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TOXICOKINETICS AND METABOLISM

Toxicokinetics of *N*-ethyl-2-pyrrolidone and its metabolites in blood, urine and amniotic fluid of rats after oral administration

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Abstract

The toxicokinetics of *N*-ethyl-2-pyrrolidone (NEP), an embryotoxic organic solvent, has been studied in Sprague–Dawley rats after oral exposure. NEP and its metabolites 5-hydroxy-*N*-ethyl-2-pyrrolidone (5-HNEP) and 2-hydroxy-*N*-ethylsuccinimide (2-HESI) were measured in plasma of pregnant and non-pregnant rats, and fetuses after NEP administration by gavage for 14 consecutive days at 50 mg/kg/day, and in plasma of non-pregnant rats after a single NEP administration. Additionally, amniotic fluid and 24-h urine samples of the pregnant rats were analyzed for NEP metabolites. Furthermore, 24-h urine samples from a repeated dose 28-day oral toxicity study in female (non-pregnant) and male rats administered developmentally non-toxic (0, 5, and 50 mg/kg/day) or toxic (250 mg/kg/day) doses of NEP were analyzed. Median peak plasma concentrations in non-pregnant rats after a single dose and repeated doses were 551 and 611 (NEP), 182 and 158 (5-HNEP), and 63.8 and 108 µmol/L (2-HESI), respectively; whereas in pregnant rats and fetuses 653 and 619 (NEP), 80.5 and 91.7 (5-HNEP) and 77.3 and 45.7 µmol/L (2-HESI) were detected. Times to reach maximum plasma concentrations for NEP, 5-HNEP, and 2-HESI were 1, 4, and 8 h, respectively, and were comparable to *N*-methyl-2-pyrrolidone (NMP) and its corresponding metabolites. In pregnant rats, plasma elimination of NEP and metabolite formation/elimination was much slower compared to non-pregnant rats and efficient placental transfer of NEP was observed. Our data, overall, suggest differences in the toxicokinetics of chemicals between pregnant and non-pregnant rats which need to be addressed in risk assessment, specifically when assessing developmental toxicants such as NEP.

Keywords *N*-Ethyl-2-pyrrolidone (NEP) · Toxicokinetics · Rats · Plasma · Urine · Amniotic fluid

Introduction

N-Ethyl-2-pyrrolidone (NEP; CAS 2687-91-4) is an amphiphilic, aprotic solvent used in a variety of industrial and technical applications. NEP is classified as a reproductive toxicant (Cat. 1B, H360D) in the European Union (EC 2013).

Human metabolism of NEP has been previously investigated and renal conversion factors of the major urinary metabolites, 5-hydroxy-*N*-ethyl-2-pyrrolidone (5-HNEP) and 2-hydroxy-*N*-ethylsuccinimide (2-HESI), were reported (Koch et al. 2014). Based on these metabolites, toxicologically based biological guidance values have recently been derived (UBA 2015) and human biomonitoring of NEP exposures has been performed in occupational and environmental settings (Koslitz et al. 2014; Ulrich et al. 2018). In contrast, basic toxicokinetic data in rats and, most importantly, data on the placental transfer of NEP and its metabolites are missing. For *N*-methyl-2-pyrrolidone (NMP), a structurally related *N*-alkyl-pyrrolidone, such toxicokinetic data in rats have previously been reported including NMP and metabolite levels in plasma (Carnerup et al. 2005). To fill the gap in knowledge for NEP, we studied the toxicokinetics and placental transfer of NEP and its main metabolites in pregnant rats after oral administration on GD 6–19, and in

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non-pregnant rats following single and 14 repeated administrations. In addition, urinary excretion of NEP metabolites was investigated in pregnant rats and in male and female rats after repeated doses over a time period of 28 days.

Materials and methods

Chemicals and reagents

N-Ethyl-2-pyrrolidone (CAS 2687-91-4; 99.4% GC) for dosing was obtained from Tokyo Chemical Industry (Tokyo, Japan); whereas for NEP analysis it was obtained from Tokyo TCI Germany (Eschborn, Germany). Acetonitrile, methanol and water (CHROMASOLV™, LC–MS grade) were purchased from Honeywell Riedel-de Haën (Seelze, Germany). Acetic acid (puriss.) was obtained from Sigma-Aldrich (Steinheim, Germany). Chemicals and reagents used for the analysis of NEP metabolites (including stable isotope labeled internal standards for each metabolite) have been previously described (Schindler et al. 2012).

Animals

Male and female adult Sprague–Dawley rats were obtained from Charles River Laboratories (Saint-Germain-sur-L'Arbresle, France). The animals were allowed to acclimatize to laboratory conditions for 1–2 weeks. Pregnant time-mated rats were obtained by housing primiparous females with males overnight. The day sperm was detected in the vaginal smear was considered to be day 0 of gestation (GD0). Animals were housed individually in clear polycarbonate cages and were kept at 21 ± 2 °C with a relative humidity of 50 ± 5% and a 12-h light/dark photocycle. Filtered tap water and commercial food pellets (UAR Alimentation, Villemoisson, France) were available ad libitum. Rats assigned to urine collection were placed in individual metabolic cages for about 1 day prior to the initiation of the studies for habituation. Within each study, rats were, based on their body weight, assigned to treatment groups by stratified randomization to ensure equal weight distribution among groups.

