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Concentrations of *N*-methyl-2-pyrrolidone (NMP) and its metabolites in plasma and urine following oral administration of NMP to rats

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Abstract

The primary aims were to study the metabolism in rats and to determine the biological levels after one oral developmentally toxic dose of *N*-methyl-2-pyrrolidone (NMP), a widely used industrial chemical. Non-pregnant female Sprague-Dawley rats were given an oral single dose of either a non-toxic dose of 125 mg NMP/kg (group 1) by gavage or a developmentally toxic dose of 500 mg/kg (group 2). Blood plasma (7 rats per time point) and urine (10 rats per time point) were sampled up to 72h after administration and analyzed using mass spectrometry. In both plasma and urine NMP, 5-hydroxy-*N*-methyl-2-pyrrolidone (5-HNMP), *N*-methylsuccinimide and 2-hydroxy-*N*-methylsuccinimide (2-HMSI) and 2-pyrrolidone (2-P) were identified. In urine 48% of the administered dose was recovered as 5-HNMP and 2-5% as 2-HMSI. The total recovery in urine was 53-59%. The peak concentrations for NMP in plasma were 1.2 and 6.9 mmol/l, 0.42 and 0.76 mmol/l for 5-HNMP, 0.07 and 0.31 mmol/l for MSI and for 2-HMSI the concentrations were 0.02 and 0.05 mmol/l for group 1 and 2, respectively. In summary, the same metabolites were found in rats as in humans and the biological levels were reported for NMP and its metabolites after oral exposure to a developmentally toxic dose and one non-toxic dose of NMP.

1. Introduction

N-methyl-2-pyrrolidone (NMP, CAS no. 872-50-4) is a small cyclic amide totally miscible in water as well as in most organic solvents. Due to its strong and selective power as a solvent, it is a widely used compound. *E.g.*, NMP is used in the petrochemical industry, in the microelectronics fabrication industry and in the manufacturing of various compounds such as pigments, cosmetics, drugs and pesticides (International program on Chemical Safety, 2001). It is also used as a graffiti remover (Anundi *et al.*, 1993 and 2000; Langworth *et al.*, 2001) and has been investigated as a transdermal enhancer in the pharmaceutical industry (Barry and Bennett, 1987; Priborský *et al.*, 1988).

In the rat, an extensive oral (Midgley *et al.*, 1992) and percutaneous (Midgley *et al.*, 1992; Payan *et al.*, 2003) uptake has been shown. The metabolism is unclear but Wells *et al.* (1992) showed that the major urinary metabolite was 5-hydroxy-*N*-methyl-2-pyrrolidone (5-HNMP).

In humans, NMP is easily absorbed in the airways (Åkesson and Paulsson, 1997), from the gastrointestinal tract (Åkesson and Jönsson, 1997) and through the skin (Ursin *et al.*, 1995; Akrill *et al.*, 2002; Åkesson *et al.*, 2004). Furthermore, a human metabolic pathway have been described (figure 1), where NMP is hydroxylated to 5-HNMP, further oxidized to *N*-methylsuccinimide (MSI) which is then hydroxylated to 2-hydroxy-*N*-methylsuccinimide (2-HMSI).

A human stillbirth after an occupational exposure, including skin contact, to an unknown level of NMP has been described in a case report (Solomon *et al.*, 1996). Furthermore, in rats, NMP have been reported to be a developmentally toxic compound after dermal and oral administration (Becci *et al.*, 1982; Hass *et al.*, 1995; Saillenfait *et*

al., 2002). Retarded growth has been reported after inhalation exposure (Saillenfait *et al.*, 2003). It is unclear whether it is NMP itself or some of its metabolites that cause this effect.

Many fertile women are exposed in the industry and it is therefore important to develop good methods for exposure assessment of NMP. Several methods for biological monitoring of NMP exposure have been described where NMP and all of its identified metabolites have been suggested as biomarkers of exposure (NMP; Åkesson and Paulsson, 1997, 5-HNMP; Åkesson and Jönsson, 2000, MSI; Jönsson and Åkesson, 2001, 2-HMSI; Jönsson and Åkesson, 2003). For all of these compounds the relationship between air and biomarker levels in humans are known. Thus, it is possible to make comparisons between the biomarker levels and the existing occupational exposure limits in air. However, it would also be useful for risk assessments to know the levels of NMP and its metabolites in rats following a developmentally toxic dose of NMP.

