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Transient enterohepatic circulation and enhanced biliary versus urinary excretion of the cytostatic drug bischolylglycinate-chloroplatinum(II) (Bamet-H2)[1]

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Abstract

Using intraluminal perfusion of "in situ" ileum in anaesthetized rats bearing catheters into the portal vein and the common bile duct that permitted portal blood and bile sample collection, and in conscious rats in which a permanent intraarterial catheter had been implanted to carry out sequential blood sampling, the existence of enterohepatic circulation of the cytostatic drug Bamet-H2–Na[Pt(cholylgycinate-*O*,*N*) (cholylgycinate-*O*) Cl]–was investigated. Total platinum in serum, bile, ileum, liver, urine and feces was measured by flameless atomic absorption spectroscopy. After i.v. and i.g., 1 μmol Bamet-H2 or cisplatin administration, model-independent methods based on the theory of statistical moments were used in order to characterize the pharmacokinetics of Bamet-H2. The area under the curve from 0 to 168 h after i.v. administration (AUC_{168}) was significantly higher (+ 27%) for Bamet-H2 than for cisplatin. However, extrapolation from 0 to infinity indicated that AUC_{∞} was lower for Bamet-H2 (− 23%) than for cisplatin. The clearance (Cl_{∞}) for cisplatin was consistently lower (− 23%) than for Bamet-H2. Ultrafiltration of serum collected at 168 h revealed that an important part of the Bamet-H2 (67%) and cisplatin (53%) remaining in the serum at this time was in the nonultrafiltrable form, i.e. probably bound, in part irreversibly, to serum macromolecules. When the rats received i.g. 1 μmol cisplatin or Bamet-H2, peak in serum concentrations of total platinum were markedly higher (6-fold) after Bamet-H2 than after cisplatin administration. The area under the curve was, also in this case, significantly higher for Bamet-H2 than for cisplatin (+ 98%). This was in part due to the enhanced intestinal absorption of Barnet-H2, as confirmed in experiments on perfused rat ileum, where an increase in portal serum Bamet-H2 concentrations was found. Moreover, markedly higher amount of the drug was found in ileum, liver and bile after perfusion with media containing Bamet-H2 as compared with experiments where cisplatin instead of

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Bamet-H2 was added to perfusion media. Moreover, after i.v. administration to conscious rats, excretion of Bamet-H2 by the kidney was lower (− 51%) than that of cisplatin, while elimination of the former compound into feces was higher (+ 189%) than that of the latter. In sum, these results indicate that in addition to the previously reported cytostatic activity of Bamet-H2, this complex has interesting cholephilic characteristics, such as reduced urinary excretion together with enhanced intestinal absorption and biliary secretion, which are probably endowed by one or both cholylglycyl moieties bound to the platinum(II) atom in the Bamet-H2 molecule.

Keywords: Bile Acid; Cancer; Chemotherapy; Glycocholate; Liver; Kidney; Metal; Pharmacokinetics; Tumor

1. Introduction

The success of *cis*-diamminedichloroplatinum(II) (cisplatin) and its analogs in the treatment of a variety of solid tumors (Loeher and Einhorn, 1984) has encouraged the search for new cisplatin derivatives with a view to improving the therapeutic index of the compound, which is reduced due to dose-limiting toxicity, i.e. nephrotoxicity, myelotoxycity, neurotoxicity, nausea and vomiting.

