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ABOLITION OF VALPROATE-DERIVED CHOLERESIS IN THE MRP2 TRANSPORTER-DEFICIENT RAT

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Running Title:

Valproate-induced choleresis is mediated by Mrp2

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Abbreviations: VPA, valproic acid; NaVPA, sodium valproate; VPA-G, valproate-acyl-glucuronide; GC/MS, gas chromatography-mass spectrometry

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Abstract

Valproic acid (VPA) is a major therapeutic agent in the treatment of epilepsy and other neurological disorders. It is metabolized in humans and rats primarily along two pathways: direct glucuronidation to yield the acyl glucuronide (VPA-G) and β-oxidation. We have shown much earlier, in the Sprague-Dawley rat, that i.v. administration of NaVPA caused a marked choleresis (mean 3.3 times basal bile flow after doses of 150 mg/kg), ascribed to the passive osmotic flow of bile water following excretion of VPA-G across the canalicular membrane. Active biliary pumping of anionic drug conjugates across the canalicular membrane is now believed to be attributable to transporter proteins, in particular Mrp2, which is deficient in the TR^- (a mutant Wistar) rat. In the present study, normal Wistar and Mrp2-deficient TR^- rats were dosed i.v. with NaVPA at 150 mg/kg. In the Wistar rats, there was a peak choleretic effect of about 3.2 times basal bile flow, occurring at about 30-45 min post dose (as seen previously with Sprague-Dawley rats). In TR^- rats given the same i.v. dose, there was no evidence of post-dose choleresis. The choleresis was correlated with the excretion of VPA-G into bile. In Wistar rats, $62.8 \pm 7.7\%$ of the NaVPA dose was excreted in bile as VPA-G, whereas in TR^- rats, only $2.0 \pm 0.6\%$ of the same dose was excreted as VPA-G in bile (with partial compensatory excretion of VPA-G in urine). This study underlines the functional (bile flow) consequences of biliary transport of xenobiotic conjugated metabolites.

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Valproic acid (VPA) is a commonly prescribed anti-epileptic drug, which is metabolized primarily by direct glucuronidation to form the acyl glucuronide (VPA-G) as well as by β-oxidation and a myriad of other more minor pathways (Granneman GR et al., 1984; Dickinson et al., 1989; Rettenmeier et al., 1989; Fischer et al., 1992).

Previously, we showed that the i.v. administration of VPA to rats, dogs and monkeys was associated with a startling choleretic effect: in Sprague-Dawley rats administered 15 and 150 mg NaVPA/kg i.v., peak increases in bile flow were approximately 1.5 and 3.3 fold respectively, within one hour of drug administration (Dickinson et al., 1979). In monkeys, the magnitude of the choleretic increments induced by NaVPA and the concentration of conjugated VPA excreted in such bile increments were similar to those found in rats (Dickinson et al., 1980). Additionally, whilst plasma VPA concentrations were correlated with the magnitude of choleresis, a better correlation was found between the bile VPA-conjugate concentrations and the magnitude of choleresis. We later ascribed the choleretic effect to be due to the osmotic effect of bile water flow driven by the excretion of VPA metabolites (primarily VPA-G) across the canalicular membrane into bile (Dickinson et al., 1982). Other researchers have also commented on such drug-induced choleretic effects (Watkins and Klaasen, 1982) and speculated on the underlying mechanism(s).

Advances in the past decade have implicated ATP-dependent transport proteins, in this case the Mrp2 organic anion transporter, as being primarily responsible for the export of intracellular organic anions (including intrahepatically-synthesized glucuronides) across a steep concentration gradient into bile. The role of the Mrp2 transporter in the excretion of conjugates from hepatocytes to the biliary tree has been elucidated in part with the advent of mutant rat strains deficient in this transporter. Similarly to the human genetic deficiency for

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Dubin-Johnson Syndrome, TR^- rats are a mutant strain of Wistar rats which are deficient in the hepatic apical transporter protein Mrp2 [MRP2 or ABCC2 in humans] (Jansen et al., 1985) due to a point mutation in the gene encoding for this protein. Mrp2 transporter proteins in hepatic cells are responsible for the ATP-dependent active transport of organic anions, in particular glutathione, glucuronide and sulphate conjugates of endogenous and exogenous compounds. Mrp2 is also associated with bile-acid-independent changes to bile flow, due to the Mrp2-mediated efflux of glutathione (Oude Elferink, 1989).