In this work we study the metabolic pathway for NMP in rats. Furthermore, we determine the biological levels after one oral dose that was developmentally toxic, and one oral dose that was developmentally non-toxic. We also study the biological levels in rat urine after multiple administration of NMP.

2. Material and Methods

2.1 Chemicals

The NMP (99.5%) used for the administration was purchased from Merck (Darmstadt, Germany). For the work-up and the analysis of plasma and urine, NMP was manufactured by Lab-Scan (Dublin, Ireland). MSI was from Aldrich (Gillingham, UK)

and 2-HMSI was from Aldrich (Milwaukee, WI, USA) while 2-P was from Janssen Chimica (Geel, Belgium). 5-HNMP and the deuterium labelled compounds [$^2\text{H}_4$]-5-HNMP, [$^2\text{H}_4$]-MSI and [$^2\text{H}_3$]-2-HMSI were synthesized by Synthelec (Lund, Sweden). Deuterium labeled NMP ([$^2\text{H}_9$]-NMP) was manufactured by Cambridge Isotope Laboratories (Andover, MA, USA). Methanol and ethyl acetate were from Lab-Scan (Dublin, Ireland), 99.5% ethanol from Kemetyl AB (Haninge, Sweden) and glacial acetic acid from Merck (Darmstadt, Germany).

2.2 Study design

2.2.1 Animals

Female non-pregnant Sprague-Dawley rats (Iffa Credo, Saint Germain sur L'Arbresle, France) weighing 180-235g were used for all the studies. The animals were acclimatized to laboratory conditions for at least 5 days prior initiating the studies. The animal rooms were maintained at $21 \pm 2^\circ\text{C}$ with a relative humidity of $45 \pm 5\%$ and with a 12-hr light/dark cycle. Commercial food pellets (UAR Alimentation, Villemoisson, France) and filtered tap water were available *ad libitum*.

2.2.2 Single Oral Administration

The metabolism and the biological levels in plasma and urine in rats were studied. The rats were given a single oral dose of NMP by gavage with distilled water as vehicle (5 ml/kg). For plasma sampling 42 rats were exposed to a non-toxic level of 125 mg/kg (group 1) and 42 rats were exposed to 500 mg/kg (group 2), which corresponds to a

developmentally toxic level found by Saillenfait *et al.* (2002). Each animal was fasted 16 hr prior to dosing, and food was returned 4 hr after dosing. Blood was collected at 1, 2, 4, 12, 24 and 48 h post-dose. At every time point 7 rats/group were killed, by bleeding the abdominal aorta under isoflurane anaesthesia. Blood was collected in heparinized tubes and plasma was immediately separated by centrifugation at 3500 rpm for 10 min. Samples were frozen (-20°C) until analysis. For urine sampling, 10 rats were exposed to 125 mg/kg (group 1) and 10 rats were exposed to 500 mg/kg (group 2) under the same treatment conditions. After dosing, the animals were immediately placed in individual metabolism cages for the collection of the urine at +4°C. Urine was collected three times a day in six- or twelve-hour intervals during three days.

2.2.3 Repeated oral administration

The biological levels in urine were studied in rats given three repeated doses of NMP. The rats were given three oral doses of NMP in 24-h intervals by gavage with distilled water as vehicle. A dose of 125 mg/kg was given to 10 rats and another 10 rats were given a dose of 500 mg/kg. Animals were fasted overnight only before the first treatment. Urine was collected as described above but three times a day in six-or twelve-hour intervals during five days. No plasma was collected.