Repeated oral administrations to pregnant rats

Pregnant rats received a daily dose of NEP by gavage (5 ml/kg of a 10 mg/mL aqueous NEP solution) on 14 consecutive days, from GD6 to GD19. In plasma (maternal and fetal) NEP and its metabolites 5-HNEP and 2-HESI were analyzed; whereas in urine and amniotic fluid samples only 5-HNEP and 2-HESI were analyzed. Rats were administered NEP at a maternally toxic (i.e., reduction in body weight), but developmentally non-toxic dose of 50 mg/kg/day ('exposed group') (Saillenfait et al. 2007a) and a concurrent control group of eight animals received the vehicle (distilled water). Maternal body weight was recorded on GD0 and then every day during the treatment period. Volumes of administration were adjusted to maternal body weight. Blood (maternal and fetal) and amniotic fluid from the exposed group were collected 1, 4, 8, 16, and 24 h after the final dose with four animals at 1 h, five animals at 4, 8, and 16 h and seven animals at 24 h. In case of the control group, samples were collected at 1 h (two animals), 4 h (two animals), and 24 h (four animals). Dams were euthanized by bleeding the abdominal aorta under isoflurane anesthesia. The fetuses were euthanized by decapitation and trunk blood was obtained. The blood samples were collected in heparinized tubes or capillaries and immediately centrifuged at 3500 rpm for 10 min to collect the plasma. All samples of fetal blood or amniotic fluid within a litter were pooled at the time of collection.

In addition, 24-h urine samples were collected for the NEP-treated rats (all seven rats) and controls (three out of four animals). For this purpose, the 24 h time point was used and the collection was performed before the first treatment (T0), after the first treatment (T1, divided into 0–6 h and 6–24 h fractions: T1/0–6; T1/6–24), after the seventh treatment (T7), and after the last treatment (T14, again divided into 0–6 h and 6–24 h fractions: T14/0–6; T14/6–24). Animals were immediately placed in individual stainless-steel metabolic cages after treatment. Metabolic cages were designed to separate urine and feces. Urine was collected at + 4 °C and all samples were stored at −20 °C until analysis.

Single and repeated oral administrations to non-pregnant rats

For the analysis of plasma levels of NEP and its metabolites 5-HNEP and 2-HESI in non-pregnant rats, animals received a single administration or 14 consecutive administrations of NEP at a dose of 50 mg/kg/day (13 administrations in case of the 16 h time point group). Blood was collected 1, 4, 8, and 16 h after the final dose, with 3 animals at each time point. Apart from that, the study was conducted as described for the pregnant rats (vide supra), except for that no urine samples were collected.

Nevertheless, urine samples from a standard repeated dose 28-day oral toxicity study in female (non-pregnant) and male rats were available (Saillenfait et al. 2016). These samples were also analyzed for 5-HNEP and 2-HESI and the results were compared to those obtained in pregnant rats. Rats were administered NEP by gavage on 28 consecutive days at the non-developmentally toxic levels of 5 mg/kg/day (< LOAEL for maternal toxicity) and 50 mg/kg/day (LOAEL for maternal toxicity), as well as 250 mg/kg/day (LOAEL for developmental toxicity) (Saillenfait et al.

2007a). A control group received the vehicle. There were each five males and females per group. Body weights of rats were recorded before each treatment on treatment days (T) 1, 3, 5, 7, 10, 14, 17, 21, 24, 27, and 28. The volume of administration was adjusted to the most recent body weight recorded. 24-h urine samples were collected the day before the first treatment (T0), and after 1, 2, 5, 7, 14, 21, 27, and 28 oral administrations of NEP (T1–T28). Samples from T1, T27, and T28 were divided into fractions 0–6 h and 6–24 h.

Chemical analyses

For the determination of NEP in plasma, the samples were diluted (minimum factor of 5) with water and 300 µL were mixed with 300 µL 0.05% acetic acid. After centrifugation (3450*g*; 10 min), 50 µL were analyzed by HPLC on a partially porous polymer-coated silica gel column with C_{27} modification (Capcell Core AQ, 3.0 × 150 mm, 2.7 µm; Shiseido, Chuo-ku, Tokyo, Japan) coupled with online matrix depletion and analyte enrichment via online-SPE (Oasis HLB, 2.1 × 20 mm, 25 µm; Waters, Eschborn, Germany). NEP was detected by ESI-MS/MS in positive ion mode using MRM detection mode. The limit of quantification (LOQ) was 0.5 µg/L (4.4 nmol/L). For further details on NEP analyses in plasma (including validation and quality control) see Supplementary Material.