The purpose of this study was to investigate whether the remarkable choleretic effects seen after i.v. administration of NaVPA were attributable, in whole or part, to the hepatic Mrp2 apical organic anion transporter, by studying Wistar and TR^- rats.

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Materials and Methods

Sodium valproate (NaVPA), nonanoic acid and heparin (porcine–derived) were purchased from Sigma Chemical Company (Sydney, Australia). Isoflurane (Forthane) and pentobarbital sodium (Nembutal) were purchased from Abbott Australasia Pty Ltd (Kurnell, Australia) and Rhone Merieux Australia Pty Ltd (Pinkenba, Australia), respectively. HPLC-grade chloroform and methanol were purchased form EM Science (Gibbstown, NJ) and Unichrom (Seven Hills, Australia), respectively. All other reagents and solvents were of analytical grade.

Animals. Male Wistar and TR^- rats (340-400 g) were purchased from The University of Queensland Central Animal Breeding House (Pinjarra Hills, Australia) and Westmead Millennium Institute (Westmead, Australia) respectively, and were housed in a 12 h light/dark cycle in a well ventilated room at 21°C, and maintained on standard rat chow and water *ad libitum*. The University of Queensland Animal Ethics Committee granted ethics approval for this study.

Surgery. All rats had i.v. and bile duct catheters inserted whilst maintained under deep and stable isoflurane anaesthesia (2.5% in air). [Details of catheter preparation have been reported previously (Dickinson et al., 1979).] Briefly, the right jugular vein was exposed and ligated; the silastic portion of the jugular catheter (filled with heparinized saline) was inserted into the superior vena cava through a small incision at the bifurcation of the interior and exterior jugular veins. The liver and intestines were exposed by an abdominal midline incision and the bile duct cannulated. The polyethylene portion of the jugular catheter and the bile-duct catheter were passed subcutaneously to the back of the animal, exiting between the scapulae, where they were protected by a flexible stainless steel spring seated in a subcutaneous pocket

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with Michel suture clips (12 mm). Surgery was completed within 45 min. The animals were then placed in stainless steel metabolism cages, and given 1.5-2 h to recover from the inhaled anaesthetic and to stabilize bile flow.

Drug administration and sample collections. Each experimental group contained 4 rats. Experiments were initiated by an i.v. bolus over 60 s of 1 ml 0.9% saline (control) or 50 mg NaVPA/ml saline to a dose of 150 mg/kg; zero time was set as the midpoint of the 60 s bolus-dosing period. Blood samples (0.3 ml) were collected from test animals at the following times; pre-dose and 6, 12, 25, 40, 60, 80, 100, 120, 150, 180, 210, 240, 300 min post-dose. Blood volumes taken were replaced by isotonic saline (0.3 ml) followed by heparinized saline (0.1 ml), to maintain patency of i.v. cannulae. Bile was collected in 5 ml polypropylene tubes. Bile flow rate was monitored for 40 –120 min pre-dosing to ensure a stable basal bile flow for each rat. Bile was collected in increments at the following times: 0-15, 15-30, 30-45, 45-60, 60-90, 90-120, 120-150, 150-180, 180-240 and 240-300 min. Normal saline was infused i.v. in volumes equal to bile volumes collected to replace lost fluid. Urine was collected into 50 ml polypropylene tubes over ice for the 5 h duration of the experiment. Plasma, bile and urine samples were stored at –20°C prior to gass chromatography-mass spectrometry (GC/MS) analysis.

