydrolysis was performed prior to the extraction of the urine.

Initial tests showed that liquid–liquid extraction with equal volumes of urine and ethyl acetate gave an extraction recovery at 70% for MSI but only about 30% for 2-HMSI. Thus, SPE was tried for the purifications. Several different SPE columns were tested for the extraction of MSI and 2-HMSI from urine samples. The highest recoveries for 2-HMSI at 80–90% was obtained by Isolute C_8 columns and this column was therefore chosen. Bond Elut C_8 (Varian) gave recoveries at ca. 80%, Bond Elute C_{18} and Isolute C_{18} gave extraction recoveries between 60 and 70% and Bond Elute C_2 extraction recoveries at ca 50%. All SPE columns gave recoveries close to 100% for MSI. The influence of the sample volume on the extraction recovery of the Isolute C_8 columns was tested. For 2-HMSI sample volumes between 0.5 and 1.0 ml did not significantly influence the recovery (80–90%). However, at sample volumes at 2.0 ml the recovery was only about 60%. For MSI

also a sample volume of 2.0 ml gave a recovery close to 100%. We chose not to add any washing step after the retention of the MSI and 2-HMSI on the C_8 columns because of the poor retention of the 2-HMSI.

3.3. Mass spectrometry and chromatography

It has previously been described that the acid anhydrides, which are compounds with similar structures as compared to the succinimides, have high abilities to stabilise electrons [17]. Thus, we tried to use the NICI mode for the determinations and found that this mode gave a much higher sensitivity than electron impact. The NICI mass spectra of MSI and [$^2H_4$]MSI are shown in Fig. 2. The base peak in the mass spectrum of unlabeled MSI was the molecular ion [M] which has lost a hydrogen at m/z 112. In the internal standard this fragment is found at m/z 115 indicating that the loss of hydrogen/deuterium is from a ring positions. There were also minor fragments at m/z 129 (MSI) and 133 ([$^2H_4$]MSI) indicating some adducts with the ammonia moderating gas. The NICI mass spectra of 2-HMSI and [$^2H_3$]-2-HMSI are shown in Fig. 3. The base peak in the mass spectrum of unlabeled 2-HMSI was m/z 111 but m/z 112 was almost as abundant as the base peak. m/z 112 and 111 corresponds to the loss of the hydroxyl group and the hydroxyl group plus a hydrogen, respectively. Other major fragments in the spectrum were m/z 96, 100, 127 ([M−2xH]), and 128 ([M−H]). The corresponding fragments found in the spectrum of labeled 2-HMSI were m/z 99, 103, 114, 115 (base peak), 130 and 131. However, the relative abundance of the different fragments in the spectrum of 2-HMSI seemed to be dependent on how clean the ion source was. With a less clean source the fragments at m/z 127 and 128 rose as compared to those at m/z 111 and 112. A similar pattern was found for the labeled 2-HMSI.

The chromatographic behaviour of MSI was excellent with symmetrical peaks. However, the high polarity of 2-HMSI gave problems with the chromatographic behaviour. Several different analytical columns were tested. A CP-WAX 51 from Chrompack gave symmetrical peaks but such high bleeding at the elution temperature that this was not applicable for the analysis even if a thin film thickness was chosen. Different non-polar columns from J&W (DB-5.625 with 1.0 μm film thickness, DB-5 MS

