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HEPATIC UPTAKE AND BILIARY EXCRETION OF THE NEUROPROTECTANT 2,3-DIHYDROXY-6-NITRO-7-SULFAMOYL-BENZO-(F)-QUINOXALINE IN RAT LIVER

INVOLVEMENT OF AN ORGANIC ANION TRANSPORT SYSTEM

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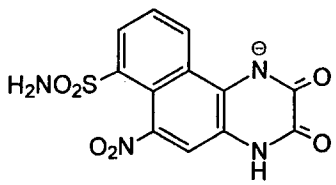
Abstract—The hepatic uptake and biliary excretion of the neuroprotective drug 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo-(f)-quinoxaline (NBQX) was studied in rat liver. In the isolated single-pass perfused rat liver preparation NBQX was infused in protein-free Krebs–Henseleit bicarbonate buffer at input concentrations ranging from 0.5 to 15 μM. The hepatic uptake could be characterized as a pseudo first-order unidirectional process with an apparent half-life of 5.8 min. Significantly higher concentrations of NBQX were measured in bile compared to perfusate with a maximal ratio at the lowest input concentration (approx. 2400-fold). Hepatic uptake and biliary excretion of NBQX exhibited saturation at increasing input concentrations, indicating an active transport mechanism. The uptake process could be described by Michaelis–Menten kinetics resulting in a $K_{m,\text{uptake}}$ of 2.2 μM. The corresponding maximal uptake rate ($V_{\text{max,uptake}}$) was calculated to be 103 nmol/min. The maximal biliary excretion rate was estimated to be 58 nmol/min. The rate-limiting factor in the overall hepatic elimination was thus biliary excretion. Co-infusion with the uricosuric drug probenecid resulted in significantly decreased hepatic uptake and biliary excretion. These data suggest that NBQX is eliminated by an organic anion transport system in rat liver which is sensitive to probenecid.

Key words: hepatic; transport; organic anion; NBQX; probenecid

NBQX† is a potent and selective glutamate antagonist at the AMPA receptor subtype in mammalian brain [1]. In several rodent animal models of stroke NBQX protects against neuronal damage or cellular degeneration even when administered up to several hours after the ischaemic injury [2–6]. The structure of NBQX is shown in Fig. 1.



NBQX

Fig. 1. Structure of NBQX in its ionized form at pH 7.4.

In mice the anticonvulsant effect of NBQX may be enhanced by co-administration of the uricosuric drug probenecid [7]. Similarly, disposition studies in rats have shown that NBQX is excreted in urine predominantly in its unchanged form via a probenecid-sensitive transport system in the kidney [8]. In these studies a significant non-renal clearance was observed, which could indicate either hepatic metabolism or excretion as an additional route of elimination. However, initial *in vitro* experiments using either rat liver microsomes or freshly isolated rat hepatocytes (unpublished results) showed no metabolism of NBQX. Since NBQX has two pK_a values of 6.4 and 10.0 it is highly ionized (approx. 90%) at physiological pH and exists as a monovalent anion. Several organic anions such as diuretics, antibiotics, and steroids are eliminated by active transport systems in the liver [9–12], although the majority are metabolized by reactions such as sulphation, glucuronidation or glutathione conjugation prior to excretion into the bile. The inhibition of both non-renal (hepatic) and renal clearance of NBQX [8] in combination with the apparent lack of metabolism could indicate that NBQX *per se* is secreted by an organic acid transport system in rat liver. The involvement of an active transport system in the hepatic elimination of NBQX

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† Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; B_{out}, biliary excretion rate; $C_{\text{bile,ss}}$, bile steady-state concentration; C_{input}, portal vein concentration; $C_{\text{output,ss}}$, effluent perfusate concentration at steady-state; E, hepatic extraction ratio; $K_{m,\text{uptake}}$, substrate concentraion at one-half of maximal hepatic uptake capacity; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo-(f)-quinoxaline; $V_{\text{max,uptake}}$, maximal hepatic uptake rate at substrate saturation concentration; $V_{\text{max,bile}}$, maximal biliary excretion rate.

was therefore studied, and this paper describes the initial characterization of NBQX hepatic uptake and biliary excretion in a single-pass perfused rat liver model.