Analytical Methods. Analysis of VPA and VPA-G in plasma, bile and urine was performed by minor modification of the validated methods described previously (Dickinson et al., 1979, 1986; Hooper et al., 1996). Briefly, to 50 µl of plasma, bile or urine was added 150 µl water, 100 µl internal standard (nonanoic acid 500 µg/ml of water), and a solution of 4 M NaCl/ 1 M HCl to a final volume of 800 µl. After mixing (vortex, 30 s) VPA was extracted into 800 µl of chloroform. The mixture was equilibrated, centrifuged and 1.5 µL of the chloroform layer

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was injected into the GC/MS. VPA-G was measured as free VPA after alkaline hydrolysis. Solutions of 50 μl plasma, bile or urine, 150 μl water and 100 μl 1 M NaOH were heated at 60°C for 30 min. After cooling samples were made to a final volume of 800 μl with a solution of 4 M NaCl/1M HCl, and VPA extracted using chloroform as described above. Plasma, bile and urine samples that had high concentrations of analytes were diluted for re-assay using blank biological fluids. In a separate analytical experiment, the concentrations of the 3-oxo and 4-hydroxy metabolites of VPA in urine were determined by GC/MS as described earlier (McLaughlin et al., 2000)

The GC/MS system comprised a Hewlett-Packard Model 7673 autoinjector, a model 5890 Series II GC, and a Model 5971 MS. System control and data acquisition were performed using the PC based HP-MS Chemstation G1034 software.

Statistical Analyses. Statistical analyses were performed using two-tailed unpaired t-test with Welch's correction (for unequal variances) as incorporated in the Prism® statistical analysis tools. $P < 0.05$ was considered as significant. Mean data are expressed as means ± SD.

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Results

Administration of NaVPA (150 mg/kg) i.v. to bile duct cannulated male Wistar rats (n = 4) induced a marked choleretic effect, increasing the mean bile flow rate to 3.2 times that of the mean basal bile flow (Fig. 1) at 30-45 min post-dose. Control rats (n = 4) administered isotonic saline i.v. had no increase in bile flow rate, only a gradual decline with time (Fig. 1). Mutant TR^- rats (n = 4) administered 150 mg/kg NaVPA i.v. showed a small insignificant increase in the mean rate of bile flow (3 of the 4 rats exhibited gradual decline in bile flow after administration of NaVPA, whilst one rat had an apparent 30% increase above basal flow rate at 15-30 min followed by a gradual decline in bile flow, as observed for the other 3 rats in this group) (Fig. 1). TR^- rats (n = 4) administered i.v. isotonic saline exhibited only a gradual decline in bile flow.

The biliary excretion rates (mg VPA equiv./h) of total VPA (predominantly VPA-G) for each bile collection time for both Wistar and TR^- rats administered 150 mg NaVPA/kg i.v. are shown in Fig 2. VPA-G excretion rate in bile of Wistar rats was well correlated with the bile flow rate (r = 0.986). The total amount of VPA-G excreted into bile over the 5 h experiment represented 62.8 ± 7.7% (Table 1) of the administered dose. This is significantly ($P<0.008$) greater than that seen for TR^- rats, where only 2.0 ± 0.6% of the same dose of NaVPA was excreted in bile as VPA-G. Similarly, a significantly ($P< 0.03$) higher percentage of the i.v. NaVPA dose was found as unconjugated VPA in the Wistar rat bile than in TR^- rat bile (1.8 ± 0.7% and 0.4 ± 0.1% respectively, Table 1).

Mean VPA plasma concentration-time profiles after i.v. administration of NaVPA were not significantly different between Wistar and TR^- rats at all time points over the 5 h experiment (Fig. 3). However, mean VPA-G plasma concentration-time profiles were markedly different

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between Wistar and TR^- rats. VPA-G concentrations in Wistar rat plasma increased slowly to a peak mean concentration of 37 ± 22 µg VPA equiv./ml at 40 min (Fig. 3). In contrast, the mean concentrations of VPA-G in plasma of TR^- rats increased rapidly to a peak of 186 ± 84 µg VPA equiv./ml at 12 min. The mean area under the VPA-G plasma concentration-time curve for TR^- rats (10500 ± 520 µg VPA equiv..min/ml) was significantly ($P<0.0001$) greater (*ca*. 5-fold) than for Wistar rats (2150± 900 µg VPA equiv..min/ml).