The NEP metabolites 5-HNEP and 2-HESI in rat urine, plasma and amniotic fluid were analyzed using a method based on Schindler et al. (2012) and adapted by Ulrich et al. (2018). In short, the metabolites were quantified by stable isotope dilution analysis, using GC–EI-MS/MS after sample clean-up by solid-phase extraction and derivatization (silylation). The LOQs were 2.0 µg/L (14 nmol/L) for 2-HESI and 2.5 µg/L (19 nmol/L) for 5-HNEP. For additional information on quality control see Supplementary Material.

Toxicokinetic calculations and statistics

Areas under the curve (AUC) for the plasma concentration vs. time curves were calculated using the log trapezoidal rule (Rowland and Tozer 2002). For each time point the median concentration of all animals was used for AUC calculations. The body weight-adjusted plasma clearance (CL) of NEP was calculated by dividing the dose (in mg/kg) by the calculated AUC (Toutain and Bousquet-Mélou 2004), assuming 100% biological availability of orally administered NEP. The plasma half-life ($T_{1/2}$) of NEP was estimated using the equation $T_{1/2} = \ln(2)/k$. The kinetic constant (k) was approximated (a) by exponentially fitting the data, and (b) via the equation $k = CL/V_D$ with a body weight-adjusted distribution volume (V_D) calculated via $V_D = D/c_0$ with D being the dose in mg/kg and the approximation $c_0 = c_{max}$ (with c_{max} being the maximum concentration of NEP measured 1 h after treatment) (Byers and Sarver 2009). Comparisons between the different study groups and pairwise comparison between time points were performed using the Mann–Whitney U test. Trends were analyzed using the Jonckheere–Terpstra test. All statistical tests were calculated with SPSS Statistics (Version 25; IBM). The level of statistical significance was set at $p < 0.05$.

Results and discussion

After exposure, NEP and its metabolites 5-HNEP and 2-HESI were detected in all analyzed matrices, i.e., plasma (maternal, fetal, non-pregnant rats), amniotic fluid, and urine; whereas NEP has been investigated in plasma samples only. In the control groups and pre-dose samples, we also detected the NEP metabolites in urine, but only at trace levels and well below those of urine samples from the dose groups. The median concentrations of 5-HNEP and 2-HESI (range) in the control groups and all study days were 0.21 µmol/L (< LOQ-39.3 µmol/L) and 0.11 µmol/L (< LOQ-7.61 µmol/L), respectively. Similar levels were found for 5-HNEP and 2-HESI in all the dose groups on T0: 0.04 µmol/L (< LOQ-0.29 µmol/L) and 0.07 µmol/L (0.03–0.18 µmol/L), respectively. In contrast, the corresponding 5-HNEP concentrations for the dose groups and over all treatment days were 0.62 mmol/L (0.07–1.75 mmol/L) at 5 mg/kg/day, 4.45 mmol/L (0.92–14.2 mmol/L) at 50 mg/kg/day, and 27.7 mmol/L (2.16–87.5 mmol/L) at 250 mg/kg/day. In case of 2-HESI the corresponding concentrations were 0.13 mmol/L (0.02–0.39 mmol/L) at 5 mg/kg/day, 0.71 mmol/L (0.07–4.11 mmol/L) at 50 mg/kg/day, and 1.62 mmol/L (0.06–5.68 mmol/L) at 250 mg/kg/day. In plasma (fetal and maternal) and amniotic fluid of the controls, NEP metabolites (5-HNEP and 2-HESI) could not be quantified. Two pregnant rats from the control group (one each from the 1 h and 4 h time point) had plasma NEP trace levels above LOQ (0.12 and 0.10 µmol/L). Plasma 5-HNEP and 2-HESI concentrations could not be reported for one litter from the 24-h group due to chromatographic interferences. Concentration/time data for NEP in plasma and 2-HESI and 5-HNEP in plasma and amniotic fluid of exposed animals are shown in Fig. 1, the respective concentrations and the derived toxicokinetic data (c_{max}, T_{max}, AUC, CL, $T_{1/2}$) are shown in Tables 1 and 2.

Plasma kinetics in non-pregnant rats

In the non-pregnant female rats, there was no obvious difference in the plasma kinetics of NEP and its two metabolites, 5-HNEP and 2-HESI, between single and repeated administrations (Fig. 1). NEP was rapidly absorbed and metabolized to 5-HNEP. 2-HESI appeared more slowly with a