MATERIALS AND METHODS

Materials. NBQX was synthesized at Novo Nordisk (Måløv, Denmark). Polyvinyl pyrrolidone (PVP12) was obtained from BASF (Ludwigshafen, Germany) and Probenecid from Nomenco (Copenhagen, Denmark). All other solvents and chemicals were of the highest commercially available grade and used without further purification. Infusion solutions of NBQX (2.0 mM) were prepared in 5% PVP and 4% glucose adjusted to pH 7.4 and filtered through a 0.22 μM Millex filter (Millipore, Denmark). For the interaction study a stock solution of NBQX (2.0 mM) and Probenecid (40.0 mM) was prepared in the same vehicle.

Isolated perfused rat liver. Male Sprague–Dawley rats of 240–280 g body weight (Mollegaards Breeding Labs, Lille Skensved, Denmark) fed on stock diet (Altromin) were used for protein and haemoglobin-free single-pass liver perfusion at 37° using Krebs–Henseleit bicarbonate buffer. The surgical procedures were as previously described [13] but the liver was kept *in situ* throughout the experiment. Livers were perfused using a MXII perfuser (MX International, Aurora, CO, U.S.A.) in the single-pass mode. To assure sufficient oxygen supply Krebs–Henseleit buffer was continuously gassed with O_2 (95%) and CO_2 (5%) and the flow adjusted to approx. 3.5 mL/min/g liver. Viability was assessed by portal pressure recordings, bile flow, LDH leakage into the perfusate and physical appearance of the liver. Following a recovery period of 20 min, the experiment was started by connecting a syringe infusion pump (Harvard Apparatus, MA, U.S.A.) to the portal vein inlet. The infusion pump was set to reach input concentrations within the range of 0.5–15 μM (17.5–525.0 nmol/min). Effluent perfusate and bile were sampled simultaneously during the experiment and analysed for intact NBQX. The hepatic extraction ratio was calculated according to Eqn (1):

$$E = C_{input} - C_{output,ss}/C_{input}, \quad (1)$$

where C_{input} is the concentration in the portal vein and $C_{output,ss}$ the concentration in perfusate leaving the liver at steady-state. Hepatic uptake was calculated as $E \times C_{input}$ (nmol/min). Biliary excretion rate was calculated using Eqn (2):

$$B_{out} = C_{bile,ss} \times \text{bile flow rate}, \quad (2)$$

where $C_{bile,ss}$ is the NBQX concentration in bile at steady-state.

HPLC analysis. NBQX was quantified by reversed-phase HPLC analysis using a 5 μm (250 × 4 mm) LiChrospher RP-18 column (Merck, Darmstadt, Germany) with a mobile phase of 0.1% TFA and acetonitrile (90:10 v/v) and 294 nm UV detection. The flow rate was 1.0 mL/min and the column temperature 35°. Perfusate samples were centrifuged at 5000 *g* for 5 min and the supernatant was injected directly. Bile samples were diluted with Krebs–Henseleit perfusate buffer before analysis. NBQX concentrations were determined using external standards in perfusate buffer.

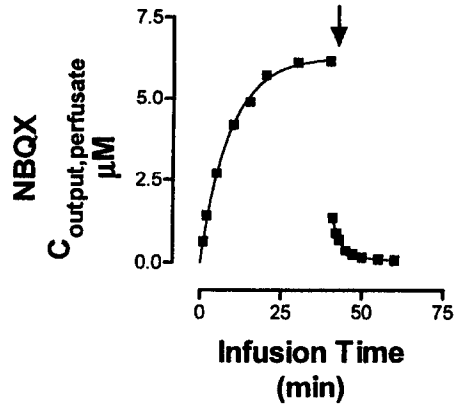

Fig. 2. Effluent perfusate concentrations of NBQX at an input concentration of 10 μM. Arrow indicates discontinuation of drug infusion with data points representing perfusate concentration at 1, 2, 3, 5, 7, 10, 15 and 20 min after infusion. Data are the means of two very similar experiments.