There were also significant ($P<0.02$) differences between the mean urinary recoveries of total VPA (predominately VPA-G in urine). Wistar rats excreted 24.4 ± 9.5% of the NaVPA dose into urine, less than half that excreted by TR^- rats (52.4 ± 11.7%) (Table 1.). In separate analytical work, the recoveries of the 3-oxo and 4-hydroxy metabolites of VPA in urine were measured and found to be not significantly different between Wistar (1.94 ± 1.52% and 0.33 ± 0.17%, respectively) and TR^- rats (2.39 ± 1.41% and 0.38 ±0.08%, respectively).

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Discussion

The basal bile flow rate of TR^- rats (13.1 µL/min, n=8) was reduced to 75% of the basal bile flow rate of Wistar rats (17.4 µL/min, n=8), as previously reported by Jansen et al. (1985). NaVPA administration (150 mg/kg i.v.) to male Wistar rats induced a dramatic increase in the biliary flow rate, approximately 3.2 times the basal bile flow rate at 30-45 min, (identical in magnitude and extent to that seen previously in male Sprague-Dawley rats (Dickinson et al., 1979)). The subsequent return to basal bile flow levels paralleled the elimination of VPA from plasma. In TR^- rats however, i.v. NaVPA administration did not increase the bile flow rate, and no correlation could then be made between plasma VPA concentrations and bile flow rate. Thus the hepatic Mrp2 transporter protein, deficient in these mutant Wistar rats, appears to be required for induction of VPA-derived choleresis.

VPA itself cannot be directly responsible for the choleretic effect in Wistar rats, as the plasma VPA concentration-time profiles for Wistar and TR- rats are not significantly different. However, the rate of VPA-G excretion into bile of Wistar rats was linearly correlated ($r = 0.986$) with the bile flow rate (compare Figs 1 and 2). In contrast, Mrp2 deficient TR^- rats excreted minimal amounts of VPA-G (2.0% of the administered NaVPA dose) into bile with no apparent choleretic effect. The presence of a small amount of VPA-G in the bile of TR^- rats implicates either another organic anion transporter protein, or passive diffusion. In this regard, it is worth noting that small amounts of VPA (also an organic anion) were found in the bile of both Wistar and TR^- rats (1.8% and 0.4%, respectively, of the administered dose).

The increase in bile flow of Wistar rats correlates with biliary excretion of the glucuronide metabolite. Whether the glucuronide itself is directly responsible for the osmotic flow of bile

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water cannot be confirmed from the current data. However, our earlier work (Dickinson at al.; 1982) found that during peak choleresis the 'anion gap' (measured difference between the positively charged [Na^+, K^+] and negatively charged [Cl^-, HCO_3^-] electrolytes was appreciably different to controls, 44.7 and 34.1 µEq/mL respectively. VPA-G like most acyl glucuronides are acidic at physiological pH and would be expected to be 99% ionized in bile, and as such VPA-G in bile could account for the 'anion gap' and Jäger et al.(2003) recently reported flavopiridol-induced choleresis (24% above basal) in isolated perfused rat livers, attributed to an increase in bile water due to the osmotic gradient created by the Mrp2-mediated excretion of flavopiridol glucuronide metabolites. It seems unlikely that the magnitude of the increase in bile flow (*ca.* 300%) seen in Wistar rats is due to another factor such as increased co-excretion of a major determinant of bile-acid independent flow eg. glutathione, as was found with the endothelin receptor antagonist bosentan (Foussier et al., 2002).