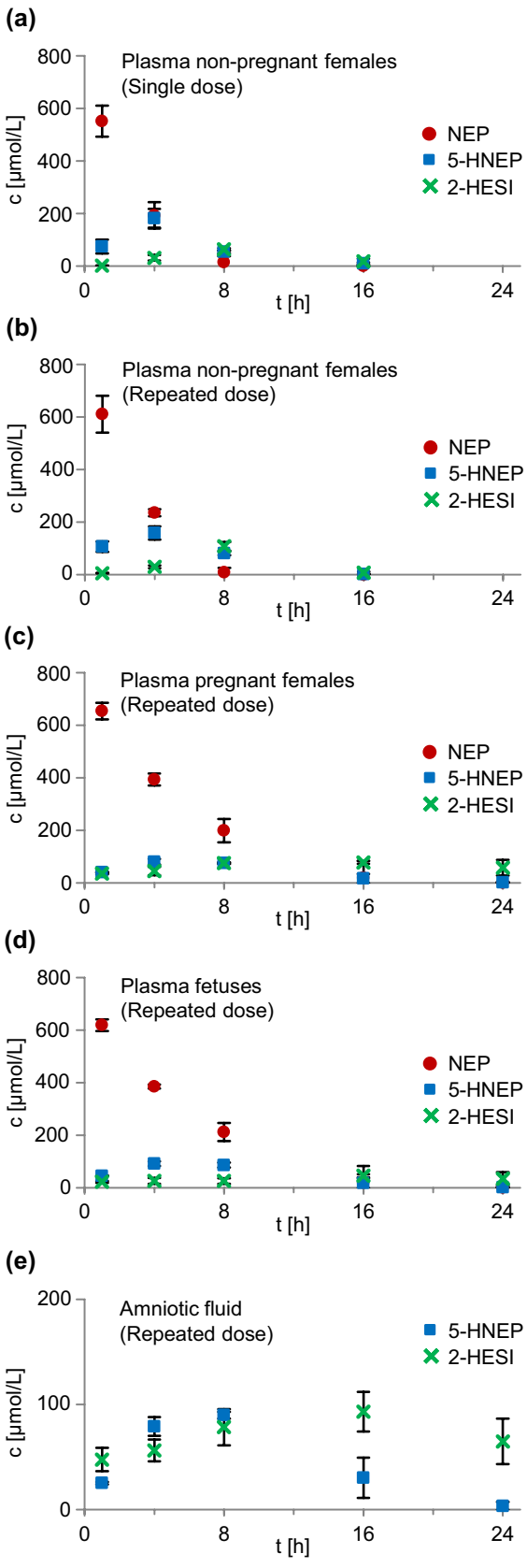

Fig. 1 Median concentrations (error bars: interquartile ranges) of NEP (circles), 5-HNEP (squares), and 2-HESI (crosses) in **a** plasma of non-pregnant female rats after a single administration of NEP, and **b** plasma of non-pregnant female rats, **c** plasma of pregnant rats, **d** plasma of fetuses (pooled per litter), and **e** amniotic fluid (pooled per litter) after 14 repeated NEP administrations

T_{max} of 8 h. Both metabolites were rapidly eliminated from plasma, with no evidence of accumulation after repeated exposures. $T_{1/2}$ for NEP was estimated to ~ 1–2 h (regarding the limited certainty of reported elimination half-lives see Supplementary Material), which is in agreement with an NMP plasma elimination half-life of 0.77 ± 0.04 h after intravenous NMP administration (Payan et al. 2002). The plasma concentration of NEP was negligible at 8 h post-dose and at 16 h for the two metabolites. 5-HNEP was present at higher concentrations than 2-HESI. After a single oral dose of 50 mg/kg/day of NEP to non-pregnant female rats, T_{max} for NEP (1 h) and its metabolites 5-HNEP (4 h) and 2-HESI (8 h) were similar to those of NMP (1 h) and its respective metabolites 5-HNMP (4 h) and 2-HMSI (6–12 h) after a single oral dose of 125 mg/kg/day (Carnerup et al. 2005). Considering the factor of 2.5 between the applied doses in these two studies, c_{max} reported by Carnerup et al. (2005) were in good agreement to those of NEP and 5-HNEP (NMP: 1.2 mmol/L—2.2 times the concentration of NEP; 5-HNMP: 0.42 mmol/L—2.3 times the concentration of 5-HNEP). However, c_{max} of 2-HMSI was much smaller (0.02 mmol/L—only 0.3 times the concentration of 2-HESI). Such a difference in ratios between NEP and NMP metabolites was also observed in urine (vide infra).

Metabolite excretion in urine of (non)pregnant rats

Body weight-adjusted (i.e., corrected for absolute dose) amounts of 5-HNEP and 2-HESI excreted in urine of pregnant and non-pregnant female rats and male rats are shown in Table 3 (dose 50 mg/kg), Table S2 (control group), Table S3 (dose 5 mg/kg), and Table S4 (250 mg/kg) and Fig. S1 (all doses). Clear differences in urinary excretion of the two NEP metabolites between dose groups were observed, with 5-HNEP levels being generally higher than 2-HESI levels at the respective time points. Differences between dose groups were statistically significant for each treatment day and when testing non-pregnant female rats and male rats separately. No significant differences between male and non-pregnant female rats were observed for excreted metabolite amounts in any of the dose groups after 1 to 28 NEP administrations, with the exception of T14 in dose group 50 mg/kg/day for both 5-HNEP and 2-HESI.