Since the use of bile acid carrier mechanisms to enhance the hepatic and small intestinal absorption of drugs was first proposed (Ho, 1987), several attempts in this direction have been made (Betebenner et al., 1991, Kramer et al., 1992, Stephan et al., 1992), among them the synthesis of Bamet-H2 (Criado et al., 1997). This complex can be considered one of the many second-generation platinum-containing compounds that have been developed in an effort to obtain new cytostatic drugs having fewer toxic side effects than cisplatin but with comparable chemotherapeutic efficacy. Bamet-H2 molecule includes two moieties of endogenous compounds with marked liver organotropism–glycocholate–to direct this platinum(II)-containing drug toward the hepatobiliary system. The existence of specific bile acid carrier proteins in the plasma membranes of hepatocyte (Azer and Stacey, 1996), which are not present in most cell types, makes these cells ideal targets for bile acid-labeled drugs. These derivatives are expected to be efficiently taken up by the liver (Kramer and Wess, 1996). The strategy also endows certain of these derivatives with enhanced hepatobiliary excretion. Owing to the previously reported cytostatic activity (Criado et al., 1997) and liver organotropism (Marin et al., 1997) of Bamet-H2, one could speculate that this compound might be potentially useful in the chemotherapy of liver tumors. Na^+-dependent bile acid uptake is to a certain extent lost in rat hepatoma cells (Kroker et al., 1978, Buscher et al., 1988, Von Dippe and Levy, 1990; Kullak-Ublick et al., 1996). However, some degree of Na^+-independent uptake is still present in liver tumor cells (Marchegiano et al., 1992; Kullak-Ublick et al., 1996), thus accounting for an efficient bile acid uptake, although lower than that of hepatocytes (Miyazaki and Namba, 1994; Kullak-Ublick et al., 1996). Recent report on the expression of the organic anion transporter, OATP in hepatocellular carcinoma which mediates chlorambucil-taurocholate uptake (Kullak-Ublick et al., 1997) further supports the potential usefulness of cytostatic agents coupled to bile acids. Bamet-H2, which is an anionic compound, has been reported to be taken up by rat hepatocytes both via Na^+-dependent and Na^+-independent pathways (Marin et al., 1997). This means that the partial loss of Na^+-dependent bile acid uptake by liver tumor cells will probably only moderately affect the ability of these cells to take up Bamet-H2.

A second possibility to be considered is the use of Bamet-H2 in regional chemotherapy. Using isolated perfused rat livers, Bamet-H2 has been shown to be rapidly taken up from the perfusate and secreted into bile (Marin et al., 1997). Thus, efficient biliary elimination is also expected to occur after Bamet-H2 leaves the tumoral area during intraarterial administration.

The existence of carrier systems for bile acids in the intestine (mainly in the ileum) account for an efficient re-uptake of molecules that have reached the gut with bile (Hofmann, 1994). Reabsorbed bile acids return to the liver via the portal vein. Thus, only a minor fraction of the bile acid pool is lost every day in the feces, while the rest remains sequestered within the so-called enterohepatic circulation. Whether Bamet-H2 shares this behavior with natural bile acids is an interesting question involving two important pharmacological issues: the re-exposure to Bamet-H2 of tumors located in the enterohepatic circuit and the ability of the body to eliminate this drug.

To gain information on the existence of enterohepatic circulation of Bamet-H2 and the relevance of biliary versus urinary excretion of this complex, experiments on both intraluminal perfusion of ''in situ'' ileum in anaesthetized rats with media-containing Bamet-H2 and administration of this drug to conscious rats were carried out. A permanent intraarterial catheter had been implanted to these animals in order to perform sequential blood sampling. At the end of these experiments, serum ultrafiltrable platinum was determined. However, in the rest of the study total platinum was used because this is related to both toxicity and anti-tumor efficacy (Campbell et al., 1983, Kelsen et al., 1985, Desoize et al., 1991, Desoize and Robert, 1994). Moreover, the use of ultrafiltrable platinum, although preferred by some investigators (Ma et al., 1994), has been questioned by others (Desoize et al., 1996) due to the ill-defined mixture of species included in this term and to the unreproducible values found by different laboratories for this parameter, since different techniques to prepare and filter the samples have been used.

2. Methods

2.1. Chemicals and animals

Cisplatin and FITC-dextran 40S were purchased from Sigma (St Louis, MO). Bamet-H2-Na[Pt(cholylgycinate-*O*,*N*) (cholylgycinate-*O*) Cl]-was synthesized and chemically characterized as previously reported (Criado et al., 1997). All other chemicals were from Merck (Darmstad, Germany).