2.3 Analytical methods

5-HNMP and 2-HMSI were analyzed according to the method presented by Carnerup *et al.* (2001). NMP, MSI and 2-pyrrolidone (2-P) were also analysed with this method. Deuterium-labelled internal standards were used for each corresponding compound

except for 2-P for which [$^2\text{H}_9$]-NMP was used. Internal standards were added to diluted plasma or urine. NMP and the metabolites were then trapped on ENV+ solid phase extraction columns, eluted with an 8:2 ethyl acetate/ethanol mixture and evaporated until dryness. Acidic water was added and the samples were then analyzed by liquid chromatography coupled to a tandem mass spectrometer (LC-MS/MS). Some minor changes were made from the original method. A heated nebulizer in the positive ion mode was used as the ion source instead of ion spray and the samples were diluted ten times with water before work-up. The mass spectrometric parameters for the compounds are shown in table 1. Furthermore, the detection limits and the precisions for NMP and its metabolites are shown in table 2. The detection limit was calculated according to Miller and Miller (1993). Chromatograms for the two main metabolites, 5-HNMP and 2-HMSI, are shown in figure 2.

2.4 Toxicokinetic calculation and statistics

The area under the plasma concentration-time curve (AUC) was calculated using the trapezoidal rule (Rowland and Tozer, 1995). Since the rats were killed at every time point of plasma sampling, the mean plasma concentrations per time point were used for the estimation of the AUC. Comparisons between groups were performed using the Mann-Whitney U-test.

3. Results

In both plasma and urine from the rats exposed to a single oral dose NMP, 5-HNMP, MSI, 2-HMSI and 2-P were identified. The recoveries of NMP and its metabolites in

urine from the rats are shown in table 3. Thus, the recoveries were 58% (range 53-64%) in group 1 and 52% (range 36-62%) in group 2. The area under the plasma concentration-time curve (AUC) and the median peak concentrations in plasma and urine and the time to reach the peaks are shown in table 4.

In the repeated administration study where doses of 125 mg/kg and 500 mg/kg over three consecutive days were given to the rats three major peaks were found for NMP and all metabolites. The total recoveries after five days are shown in table 3 and the urine concentrations are shown in table 5. The peak time and peak concentrations for the first administration were similar to those found after single dosing. The time to peak during the repeated administration did not vary significantly for any of the metabolites. There was no obvious accumulation for any of the compounds during the repeated administration. Urine concentration-time curves for 2-HMSI is shown in figure 3.

4. Discussion

The most important results were the identification of unchanged NMP and the metabolites MSI, 2-HMSI and 2-P in plasma and urine from NMP exposed rats. Furthermore, the levels of NMP and its metabolites were reported after one developmentally toxic dose and one non-toxic dose of NMP.

Previously, only 5-HNMP has been confirmed, using mass spectrometry, as a metabolite of NMP in rats (Wells *et al.*, 1992). However, Payan *et al.* (2003) suggested MSI and 2-HMSI as NMP metabolites in rats given a single dose of radioactively labelled NMP intravenously, by comparing HPLC retention times. These metabolites were confirmed using mass spectrometry in this study. Furthermore, in another study

Wells and Digenis (1988) administered radiolabelled ^3H - and ^{14}C -isomers of NMP to rats and studied the excretion of the radioactivity in the urine. In one experiment ring labelled ^{14}C -NMP was used and three peaks from radioactively labelled compounds were obtained following reversed phase liquid chromatography of the urine. Furthermore, administration of NMP labelled with tritium in the 4-position (same as the 2-position in 2-HMSI) together with methyl labelled ^{14}C -NMP revealed the same peaks for ^{14}C but only the last of these were labelled for tritium. This peak corresponded to the main metabolite and was the one later identified as 5-HNMP (Wells *et al.*, 1992). In separations of the metabolites found in this study using a similar separation as used by Wells and Digenis (1988), only 2-HMSI had a shorter retention time than 5-HNMP while the retention times for NMP, MSI and 2-P were all longer. Thus, one of the two minor peaks in that study probably corresponds to 2-HMSI. This is in accordance with the loss of tritium from the 4-position. The metabolite corresponding to the other peak probably remains to be identified. The fact that we only found approximately 55% of the administered dose of NMP in this study supports the presence of another metabolite. The loss of tritium may indicate a metabolism in the 4-position for this compound as well. Faeces and tissues were not examined in the present experiments and this could contribute to the low urinary recovery. However, in previous studies only 1-2% of the administered dose was recovered in faeces after both oral and dermal administration and only small amount were found in expired air and tissues (Wells and Digenis, 1988; Midgley *et al.*, 1992).