Compared to the 5 mg/kg/day dose group, median (T1–T28) excreted metabolite amounts of 5-HNEP and 2-HESI were 10 and 7.1 (males) and 11 and 8.1

Table 1 Median concentrations (range) of NEP and its metabolites 5-HNEP and 2-HESI in plasma and amniotic fluid samples

	Median concentrations (range) (µmol/L)				
	Time after dose				
	1 h	4 h	8 h	16 h	24 h
NEP					
Plasma—non-pregnant rats, Single dose	551 (472–590)	193 (122–223)	15.0 (4.12–48.0)	0.75 (0.24–1.20)	–
Plasma—non-pregnant rats, Repeated dose	611 (543–684)	236 (210–237)*[a]	9.37 (5.48–38.1)*[a]	0.03 (0.03–0.04)*[a]	–
Plasma—pregnant rats Repeated dose	653 (631–698)	393 (304–475)*[a]	199 (180–247)*[a]	19.3 (7.97–79.4)*[a]	0.81 (0.15–3.68)
Plasma—fetuses Repeated dose	619 (563–647)	385 (354–443)	212 (159–233)	19.4 (6.31–85.3)	0.81 (0.16–3.92)
5-HNEP					
Plasma—non-pregnant rats, Single dose	74.4 (38.2–90.8)	182 (144–216)	53.9 (45.8–55.0)	9.73 (4.70–14.5)	–
Plasma—non-pregnant rats, Repeated dose	106 (70.6–110)	158 (145–195)*[a]	81.2 (78.2–94.0)	1.26 (1.09–4.68)*[a]	–
Plasma—pregnant rats Repeated dose	40.8 (38.5–41.7)*[c]	80.5 (66.8–83.2)*[a],**	75.3 (70.6–105)**	16.7 (14.8–33.7)*[a]	2.12 (1.46–5.77)
Plasma—fetuses Repeated dose	45.3 (41.3–45.3)*[d]	91.7 (79.2–95.3)*[d],**	86.3 (77.2–119)**	17.6 (16.8–41.1)	2.06[#] (1.79–6.13)
Amniotic fluid Repeated dose	25.2 (23.5–26.4)*[c]/*[d]	79.0 (70.3–82.6)*[d]	89.8 (81.6–126)	30.2 (23.2–46.2)	3.42 (2.93–9.12)
2-HESI					
Plasma—non-pregnant rats, Single dose	1.66 (0.71–1.81)	31.5 (21.0–42.3)	63.8 (56.1–65.2)	18.3 (6.31–25.5)	–
Plasma—non-pregnant rats, Repeated dose	5.49 (5.46–8.16)	29.9 (24.2–35.7)	108 (97.9–130)*[a]	7.60 (6.68–15.8)*[a]	–
Plasma—pregnant rats Repeated dose	35.8 (33.7–39.4)*[b]/*[c]	44.9 (30.5–51.9)*[b]	75.3 (55.4–77.4)*[a]/*[b],**	77.3 (63.5–81.4) **[a]/*[b],**	58.1 (34.0–77.5)
Plasma—fetuses Repeated dose	22.4 (18.3–28.8)*[b]/*[d],***	26.5 (11.0–37.3)*[b]/*[d],***	26.2 (17.8–49.2)*[b]/*[d],***	45.7 (38.0–46.7)*[b]/*[d],***	34.9[#] (18.0–78.0)***
Amniotic fluid Repeated dose	47.5 (41.8–59.5)*[c]/*[d]	56.2 (40.6–76.8)*[d]	78.4 (53.1–91.8)*[d]	93.2 (69.4–106)*[d]	64.8 (42.0–85.9)

*Significant difference (Mann–Whitney *U*, $p < 0.05$); **no significant difference (Mann–Whitney *U*, $p > 0.05$) between time points; ***concentration at 16 h significantly different (Mann–Whitney *U*, $p < 0.05$) from 1 h and 4 h but not significantly different from 8 h ($p = 0.151$); no significant difference between 16 h and 24 h (Mann–Whitney *U* test; $p = 0.429$) or any other combination of time points (1 vs. 4 h, 1 vs. 8 h, 1 vs. 24 h, 4 vs. 8 h, 4 vs. 24 h, 8 vs. 24 h; Mann–Whitney *U*, $p > 0.05$)

[#]5-HNEP and 2-HESI metabolite levels only available for 6 out of 7 litters

[a]Pregnant and non-pregnant rats (repeated dose); [b]Pregnant rats and fetuses; [c]Pregnant rats and amniotic fluid; [d]Fetuses and amniotic fluid