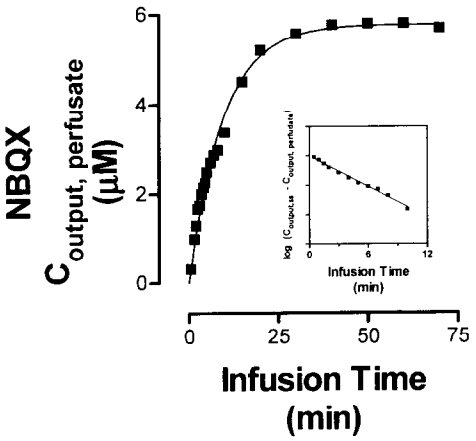

Fig. 3. Hepatic uptake of NBQX in the single-pass perfused rat liver preparation. NBQX was infused at an input concentration of 10 μM, and each point represents the mean of two determinations from a single experiment. Inset: Semilogarithmic plot of the concentration difference between perfusate $C_{output,ss}$ and C_{output}. The computer-generated curve based on a first-order process is shown.

RESULTS AND DISCUSSION

To characterize the net uptake process of NBQX in rat liver an experiment was conducted to determine whether the transport between perfusate and liver

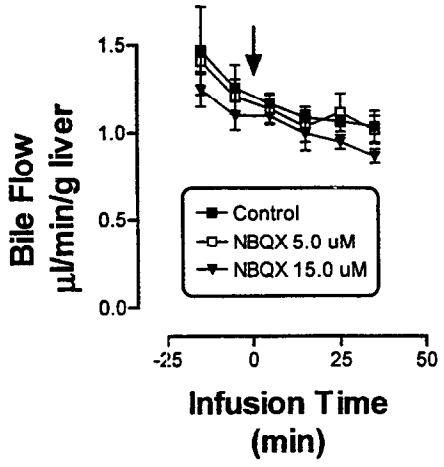

Fig. 4. Bile flow from control (no NBQX) and NBQX infusion experiments. Arrow indicates start of NBQX infusion. Data are the means (±SD) of 4–6 experiments.

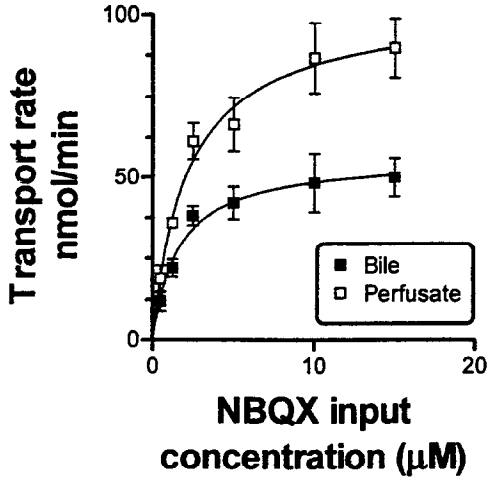

Fig. 5. Hepatic uptake (□) and biliary excretion rate (■) of NBQX in the isolated perfused rat liver as a function of input concentration. Computer-generated curves are drawn based on Michaelis–Menten kinetics. Data are the means (±SD) of 4–6 experiments.