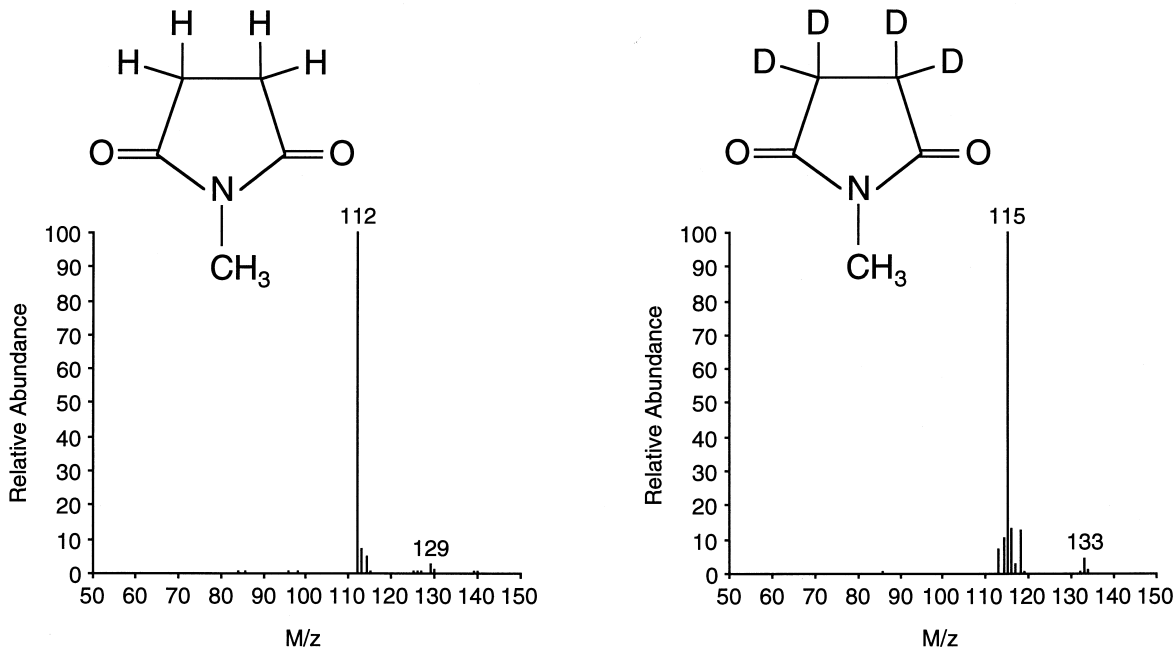

Fig. 2. Negative-ion chemical ionisation mass spectra of MSI and tetradeuterium labeled MSI.

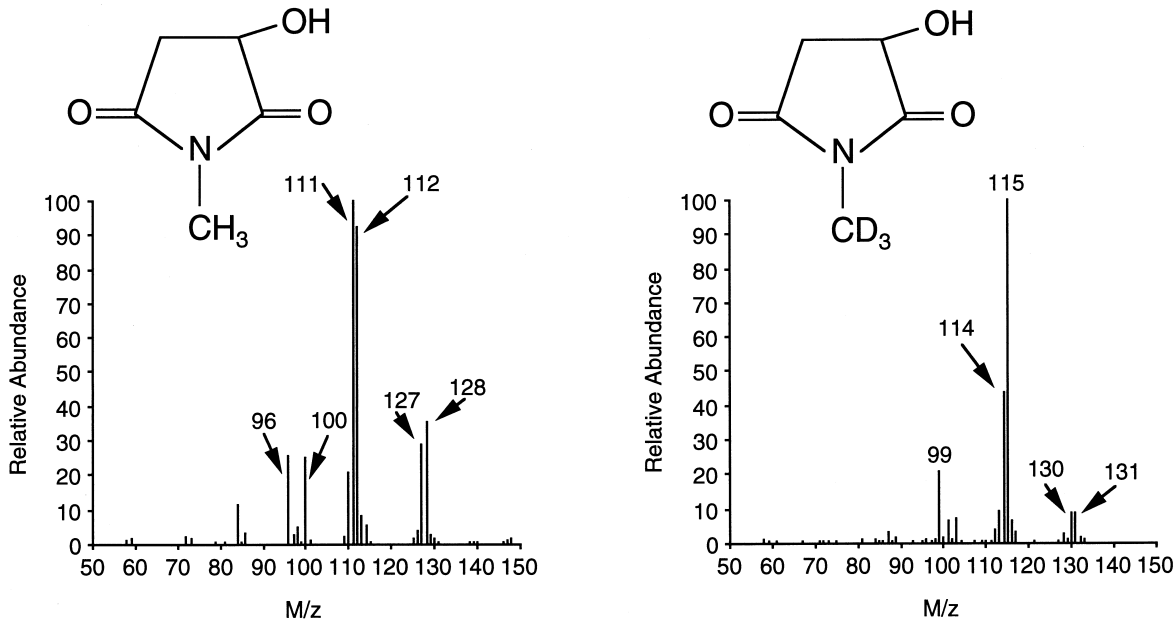

Fig. 3. Negative-ion chemical ionisation mass spectra of 2-HMSI and trideuterium labeled 2-HMSI.

with 0.25 μm film thickness and DB-1 with 0.1 μm film thickness) gave all more or less tailing peaks. However, it was decided that the best choice was the DB-5 MS. For urine, it was possible to use the method both for MSI and 2-HMSI. However, it was necessary to clean the injector liner and to cut the column at the injector side after some hundred injections in order to obtain 2-HMSI-peaks with only minor tailing. For plasma, the concentrations of 2-HMSI in exposed workers were so low that it was hard to obtain enough high chromatographic efficiency. Thus, the method was not further studied for the analysis of 2-HMSI in plasma. A chromatogram from a urine sample containing 50 ng MSI/ml and 1000 ng 2-HMSI/ml is shown in Fig. 4. A chromatogram from a plasma sample containing 8 ng MSI/ml is shown in Fig. 5. For the fragment at m/z 112 for MSI in urine and plasma there was only minor influences by signals from the matrix. For 2-HMSI the signals from the matrix were significant at all fragments but it was concluded that m/z 112 was the best fragment for the determinations.