2-P was identified as a novel NMP metabolite in our study. This metabolite is probably formed directly from NMP by demethylation but should probably be of minor

importance since the plasma level was small compared to NMP and the other metabolites. It remains to establish if 2-P is also a metabolite of NMP in humans.

In this study 33-54% of the administered dose was excreted as 5-HNMP. This is in accordance with results previously reported by Payan *et al.* (2002). In that study five groups of rats were intravenously administered different doses of methyl labelled ^{14}C -NMP and urinary elimination of 5-HNMP accounted for 42-55% of the administered doses. Also, Payan *et al.* reported that 4-10% of the administered dose was eliminated as NMP in the urine while Wells and Digenis (1988) found no urinary excretion of unchanged NMP. In our study, unchanged NMP was found in urine but only at low levels (1.2-3.7%). Furthermore, in this study 99-100% of the eliminated amount (51-58% of the dose) was found within 24 h of the administration. Payan *et al.* (2002) reported similar results, 85-98% of the radioactivity in urine. However, for the two highest administrations (100 and 500 mg/kg) in that study, an increased amount was eliminated in urine post 24 h, indicating that there was a delay in urinary elimination. A delay in time to reach the peak concentration was also found for 5-HNMP in plasma and Payan *et al.* suggested this to be a result of a saturable elimination process of NMP. The AUC for NMP in that study was also significantly higher for the two higher doses than for the lower. A delay in time to reach the maximal concentration for 5-HNMP, MSI and 2-HMSI was also found in plasma in our study, after administration of the higher dose. High concentrations of NMP in plasma, and relatively higher AUC for NMP and MSI at the higher dose compared to the lower one was also found in our study.

Repeated dosing of chemicals are important for studies of toxicokinetics. In addition, repeated dosing of NMP or its metabolites during several days may be an

interesting regime for studying the developmental toxicity of these compounds. Thus, we investigated whether this regime caused any accumulation of any of the metabolites in rats as has been found in humans. However, there were no obvious evidence for accumulation but since only urine was sampled in this study it is not possible to draw a certain conclusion. Still, we think that the lack of tissue distributed radioactivity in other studies (Wells and Digenis, 1988; Midgley *et al.*, 1992) together with the rapid elimination of radioactivity found by Payan *et al.* (2002) indicate no or only little accumulation. In humans, repeated exposure to NMP will result in an accumulation of at least 2-HMSI due to its long half-life (Jönsson and Åkesson, 2003). Thus, the elimination processes seem to be faster in rats than humans.

The same metabolites that previously have been identified in humans were also identified in rats. However, there were some notable differences. The mean urinary excretion of 5-HNMP and 2-HMSI in volunteers orally exposed to NMP corresponded to 44% and 20%, respectively, of the administered dose. The mean total recovery was 65% (Åkesson and Jönsson, 1997). In rats, the relative excreted amount of 5-HNMP was higher than in humans while it was 4-9 times lower for 2-HMSI. The differences between rats and humans were also obvious from the plasma values. Thus, only small differences were found when comparing the AUC or the plasma concentrations of 2-HMSI from rats in this study with a previous study by Åkesson *et al.* (2004) where male and female volunteers were topically exposed to 300 mg of NMP. Thus, in that paper, these women had a median AUC for 2-HMSI of 230 $\mu\text{mol}\cdot\text{h/l}$, while rats exposed to a dose corresponding to the NOAEL (125 mg/kg), showed a median AUC of only 250 $\mu\text{mol}\cdot\text{h/l}$. The corresponding values for the plasma concentrations were 5.4 and 20 $\mu\text{mol/l}$,