was unidirectional, i.e. irreversible elimination from perfusate, or bidirectional across the hepatocyte plasma membrane. Following infusion of NBQX for 40 min, the infusion was stopped and efflux perfusate concentrations were measured. As seen from Fig. 2, NBQX concentrations dropped instantly and reached levels of less than 0.1 µM within 10 min. This indicated insignificant efflux of NBQX and the uptake process could be regarded as unidirectional (irreversible) under the present experimental conditions. An apparent half-life for the hepatic uptake process was estimated based on this assumption. NBQX was infused at an input concentration of 10 µM (Fig. 3). Multiple outflow perfusate samples were analysed for NBQX over a period of 70 min. The outflow perfusate concentrations, which represent the sum of both hepatic influx and efflux (negligible) of NBQX, reached a steady-state level after approx. 30 min. As seen from the outflow concentrations, the perfused liver was apparently viable for at least 70 min following the 20 min recovery period. This was also shown by constant bile flow throughout the subsequent experiments (range 1.0–1.5 µL/min/g liver; Fig. 4).

A semilogarithmic plot of the difference between effluent concentration at steady-state ($C_{output,ss}$) and that at time before steady-state (C_{output}) against time yielded a straight line, indicating that uptake of NBQX into the liver is a first-order process with an apparent rate constant of 0.12 min and a half-life of 5.8 min. Consequently, subsequent kinetic experiments were conducted under steady-state conditions to measure effluent perfusate and bile concentrations after 35 and 40 min of drug infusion.

In a series of experiments the input concentration of NBQX was increased from 0.5 µM to 15 µM. The hepatic extraction ratio showed a dose-dependent decrease (see Table 1). Thus, NBQX has an intermediate to high hepatic extraction ratio [14], similar to what was observed *in vivo* where the drug had an hepatic clearance of 2.3 L/kg/hr [8] compared to an estimated hepatic blood flow of approx. 4 L/kg/hr [15, 16]. At low input concentrations the bile to perfusate concentration ratio was as high as 2400-fold (Table 1). Surprisingly, the bile flow remained almost constant throughout the infusion period despite high concentrations of NBQX in bile. A likely choleresis produced by NBQX could be counteracted by a direct effect (cholerestatic) on bile formation by the compound. This suggests that NBQX belongs to a group of xenobiotics of the category B type, all of which exhibit significant biliary excretion with bile to plasma concentration ratios between 10 and 1000 [17].

Both the net hepatic uptake and biliary excretion processes exhibited saturation at input concentrations exceeding approx. 2.5 µM. Graphical Eadie–Hofstee plots of V/S vs S (data not shown) were linear, indicating the involvement of a single transport system, or possibly two with similar K_m values, at

Table 1. Effluent perfusate concentration, bile concentration and hepatic extraction ratio of NBQX at steady-state in the single-pass perfused rat liver as a function of input concentration*

C_{input} (µM)	$C_{output,ss}$ (µM)	$C_{bile,ss}$ (µM)	E
0.5	0.07 ± 0.02	1185 ± 65	0.86 ± 0.02
1.25	0.20 ± 0.02	2171 ± 107	0.84 ± 0.02
2.50	0.36 ± 0.12	4005 ± 207	0.85 ± 0.05
5.0	2.66 ± 0.30	4428 ± 405	0.44 ± 0.04
10.0	7.29 ± 0.47	6143 ± 257	0.27 ± 0.04
15.0	12.45 ± 1.20	6366 ± 550	0.17 ± 0.03

* Mean ± SD; N = 4–6.