In TR^- rats as compared to Wistar rats, a much greater proportion of the administered dose was excreted as VPA-G in urine (50% vs 23%, Table 1). This presumably reflects enhanced secretion of VPA-G formed in the TR^- rat liver into blood as a result of blocked secretion via Mrp2 into bile. A contribution from up-regulation of Mrp3 (an organic anion transporter located on the sinusoidal membrane of hepatocytes) in the TR^- rats (Hirohashi et al., 1999, Cao et al., 2002, Donner and Keppler, 2001) may contribute to the secretion of VPA-G into the blood of TR- rats. Clearly, the kidneys are the primary site of excretion of VPA-G in Mrp2 deficient bile-exteriorized TR^- rats. Although Mrp2 transporters are also found in renal cells, the work of de Vries et al. (1989) using napthol-B-D-glucuronide as a model compound, showed that the genetic defect in TR^- rats is restricted to impaired hepato-biliary excretion.

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Considerably less of the dose was recovered in total bile plus urine in TR- rats than in Wistar rats as VPA plus VPA-G in 5 h (55% vs 89%, Table 1). Presumably, a contribution to the low recovery in TR^- rats is the small urinary collection time (although Wistar rat plasma concentrations of VPA-G were not detectable after 120 min, in TR^- rat plasma VPA-G was still detectable (0.7 μg/ml) at 300 min). Additionally, hepatic Mrp3 anion transporter protein excretion of VPA-G into the systemic circulation may represent a rate-limiting step in animals deficient in the hepatic Mrp2 transporter, though the rate of clearance of VPA from plasma is identical for both Wistar and TR^- rats, indicating no rate-limiting steps in hepatic uptake and metabolism of VPA. Jäger and colleagues (2003) reported a similar but lesser discrepancy in flavopiridol recovery from Wistar and TR- isolated perfused rat livers, (44% and 34%, respectively) which was speculated to be due to the higher average liver weights in TR- rats.

In summary, we have demonstrated that the choleresis induced by i.v. NaVPA administration to rats is highly correlated with the ATP-dependent excretion of VPA-G into bile mediated by the Mrp2 transporter protein. Although the magnitude of biliary excretion of VPA-G and accompanying choleresis (up to 3.2 times basal bile flow) are quite remarkable, the effect appears to be due uniquely to the Mrp2 transporter.

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Footnotes

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Legends for figures

Fig. 1. Bile flow in bile-exteriorized Wistar (squares) and TR^- (triangles) rats given an i.v. dose of 150 mg of NaVPA per kg (closed symbols) or normal saline control (open symbols) at time zero. Results are means ± SD (n=4). The volume of bile produced in fixed time intervals was used to calculate flow.

Fig. 2. Biliary excretion rate (mg VPA equiv./hr) of VPA-G (closed symbols) and VPA (open symbols) for bile-exteriorized Wistar (squares) and TR^- (triangles) rats administered 150 mg/kg NaVPA i.v. Results are means ± SD (n=4). For each timed bile sample collection, the excretion rate was determined by dividing the total amounts of VPA and VPA-G in the bile collection by the collection time.

Fig. 3. Plasma concentrations of VPA-G (closed symbols) and VPA (open symbols) in bile-exteriorized Wistar (squares) and TR^- (triangles) rats after i.v. administration of 150 mg/kg NaVPA at time zero. Results are means ± SD (n=4). There are no differences in the plasma concentration profiles of VPA for both rat strains but the plasma concentrations of VPA-G in TR- rats are higher than those seen in Wistar rats.

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Table 1. %Recovery of VPA dose as VPA and VPA-G in Urine and Bile

	Wistar Rat (Mean ± SD)	TR^- Rat (Mean ± SD)
VPA in Bile	1.8 ± 0.7	0.4 ± 0.1*
VPA-G in Bile	62.8 ± 7.7	2.0 ± 0.6**
Total in Bile	64.6 ± 8.3	2.4 ± 9.5***
VPA in Urine	1.9 ± 1.2	2.6 ± 0.6
VPA-G in Urine	22.5 ± 8.3	49.8 ± 0.7**
Total in Urine	24.4 ± 9.5	52.4 ± 11.7*
Total Recovery as VPA and VPA-G	89.0 ± 17.8	54.8 ± 12.4**