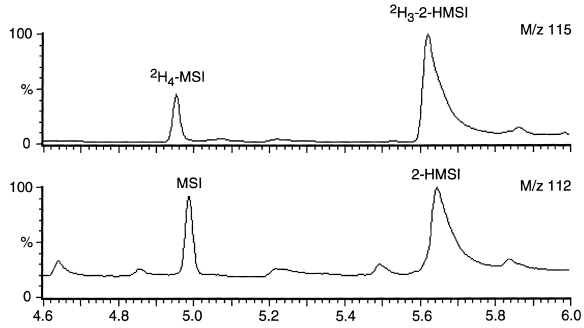

Fig. 4. Chromatogram from a urine sample containing 50 ng MSI/ml and 1000 ng 2-HMSI/ml.

3.4. Quantitative analysis

3.4.1. Calibration graph

Equations describing the calibration graphs of MSI in urine in the range 3–2000 ng/ml (n=9) and in the range 1–2000 ng/ml for plasma (n=9) are shown in Table 1. For determinations below 300 ng/ml graphs in this concentration range were used. In addition, equations describing the calibration graphs of 2-HMSI in urine in the range 200–40 000 ng/ml (n=9) are shown in Table 1. For determinations below 5000 ng/ml graphs in this concentration range were used. All calibration graphs were linear over the whole range of concentrations.

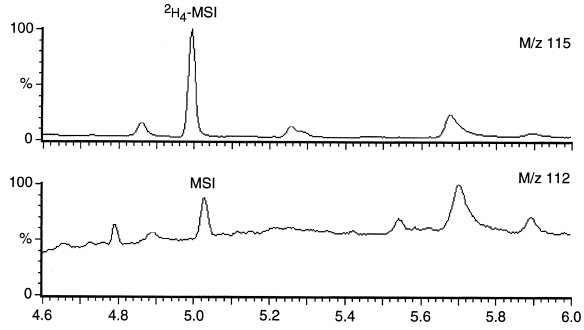

Fig. 5. Chromatogram of a plasma sample containing 8 ng MSI/ml.

3.4.2. Detection limit

Plasma and urine were collected from ten volunteers who were presumed to be unexposed to NMP, 5-HNMP, MSI and 2-HMSI. Internal standards were added and the samples were analysed according to the method described above. The detection limit was calculated as reported by Miller and Miller [18] as the concentration corresponding to the mean plus three times the standard deviation of the peak area ratios with the same retention time as MSI and 2-HMSI. The detection limit for MSI in urine was 3 ng/ml and in plasma 1 ng/ml. The detection limit for 2-HMSI in urine was 200 ng/ml.

3.4.3. Precision

The intra-day precision of the urine method was determined from the analysis of urine samples from ten different subjects spiked with 50 ng/ml or 400 ng/ml of MSI and with 1000 or 8000 ng/ml of 2-HMSI. The intra-day precision of the plasma methods was determined from the analysis of plasma samples from ten different subjects spiked with 60 ng/ml or 1200 ng/ml of MSI. The coefficients of variation (C.V.s) were determined for the ratio between the area for MSI or 2-HMSI and their internal standards. The results are shown in Table 2.

The between-day precision was studied by analysing two urine samples ten times during a period of five weeks. One of the samples contained 100 ng MSI and 18 000 ng 2-HMSI per ml and the other sample contained 1000 ng MSI and 10 000 ng 2-HMSI per ml. In addition, two plasma samples containing 100 and 900 ng MSI/ml were analysed seven times during a period of five weeks. The C.V. for the determinations are shown in Table 3.

3.4.4. Recovery

The recovery for the overall method was investigated by working-up ten different urine or plasma samples spiked with MSI and 2-HMSI. For urine, two sets of samples with the concentrations 50 ng

Table 1
Equations describing the calibration graphs for MSI and 2-HMSI in urine or plasma

Compound	Matrix	Concentration range (ng/ml)	Slope (ng/ml)	Intercept	Correlation coefficient
MSI	Urine	3–2000	0.010	0.010	0.999
MSI	Plasma	1–2000	0.010	0.011	0.999
2-HMSI	Urine	200–40 000	0.00023	0.050	0.998

Table 2
Intra-day precisions in the analysis of urine and plasma samples spiked with different amounts of MSI and 2-HMSI

Compound	Matrix	Concentration (ng/ml)	Intra-day precision[a] (%)	Number of samples
MSI	Urine	50	6	10
MSI	Urine	400	2	10
MSI	Plasma	60	2	10
MSI	Plasma	1200	2	10
2-HMSI	Urine	1000	3	10
2-HMSI	Urine	8000	5	10

[a] Given as coefficients of variation.