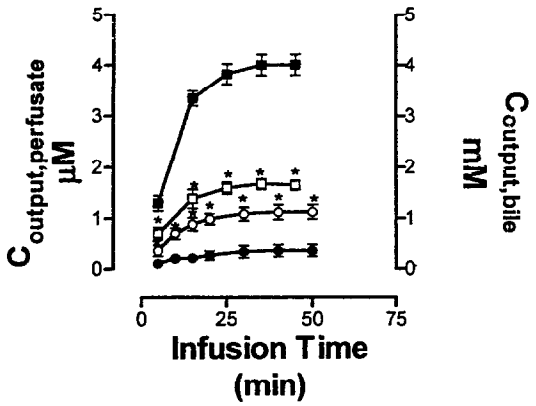

Fig. 6. Influence of Probenecid on hepatic uptake and biliary excretion of NBQX in the isolated perfused rat liver following either infusion of 2.0 µM NBQX alone or co-infusion with 40.0 µM Probenecid. (●) NBQX perfusate concentration C_{output} without Probenecid; (○) NBQX perfusate concentration C_{output} with Probenecid; (■) NBQX bile concentration C_{bile} without Probenecid; (□) NBQX bile concentration C_{bile} with Probenecid. Note that perfusate concentrations are in µM and bile concentrations are in mM. Data are the means (±SD, N=6). *Significantly different from control ($P < 0.001$; two-sample t-test).

hepatic sinusoidal and canalicular membranes, respectively. Kinetic estimates from these graphical plots were then used as initial values in a non-linear regression analysis to fit the Michaelis–Menten equation to the data (Fig. 5). From the computer-generated curve-fit a $V_{max,\ uptake}$ was determined to 103 nmol/min with an apparent Michaelis–Menten constant ($K_{m,\ uptake}$) of 2.2 µM. Similarly, a $V_{max,bile}$ of 58 nmol/min was determined. Since a more accurate determination of $K_{m,bile}$ would require studies using bile canalicular membrane vesicles, an apparent affinity of NBQX for the canalicular transport system was estimated in this study with a value of 1.9 µM. From these kinetic parameters, it appears that biliary excretion is the rate-limiting step in hepatic elimination of NBQX in rat liver.

Based on the recent findings that the uricosuric drug Probenecid enhances the anticonvulsant effect of NBQX in mice [7] and, in addition, decreases both NBQX renal and non-renal (hepatic) clearance in rats [8], the effect of Probenecid co-infusion on overall hepatic elimination was studied. At 20-fold higher concentrations, Probenecid significantly decreased both hepatic uptake and biliary excretion (Fig. 6). Steady-state outflow perfusate concentrations increased approx. 3.2-fold, whereas bile concentrations decreased 2.5-fold following co-infusion. The present data seem to meet some of the properties associated with an active carrier-mediated transport of NBQX into bile: (1) secretion against a substantial concentration gradient; (2) substrate saturation with a defined maximal transport T_m; and (3) inhibition by a known cholephilic drug [9]. Since NBQX is not biotransformed prior to secretion into bile a direct metabolic interaction with Probenecid, e.g. glucuronidation, could be excluded; it is known that Probenecid is extensively conjugated before biliary excretion [18]. The fact that 20-fold higher concentrations only inhibited NBQX biliary elimination by 60% could indicate either that NBQX has higher affinity for the organic anion carrier or that more than one transport system is involved, only one of which is inhibited by Probenecid. Presently, distinct biliary anion transport systems have been postulated which include a multispecific organic anion transporter protein and a more specific one for bile acids [9–12].

Similarly, it is difficult to describe conclusively the net hepatic uptake process of NBQX in terms of a single carrier system on the sinusoidal membrane. Several hepatic uptake systems with affinity for organic anions have been described [10, 19] and NBQX could exhibit affinity for more than one. The possibility that NBQX is taken up by passive diffusion and then binds to intracellular proteins such as ligandin, Y and Z proteins, bile acid binding proteins, and others is unlikely given its physico-chemical properties. NBQX is highly ionized and has a negative partition coefficient at physiological pH ($\log D_{7.4} - 1.08$ [8]). If Probenecid competed with NBQX for intracellular binding sites, this would only temporarily cause a disturbance in steady-state clearance, i.e. increased efflux to both bile and perfusate [20]. Furthermore, since the efflux of NBQX into perfusate could be regarded as insignificant, the observed increase in perfusate concentrations is more likely a result of competition for uptake sites at the sinusoidal plasma membrane.

In conclusion, the present data suggest that the neuroprotective drug NBQX is eliminated in rat liver by organic anion transport systems on both the sinusoidal plasma and canalicular membrane, and that these processes are partly inhibited by Probenecid.

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