(non-pregnant females) times higher in the 50 mg/kg/day dose group (factor 10 between doses); whereas they were 69 and 16 (males) and 73 and 21 (non-pregnant females) times higher in the 250 mg/kg/day dose group (factor 50 between doses). For NMP, Carnerup et al. (2005) reported a nearly complete urinary elimination of 5-HNMP and 2-HMSI (99–100%) within 24 h after a single oral NMP dose in non-pregnant female rats. Even in the 500 mg/kg/day dose group of Carnerup and co-workers a nearly complete elimination was observed although maximum plasma levels of both metabolites and maximum urinary 2-HMSI levels were reached with delay in this higher dose. The respective median recoveries (renal conversion factors) for 5-HNMP and 2-HMSI were 48% and 5.1% (at 125 mg/kg NMP) and 48% and 2.3% (at 500 mg/kg NMP). Accordingly, in our case of NEP, a nearly complete urinary elimination of the corresponding metabolites (5-HNEP and 2-HESI) within 24 h after dosage can be assumed. In our study, median 24-h renal conversion factors ($F_{ue\ 24\ h}$, i.e., percentage of dose recovered as the respective metabolite in urine within 24 h) after the first treatment for 5-HNEP and 2-HESI were 36.5% and 8.1% (ratio 5-HNEP/2-HESI: 82/18; males; 5 mg/kg/day), 29.2% and 6.1% (83/17; females; 5 mg/kg/day), 37.8% and 6.5% (85/15; males;

Table 2 Toxicokinetic parameters for NEP and its metabolites 5-HNEP and 2-HESI in plasma for the dose group 50 mg/kg/day: peak concentration (c_{max}) – median and range; time of peak concentration (T_{max}); areas under the curve (AUC) for the plasma concentration vs. time curves; body weight-adjusted plasma clearance (CL); estimated plasma half-life ($T_{1/2}$)

	c_{max} (µmol/L)	T_{max} (h)	AUC (µmol h/L)	CL (L/(h kg))	$T_{1/2}$ (h)
NEP					
Non-pregnant rats Single dose	551 (472–590)	1	1616	0.27	~1–2
Non-pregnant rats Repeated dose	611 (543–684)	1	1781	0.25	~1–2
Pregnant rats Repeated dose	653 (631–698)	1	3666	0.12	~2–4
Fetuses Repeated dose	619 (563–647)	1	3640	0.12	~2–4
5-HNEP					
Non-pregnant rats Single dose	182 (144–216)	4	~1050[b]	n.a.[d]	n.a.[d]
Non-pregnant rats Repeated dose	158 (145–195)*[e]	4	~1050[b]	n.a.[d]	n.a.[d]
Pregnant rats Repeated dose	80.5[a] (66.8–83.2)*[e]	4–8	881	n.a.[d]	n.a.[d]
Fetuses Repeated dose	91.7[a] (79.2–95.3)	4–8	988	n.a.[d]	n.a.[d]
2-HESI					
Non-pregnant rats Single dose	63.8 (56.1–65.2)	8	~550[b]	n.a.[d]	n.a.[d]
Non-pregnant rats Repeated dose	108 (97.9–130)*[e]	8	~650[b]	n.a.[d]	n.a.[d]
Pregnant rats Repeated dose	77.3[a] (63.5–81.4)*[e]/*[f]	8–16	≫ 1500[c]	n.a.[d]	n.a.[d]
Fetuses Repeated dose	45.7[a] (38.0–46.7)*[f]	(16 (− 24))[h]	≫ 800[c]	n.a.[d]	n.a.[d]

*Significant difference (Mann–Whitney U test, $p < 0.05$)

[a]Reported for time point with highest median level; [b]Elimination incomplete; [c]Elimination far from complete; [d] *n.a.* not applicable; [e]Pregnant and non-pregnant rats; [f]Pregnant rats and fetuses

Table 3 Body weight-adjusted amounts (median and range) of 5-HNEP and 2-HESI excreted in urine within 24 h for the dose group 50 mg/kg/day

Treatment	5-HNEP [µmol/(kg 24 h)]			2-HESI [µmol/(kg 24 h)]		
	Pregnant females	Non-pregnant females	Males	Pregnant females	Non-pregnant females	Males
T1	167 (138–174)	176 (110–188)	167 (148–242)	32.2 (25.3–40.8)	33.1 (29.2–37.8)	28.7 (22.9–41.2)
T5	–	176 (145–216)	166 (155–216)	–	26.1 (24.3–42.5)	24.5 (15.2–30.8)
T7	172 (86.2–192)	193 (167–203)	173 (127–207)	34.3 (23.4–56.6)	29.4 (25.0–45.9)	25.2 (8.25–28.4)
T14	100 (70.1–120)	196 (186–215)	173 (145–186)	51.4 (35.9–69.7)	41.3 (32.9–71.9)	18.6 (15.2–37.3)
T21	–	170 (151–271)	199 (164–220)	–	35.6 (26.2–65.3)	33.8 (20.1–49.8)
T27	–	197 (157–210)	204 (169–249)	–	44.5 (35.6–84.9)	35.9 (21.7–48.4)
T28	–	197 (163–233)	162 (158–274)	–	49.2 (46.8–93.5)	44.2 (22.6–64.2)
T1–28	154 (70.1–192)	191 (110–271)	174 (127–274)	35.9 (23.4–69.7)	36.3 (24.3–93.5)	28.0 (8.25–64.2)