Table 3
Between-day precisions in the analysis of urine and plasma samples with different amounts of MSI or 2-HMSI

Compound	Matrix	Concentration (ng/ml)	Between-day precision[a] (%)	Number of samples
MSI	Urine	100	4	10
MSI	Urine	1000	3	10
MSI	Plasma	100	4	7
MSI	Plasma	900	3	7
2-HMSI	Urine	10 000	2	10
2-HMSI	Urine	18 000	4	10

[a] Given as coefficients of variation.

MSI and 1000 ng 2-HMSI per ml or 400 ng MSI and 8000 ng 2-HMSI per ml were tested. For plasma, two sets of samples with the concentrations 50 or 500 ng MSI per ml were tested. No internal standard was added before the work-up. However, prior to the analysis, an ethyl acetate solution containing the internal standards was added. Comparisons were made with standard solutions of MSI and 2-HMSI made directly in ethyl acetate and with the same concentrations of internal standards added. The recoveries and the precisions in these are shown in Table 4.

3.4.5. Comparison between methods

Urine samples in the range 400–20 000 ng 2-HMSI/ml from NMP exposed subjects were determined by the method described by Jönsson and Åkesson [15] and by the present method (Fig. 6). The linear regression with a slope close to 1 and almost no intercept suggests that both methods have a high accuracy. This is further supported by the fact that a comparison by paired *t*-test gave no statistical-

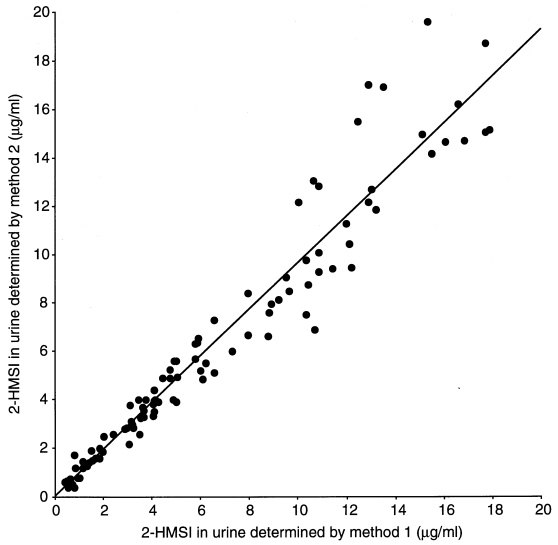

Fig. 6. Comparison between 101 determinations of 2-HMSI in urine by the present method (method 1; *x*-axis) and the method described by Jönsson and Åkesson [15] (method 2; *y*-axis). The equation of the line is $y=0.96x-0.054$ with a correlation coefficient of 0.97.

Table 4
Recovery of MSI and 2-HMSI from urine and plasma for the overall method

Compound	Matrix	Concentration (ng/ml)	Recovery (%)	Precision[a] (%)	Number of samples
MSI	Urine	50	117	7	10
MSI	Urine	400	109	3	10
MSI	Plasma	50	101	2	10
MSI	Plasma	500	91	3	10
2-HMSI	Urine	1000	81	13	10
2-HMSI	Urine	8000	89	5	10

[a] Given as coefficients of variation.

ly significant difference between results of the two methods. However, Fig. 6 also suggests a rather high imprecision in one or both methods which seems to be somewhat larger than what is indicated by the between-day precision of about 7% for the method by Jönsson and Åkesson [15] and about 3% for the present method. For MSI there is no alternative method available for a comparison of the determinations.

3.5. Application

Urine and plasma from a subject experimentally exposed for 8 h to 10 mg NMP/m^3 (Swedish occupational exposure limit: 200 mg/m^3) was collected immediately after the end of exposure. The levels of MSI in the urine and plasma samples were 300 and 200 ng/ml, respectively. The level of 2-HMSI in the urine sample was 3400 ng/ml.

4. Conclusions

A simple and fast method for simultaneous determination of MSI and 2-HMSI in urine or for determination of MSI in plasma has been developed. The method has high precision and excellent recovery. The detection limits are sufficiently low for determinations of MSI and 2-HMSI in urine and MSI in plasma from workers exposed to NMP in levels found in the work environment. Thus, the method may be used for biological monitoring of exposure to NMP.

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