Male Wistar rats (200 g, five animals for each experimental group) were from the Animal House at the University of Salamanca, Spain. They were fed with commercial rat pelleted food (Panlab, Madrid, Spain) and water ad libitum. Temperature (20°C) and light/dark cycle (12 h: 12 h) in the room were controlled. All animals received humane care as outlined in the ''Guide for the Care and Use of Laboratory Animals'' (National Institute of Health Publication No. 8023, revised 1985).

2.2. Drug administration and sample collection in studies on conscious rats

After intraperitoneal anaesthesia with 50 mg/kg body weight sodium pentobarbital (Nembutal N.R., Abott, Madrid, Spain) a median laparotomy was performed and a flexible catheter (0.9 mm diameter, Introcan 22/G1, Braun, Barcelona, Spain) was implanted into the abdominal portion of the aorta artery, which did not greatly affect blood flow through this point. The catheter was fixed with cyanoacrylate, exteriorized through the back part of the neck and fixed with silicone. The abdominal incision was then closed. Operations were performed under sterile conditions. Rats were allowed to recover from anesthesia in a warmed cabinet. The surgically prepared rats were housed in individual metabolic cages where they had free access to food and water. At least 4 days after undergoing catheter implantation, a single dose of 1 μmol Bamet-H2 or cisplatin dissolved in 0.5 ml sterile 0.9% NaCl solution was given through the penial vein (i.v.) or via a polyethylene tube temporally placed in the esophagus (i.g.), under light ether anaesthesia. Blood samples were collected through the permanently implanted catheter at different time intervals over the following 7 days. Serum was immediately separated by centrifugation (13000 rpm for 5 min) and digested with a nitric acid solution (60%; vol:vol) overnight at room temperature, then heated at 80°C for 2 h and finally at 150°C until dryness. Metabolic cages permitted urine and feces separation. Sam-

ples collected during the experiments were measured by volume (urine) or weight (feces) and digested as described above. An extra sample of blood was collected at the end of the experiment. This was ultrafiltered using filters with a 10 kD cut off (Amicon Microcon-10, Lexington, MA). The resulting ultrafiltrate was digested as described above.

2.3. "In situ" rat ileum perfusion

Fasted rats were anaesthetized before cannulating the common biliary duct (PE-10, Biotrol Pharma, Paris, France). Two polypropylene tubes (3.2 mm diameter, Cole-Palmer, Niles, IL) were implanted in the small intestine at 15 cm and 3 mm from the ileal-cecal valve, which had been previously ligated. The former tube was used as inlet and the latter as outlet. Intraluminal perfusion was carried out at 1 ml/min using a peristaltic pump. Perfusion medium was glucose-free Krebs–Henseleit solution (120 mM NaCl, 5 mM KCl, 0.65 mM $MgSO_4$, 1.17 mM KH_2PO_4, 1.29 mM $CaCl_2$, 25 mM $NaHCO_3$, pH 7.4) to which 100 mg/l gentamycin was added. The ileum was first washed until the outflowing perfusate had become colorless. An additional open perfusion for 15 min was carried out. The perfusion system was then shifted from open to closed circulation. After 10 min recirculation the perfusion medium was replaced by 10 ml of fresh Bamet-H2- or cisplatin-containing Krebs–Henseleit solution. The ileum was perfused with this recirculating medium for 60 min at 38°C. Bile samples were collected at 15-min intervals throughout the experimental period, at the end of which the liver and the perfused ileum were washed, weighed, homogenized and digested in nitric acid. In a different set of experiments a catheter (Introcan-W, Braun, Melsungen, Germany) was implanted into the portal vein and fixed with cyanoacrylate (Loctite España, Madrid, Spain) in order to carry out portal blood sampling during ileum perfusion. In previous experiments the perfusion medium included 50 mg/l non-absorbable 40 kDa FITC-dextran in order to evaluate the magnitude of net water exchange between the perfusate and the mucosa of the ileum under these particular experimental circumstances. No changes in either FITC-dextran concentrations or in the final perfusion medium volume were found (data not shown).