respectively. The small differences found may be of importance if it is proved that 2-HMSI may induce developmentally toxic effects. After administration of the higher dose (500 mg/kg) in rats, corresponding to two times the LOAEL, the AUC and plasma concentrations were 780 $\mu\text{mol}\cdot\text{h/l}$ and 50 $\mu\text{mol/l}$, respectively. Thus, the systemic dose of 2-HMSI in rats was only 3-9 times larger than in female volunteers exposed to a dose that corresponds to an 8-h exposure of approximately 10 mg NMP/m³. On the other hand, if NMP itself is proved to be developmentally toxic, the dose in rats was 970-1200 times larger. In that case, occupational exposure of NMP should probably be of minor risk of inducing developmentally toxic effects. The high levels obtained for NMP and 5-HNMP may indicate that one of these compounds are responsible for the toxic effects but this remains to be determined. However, extrapolation of animal results to humans should be interpreted with caution. Possible differences between oral, dermal, and inhalation routes of exposure should be considered. The pregnant state itself could also result in changes, both in the routes of absorption but also more particularly in the rates of metabolism.

In conclusion, we have reported levels of NMP and its metabolites in rats orally exposed to one developmentally toxic dose and one non-toxic dose of NMP. However, to be able to interpret these levels in terms of risk it is essential to perform more studies, *e.g.*, it is necessary to have knowledge about which metabolite of NMP that causes the developmental toxicity. Such studies are in progress.

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Footnotes*Abbreviations:*

2-HMSI	2-hydroxy- <i>N</i> -methylsuccinimide, page 1 (title page)
5-HNMP	5-hydroxy- <i>N</i> -methyl-2-pyrrolidone, page 1 (title page)
2-P	2-pyrrolidone, page 1 (title page)
AUC	area under the curve, page 1 (title page)
CYP2E1	cytochrome P450 isoform 2E1, page 1 (title page)
MSI	methylsuccinimide, page 1 (title page)
NMP	<i>N</i> -methyl-2-pyrrolidone, page 1 (title page)

Table 1: Mass spectrometric parameters for NMP, its metabolites and corresponding deuterium labeled internal standard.

Compound	Precursor	Fragment	Declustering	Collision
	ion	ion	Potential	Energy
	<i>m/z</i>	<i>m/z</i>	(V)	(V)
NMP	99.8	58.3	30	31
[² H ₉]-NMP	108.7	62.3	31	37
5-HNMP	115.8	57.2	30	31
[² H ₄]-5-HNMP	119.8	60.5	30	31
MSI	114.1	58.2	30	21
[² H ₃]-MSI	117.3	59.3	30	26
2-HMSI	129.9	58.3	30	31
[² H ₃]-2-HMSI	132.9	61.2	30	31
2-P	85.7	44.2	26	35

Table 2: Detection limits and precision for NMP and its metabolites.

Substance	Detection Limit (ng/ml)		Precision (%CV)		Spiked conc. (µg/ml)
	Plasma	Urine	Plasma	Urine	
NMP	0.99	0.64	5	4	6.3
5-HNMP	93	72	5	7	6.3
MSI	110	11	4	5	6.3
2-HMSI	2.9	0.15	4	6	6.3
2-P	15	18	4	1	5.0

Table 3: Median (range) recoveries of the administered dose for NMP and its metabolites after a single dose and after repeated doses on three consecutive days.

Dose (mg/kg)	Substance	Recovery single dose	Recovery repeated doses
		(%)	(%)
125	NMP	1.6 (1.2-3.2)	2.9 (1.6-5.1)
125	5-HNMP	48 (46-56)	58 (42-68)
125	MSI	0.1 (0.1-0.1)	1.1 (0.8-1.4)
125	2-HMSI	5.1 (3.4-8.1)	4.2 (2.2-7.7)
125	2-P	0.7 (0.7-0.9)	3.6 (2.6-7.5)
500	NMP	2.6 (1.2-3.7)	4.6 (2.1-6.1)
500	5-HNMP	48 (33-54)	48 (39-82)
500	MSI	0.1 (0.1-0.3)	1.1 (0.6-3.1)
500	2-HMSI	2.3 (1.3-2.9)	1.5 (0.7-2.3)
500	2-P	0.8 (0.6-1.0)	0.5 (0.3-4.9)

Table 4: Median peak concentrations (range), time to reach the peak and median AUC

after a single oral dose of NMP.