*P<0.05, **P<0.01, ***P<0.001

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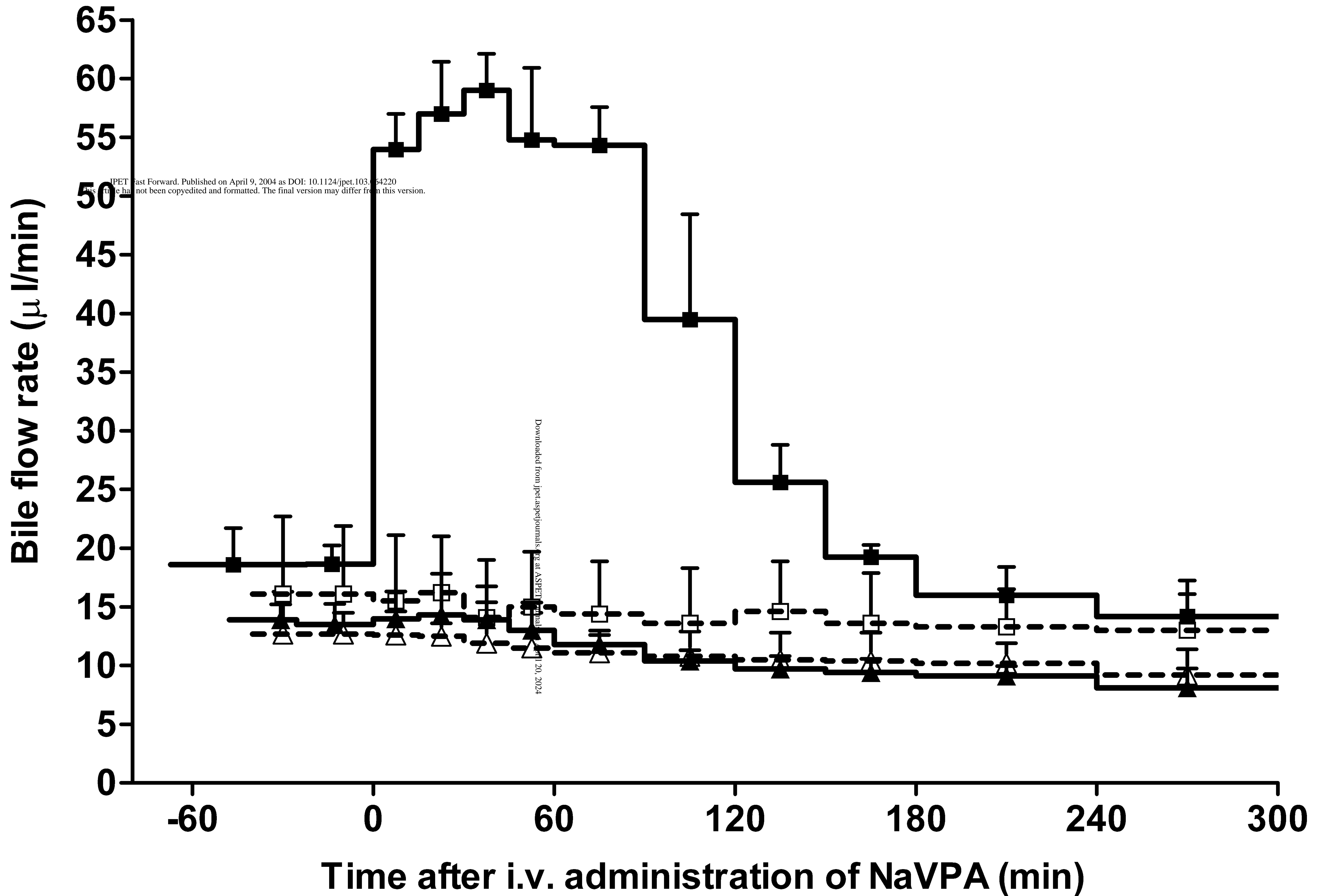