50 mg/kg/day), 39.8% and 7.5% (84/16; females; 50 mg/kg/day), 50.0% and 2.7% (95/5; males; 250 mg/kg/day), and 53.3% and 3.5% (94/6; females; 250 mg/kg/day). The tendency of higher 5-HNEP fractions with increasing dose suggested a saturation of metabolic processes leading to the formation of 2-HESI. This is in agreement with a previous study (Payan et al. 2002), which suggested a saturation of NMP metabolism after dermal and intravenous administration at high doses. Conversion factors in the 250 mg/kg/day dose group of this study and those reported for NMP were very similar.

Interspecies comparison (rodent/human) of the data

We previously reported a complete urinary elimination of 5-HNEP within 3–4 days and an incomplete 2-HESI elimination within 4 days after a single oral dose of NEP (Koch et al. 2014). For this purpose, three volunteers were given 220–252 µg NEP/kg and the median (range) $F_{ue\ 24\ h}$ was determined to 26.4% (24.0–27.8%) and 6.9% (5.2–8.9%) for 5-HNEP and 2-HESI, respectively. The corresponding median 96-h renal conversion factors were 28.9% (27.8–30.1%) and 21.6% (17.2–26.9%). For human NMP metabolism after single oral dosages (three volunteers, 100 mg absolute dose each), Akesson and Jönsson (1997) reported a complete urinary elimination of 5-HNMP and 2-HMSI within 2 and 6 days. The average dose recoveries (144-h renal conversion factors) were 44% and 20%, respectively. The mean renal conversion factors (F_{ue}) for 5-HNEP in rats were similar to those in humans (1.0- and 1.4-fold higher) at the two lower doses investigated, but 1.7- to 1.8-fold higher at 250 mg/kg/day. Conversely, F_{ue} for 2-HESI was generally lower in rats compared to humans (0.28- to 0.38-fold at the lower doses and 0.13- to 0.16-fold at 250 mg/kg/day). Similar interspecies differences have been previously reported when comparing human and rodent data for the metabolism of NMP (Carnerup et al. 2005; Akesson and Jönsson 1997). In example, F_{ue} of 5-HNMP was only 1.1-fold higher in rats compared to humans; whereas F_{ue} of 2-HMSI was much lower in rats (0.12- and 0.05-fold). Considering the suggested saturation of NEP (and NMP) metabolism at high doses, the observed differences between rats and humans in the ratios of renally excreted 5-HNEP (5-HNMP) and 2-HESI (2-HMSI) might be, at least in part, attributable to differences in the doses investigated. However, interspecies differences in toxicokinetics, e.g., a slower elimination of 2-HESI and 2-HMSI in humans, might also contribute.

Effect of pregnancy on plasma kinetics

There were marked differences between non-pregnant and pregnant rats at late gestation. After repeated administration, elimination of NEP from plasma was slower in pregnant rats compared to non-pregnant rats (Fig. 1), with AUCs and $T_{1/2}$ twice as high in pregnant rats (Table 2). Plasma NEP (median concentrations) ratios between pregnant and non-pregnant rats (repeated dose) were 1.07 (1 h), 1.67 (4 h), 21.2 (8 h), and 643 (16 h). The kinetics of the two main NEP metabolites was also affected. There were a limited and/or delayed formation of 5-HNEP and 2-HESI in pregnant rats, as suggested by an observed reduction of c_{max} by a factor of 2.0 and 1.4, respectively; whereas T_{max} was increased (Table 2). In addition, the clearance of 2-HESI from plasma was reduced in pregnant rats and a decrease of 2-HESI in plasma was only observed 24 h post-dose (Fig. 1). These results are also clearly reflected in the urinary excretion of 5-HNEP (Table 3). Here, the plasma 5-HNEP (median concentrations) ratios between pregnant and non-pregnant rats (repeated dose) were 0.38 (1 h), 0.51 (4 h), 0.93 (8 h), and 13.3 (16 h). In case of 2-HESI, the ratios were 6.52 (1 h), 1.50 (4 h), 0.70 (8 h), and 10.2 (16 h). This outcome might be related to reduced hepatic levels of several cytochrome P450 (CYP) enzymes in rats during pregnancy including CYP2E1 (He et al. 2005), an enzyme which is known to contribute to both human and rat NMP metabolism (Ligocka et al. 2003) and might also contribute to the metabolism of NEP. In addition, further physiological changes (e.g., in the distribution volume, glomerular filtration rate) are known to occur during pregnancy (Krauer 1987) and might also contribute to the observed differences in NEP toxicokinetics between pregnant and non-pregnant rats. Finally, body weight-based doses can lead to an effective overdosing of dams in late gestation, especially so in case of xenobiotics with low placental transfer (Boike et al. 1989; Payan et al. 1990). However, this is probably less of a problem here, given the even distribution of NEP and 5-HNEP between maternal and fetal plasma and between maternal plasma and amniotic fluid (vide infra).