Compound	Matrix	Group 1			Group 2		
		Concentration (mmol/l)	Time (h)	AUC (mmol*h/l)	Concentration (mmol/l)	Time (h)	AUC (mmol*h/l)
NMP	Plasma	1.2 (0.95-1.4)	1	7.1	6.9 (0.36-7.2)	2	58
5-HNMP	Plasma	0.42 (0.36-0.60)	4	4.3	0.76 (0.57-1.1)	12	13
MSI	Plasma	0.07 (0.06-0.10)	4	0.46	0.31 (0.15-0.44)	12	3.9
2-HMSI	Plasma	0.02 (0.02-0.08)	12	0.30	0.05 (0.02-0.14)	12	0.65
2-P	Plasma	0.01 (0.01-0.01)	4	0.10	0.04 (0.03-0.04)	4	0.60
NMP	Urine	1.5 (0.77-1.8)	0-6	NA ¹	3.6 (2.7-7.8)	6-12	NA
5-HNMP	Urine	41 (40-58)	6-12	NA	96 (86-113)	6-12	NA
MSI	Urine	0.14 (0.08-0.17)	6-12	NA	0.37 (0.16-0.44)	12-24	NA
2-HMSI	Urine	3.5 (2.0-8.8)	6-12	NA	4.4 (3.4-8.9)	12-24	NA
2-P	Urine	0.65 (0.57-1.0)	6-12	NA	1.5 (1.5-2.0)	6-12	NA

¹ Not applicable

Table 5: Median (range) peak concentrations¹ after three administrations in 24 h intervals.

Dose (mg/kg)	2	Substance	Peak 1	Peak 2	Peak 3
125		NMP	1.6 (0.0-2.4)	1.9 (1.6-2.5)	2.0 (1.6-3.0)
125		5-HNMP	39 (31-69)	33 (12-46)	35 (13-58)
125		MSI	0.6 (0.4-0.7)	0.7 (0.5-1.2)	0.9 (0.4-2.1)
125		2-HMSI	2.2 (0.6-4.1)	1.6 (0.5-3.0)	2.8 (1.6-6.3)
125		2-P	2.8 (1.8-5.1)	2.1 (0.6-3.2)	2.5 (0.1-4.3)
500		NMP	12 (9.8-99)	12 (11-17)	14 (9.5-19)
500		5-HNMP	111 (70-163)	129 (86-177)	166 (130-236)
500		MSI	1.5 (0.8-6.5)	3.5 (1.3-14)	4.3 (2.0-17)
500		2-HMSI	3.0 (1.2-7.1)	2.9 (1.5-6.0)	6.0 (3.8-9.7)
500		2-P	1.0 (0.9-6.1)	4.3 (1.8-5.0)	2.2 (1.9-2.4)

¹ Unit: mmol/l

Figure 1. The proposed metabolism of NMP in humans.

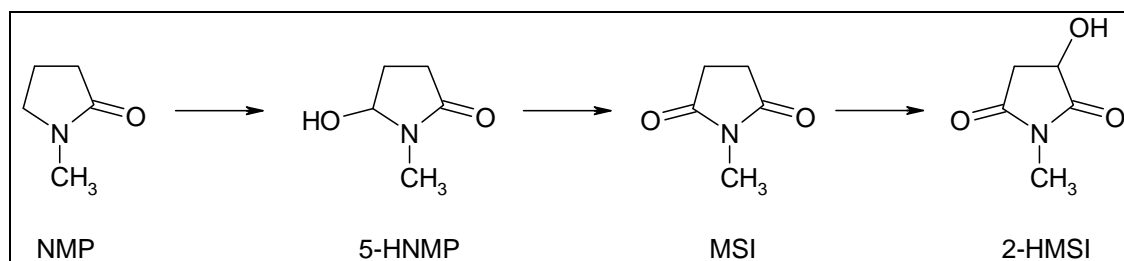


Figure 2. The chromatograms for the main metabolites, 5-HNMP (a) and 2-HMSI (b) in rat urine spiked to concentration of 1.0 $\mu\text{g/ml}$.