2.4. Analytical, pharmacokinetic and statistical methods

Total platinum content in digested samples of serum, urine, feces, liver, ileum and bile were measured by flameless atomic absorption spectroscopy (Z-8100 Polarized Zeeman apparatus with a graphite furnace, Hitachi, Pacisa, Madrid, Spain).

In order to characterize the pharmacokinetics of Bamet-H2 after i.v. and i.g. administration, model-independent methods based on the theory of statistical moments were used. Total (free + bound) Bamet-H2 and cisplatin serum concentration curves, i.e. AUC (area under the curve) and MRT (mean residence time), were calculated by numerical integration using the trapezoidal rule as described by Yamaoka et al. (1978). Because this was observed over a limited period of time (168 h), extrapolation to infinity time (∞) was carried out using a monoexponential equation. Clearance (Cl) was calculated as the absolute dose divided by the AUC and the half-life time ($T_{1/2}$) as $\ln 2/K_e$ where K_e (elimination constant) was 1/MRT.

Results are expressed as means $\pm$ S.E. To calculate the statistical significance of differences among groups, the Student t-test method was used. Statistical analyses were performed on a Macintosh PowerPC 6200/200 computer (Apple Computer, Cupertino, CA).

3. Results

Both Bamet-H2 and cisplatin disappeared rapidly from serum during the first day after i.v. administration. The process was slower for Bamet-H2 than for cisplatin for the first 48 h (Fig. 1, upper panel). Thus, serum concentrations of Bamet-H2 remained significantly higher than those of cisplatin during the first day. A second phase with slower disappearance was observed over the next 6 days. During this phase Bamet-H2

concentrations become lower than those of cisplatin. The area under the curve from 0 to 168 h (AUC_{168}) was significantly higher (+27%) for Bamet-H2 than for cisplatin. Ultrafiltration of serum collected 168 h after i.v. administration revealed that an important part of the Bamet-H2 (67.4 ± 1.0%) and cisplatin (53.2 ± 3.1%, $p < 0.05$) remaining in the serum at this time was in the non-ultrafiltrable form, i.e. probably bound, in part irreversibly, to serum macromolecules. Therefore, extrapolation from 0 to infinity afforded pharmacokinetic parameters that must be cautiously considered. In contrast to AUC_{168}, AUC_{∞} was lower for Bamet-H2 (353 ± 27 μmol/l/h, $p < 0.001$) than

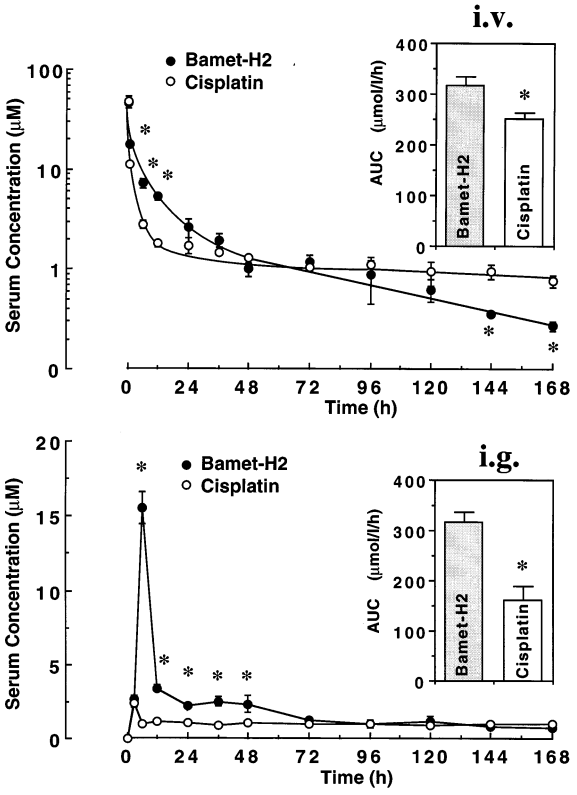

Fig. 1. Serum drug concentration following intravenous (i.v., upper panel) or intragastric (i.g., lower panel) administration of 1 μmol Bamet-H2 (●, $n = 5$) or cisplatin (○, $n = 5$). Blood was collected at the indicated times from the aorta artery through a permanently implanted catheter. Platinum contents in the digested serum samples were determined by flameless atomic absorption spectroscopy. The insets are AUC, area under the curve. Values are means ± S.E. * $p < 0.05$ on comparing Bamet-H2 to cisplatin by Student's t-test.