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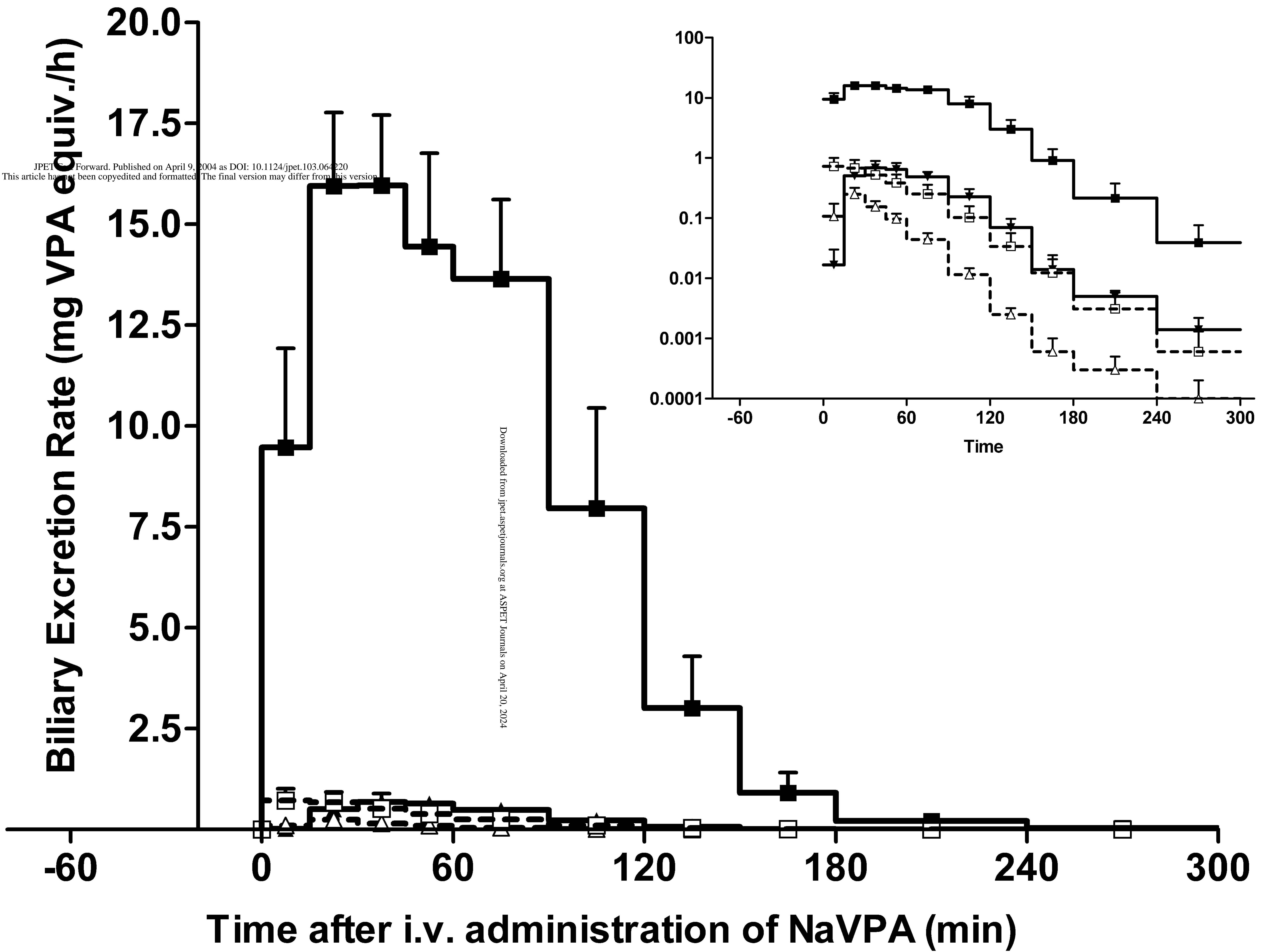