Placental transfer of NEP and its metabolites

The placental transfer of NEP on GD 19 was rapid. Both NEP and 5-HNEP in fetal plasma were similar to those in maternal plasma already 1 h after dosage (Table 1; Fig. 1). Most of the non-metabolized NEP was cleared from maternal and fetal plasma 16 h after dosage and only ~ 3% were found when compared to the level at 1 h. A further decrease to about 0.1% could be traced after 24 h thus suggesting a nearly complete clearance of NEP from plasma. The kinetic profiles of NEP and 5-HNEP were comparable in the mothers and the fetuses (Fig. 1) and concentrations (c_{max} and AUC) in maternal and fetal plasma were very similar (Table 2). The median ratios of fetal/maternal plasma NEP concentrations were 0.95 (1 h), 0.98 (4 h), 1.07 (8 h), 0.99 (16 h), and 1.00 (24 h). The corresponding ratios of 5-HNEP were 1.11 (1 h), 1.14 (4 h), 1.15 (8 h), 1.05 (16 h), and 0.97 (24 h). This result is of particular concern because NEP has developmental effects which are comparable to those of NMP in rats and the effects of NMP had been mainly attributed to the parent compound rather than its metabolites (Flick et al. 2009; Saillenfait et al. 2007b). There was no accumulation of NEP or 5-HNEP in the fetus or amniotic fluid. When considering a potential saturation of NEP metabolism (as discussed for non-pregnant rats above), combined exposures with other xenobiotics (e.g., NMP) which are metabolized via common metabolic pathways might also

change NEP elimination kinetics, thus possibly increasing its toxicity.

In contrast to 5-HNEP, significantly lower 2-HESI concentrations were found in fetal plasma compared to the dams with the exception of the 24-h time point (Table 1; Fig. 1). The fetal/maternal ratios of the median plasma 2-HESI concentrations were 0.63 (1 h), 0.59 (4 h), 0.35 (8 h), 0.59 (16 h), and 0.60 (24 h). The results suggest a low-to-moderate placental transfer of 2-HESI and/or a limited metabolic capacity of the fetal cytochrome P450 (CYP450) oxidase system. In example, marked differences between fetal and adult CYP450 content and activity levels have been previously described, including reduced hepatic CYP2E1 levels (Hines 2008; Johnsrud et al. 2003). Similarly, differences in the substrate specificity between fetal and adult CYP2E1 have been reported (Carpenter et al. 1997). Therefore, assuming a reduced CYP2E1 activity towards NEP in fetuses, the otherwise similar 5-HNEP levels (especially ≥ 8 h after dosage) in fetal and maternal plasma could also point to a good placental transfer of 5-HNEP itself rather than fetal metabolism of NEP only.

NEP metabolites in amniotic fluid

Both, 5-HNEP and 2-HESI, were found in amniotic fluid at the end of pregnancy. Concentration/time data for 5-HNEP concentrations were similar to those in plasma from dams and fetuses (Fig. 1; Table 1). 5-HNEP was nearly completely cleared from amniotic fluid after 24 h. 2-HESI concentrations in amniotic fluid were slightly but consistently higher than maternal plasma concentrations and the ratios of median 2-HESI concentrations of maternal plasma vs. amniotic fluid were 0.75, 0.80, 0.96, 0.83, and 0.90 at 1, 4, 8, 16, and 24 h, respectively. In contrast, the concentrations of 2-HESI in amniotic fluid were clearly higher compared to those in fetal plasma. The ratios of median 2-HESI concentrations of fetal plasma vs. amniotic fluid were 0.47 (1 h), 0.47 (4 h), 0.33 (8 h), 0.49 (16 h), and 0.54 (24 h). Moreover, 2-HESI was not completely cleared from amniotic fluid within 24 h. Interactions between the fetus and the amniotic fluid (e.g., through fetal urine production and deglutition), and differences in pH between fetus, amniotic fluid, and maternal blood may contribute to this distribution pattern of 2-HESI in the maternal–fetal compartment.

Conclusions

Overall, our data show similarities between the metabolism of NEP and NMP in non-pregnant rats. Specifically, our study proofs efficient placental transfer of NEP and most likely also 5-HNEP based on our additional data in different compartments of the maternal/fetal unit in pregnant rats. Finally, data comparison between pregnant and non-pregnant rats shows a much slower plasma elimination of NEP and a delayed formation of 5-HNEP and 2-HESI in pregnant rats compared to non-pregnant rats, thus suggesting that altered toxicokinetics during pregnancy needs to be addressed in risk assessment.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The animal facilities used in the study have been accredited by the French Ministry of Agriculture. All animal experiments complied with European Union Directive 2010/63/EU and French legislation for the protection of animals used for scientific purposes and were approved by the local ethical committee and the French Ministry of Superior Education and Research.

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