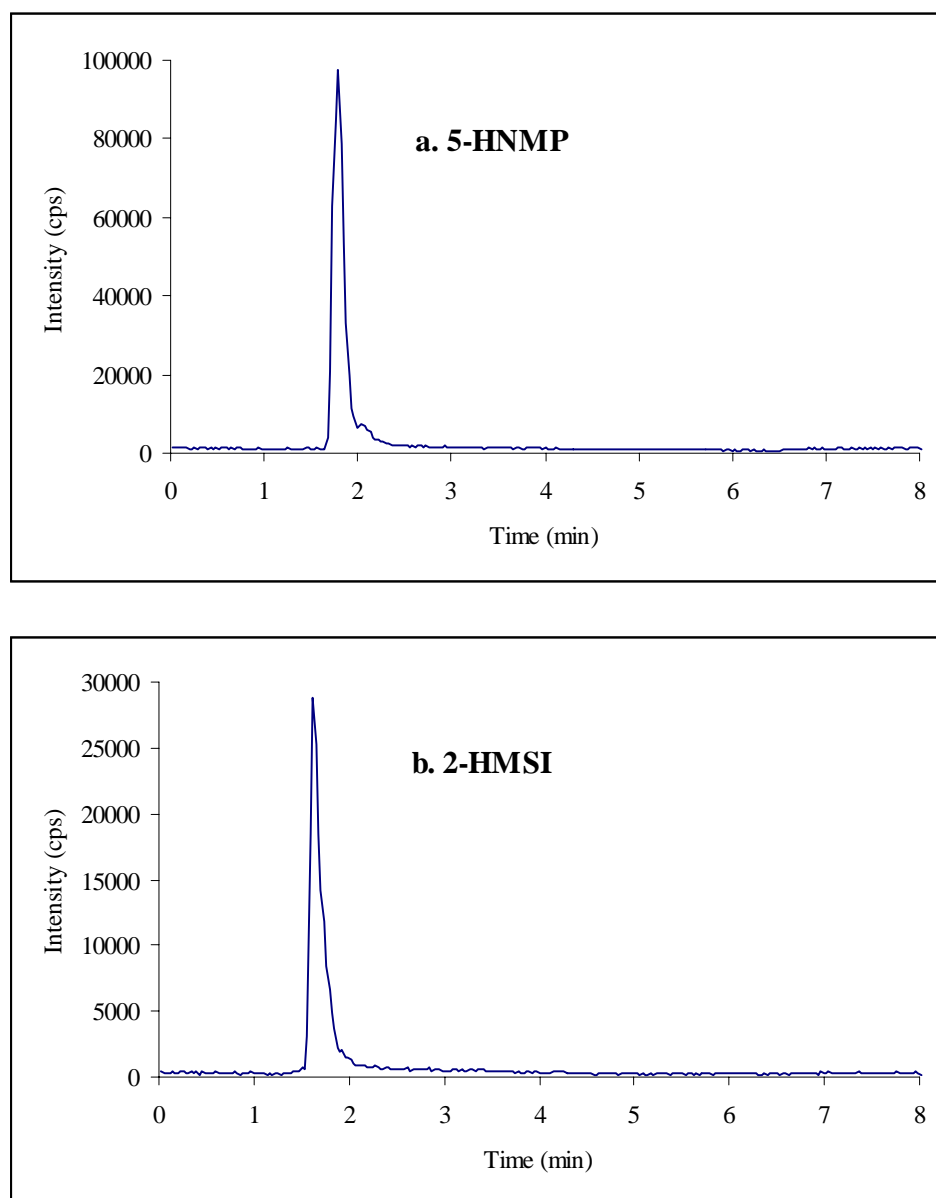


Figure 3. The urine concentration vs. time for 2-HMSI in rats given 125 mg/kg (◆) and 500 mg NMP/kg (■) by gavage at 0, 24 and 48 h.