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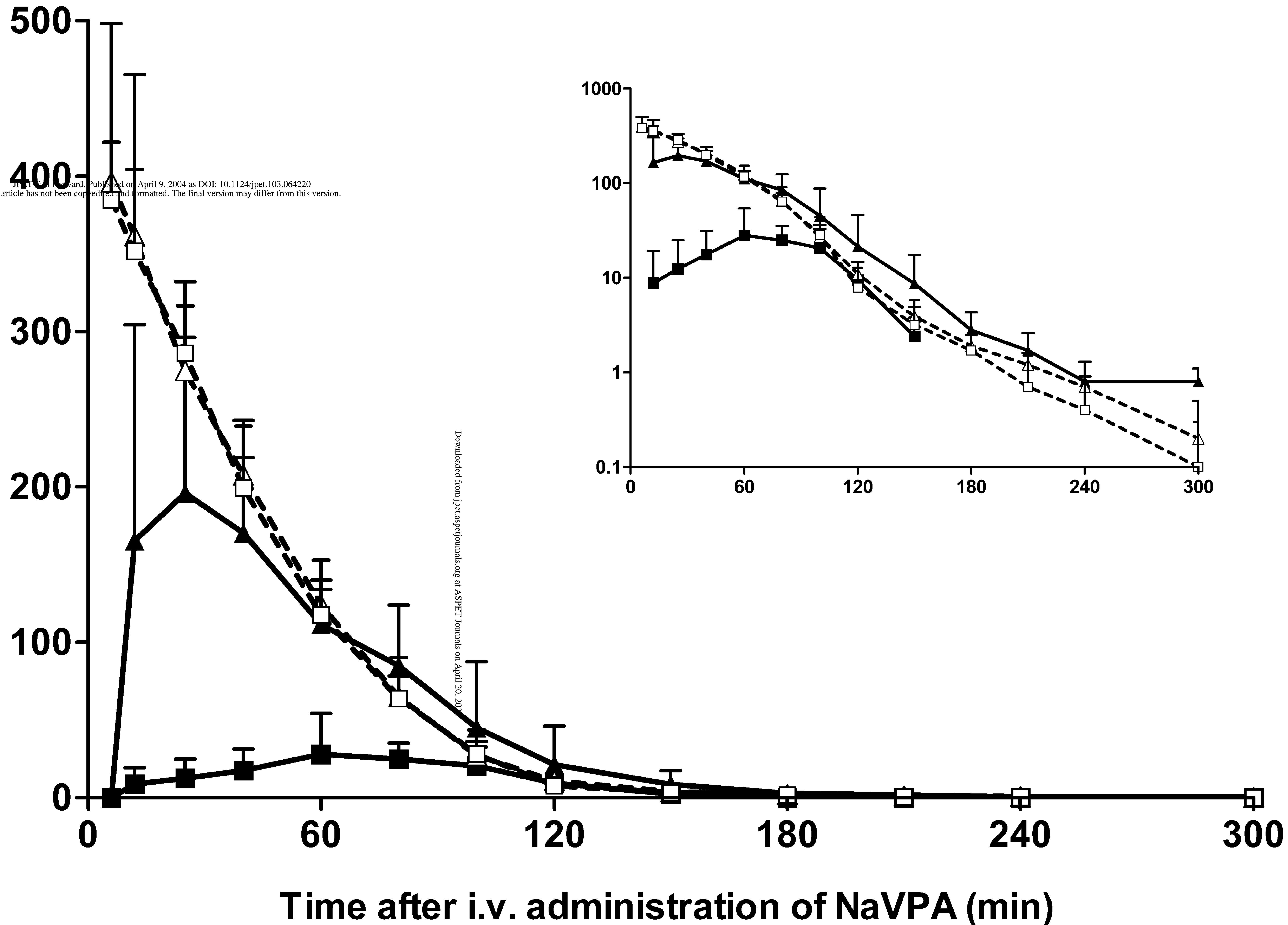

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