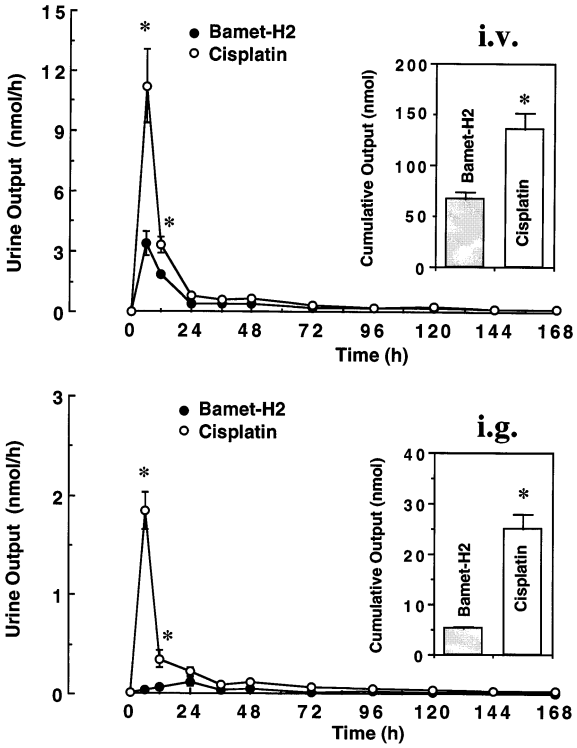

Fig. 2. Drug excretion into urine following intravenous (i.v., upper panel) or intragastric (i.g., lower panel) administration of 1 μmol Bamet-H2 (●, $n = 5$) or cisplatin (○, $n = 5$). Using metabolic cages, urine was collected at the indicated times. Volume was measured and platinum contents in digested urine samples were determined by flameless atomic absorption spectroscopy. The insets are cumulative output into urine. Values are means ± S.E. * $p < 0.05$ on comparing Bamet-H2 to cisplatin by Student's t-test.

for cisplatin (459 ± 22 μmol/l/h). The clearance (Cl_{∞}) for cisplatin (2.17 ± 0.07 ml/h) was consistently lower (−23%, $p < 0.05$) than for Bamet-H2 (2.83 ± 0.09 ml/h). Both the mean residence time (MRT) and serum half-life time ($T_{1/2}$) were significantly shorter (−73% and −73%, respectively, both $p < 0.001$) for Bamet-H2 (MRT = 60 ± 6 h; $T_{1/2}$ = 41 ± 3 h) than for cisplatin (MRT = 220 ± 13 h; $T_{1/2}$ = 153 ± 9 h).

Following i.g. administration, a peak in serum drug concentrations was obtained in few hours (Fig. 1, lower panel); this was almost 6-fold higher for Bamet-H2 (≈ 16 μM) than for cisplatin (≈ 2.5 μM). The area under the curve was, also in this case, significantly higher for Bamet-H2 than for cisplatin (+98%).

After i.v. and i.g. administration of Bamet-H2 or cisplatin, excretion into urine of the former was always much lower than that of the latter (Fig. 2). The amount of both drugs excreted by the kidney was higher when they were given i.v. (13.6% and 6.7% of the dose administered for cisplatin and Bamet-H2, respectively). By contrast, the proportion of cisplatin vs Bamet-H2 eliminated into feces depended upon the administration route (Fig. 3). After i.v. injection lower amount (20.0% of the dose administered) of cisplatin was found in feces as compared to Bamet-H2 (57.7% of the dose administered). When these compounds were administered i.g. the amount of Bamet-H2 in feces was not significantly modified (52.1% of the dose

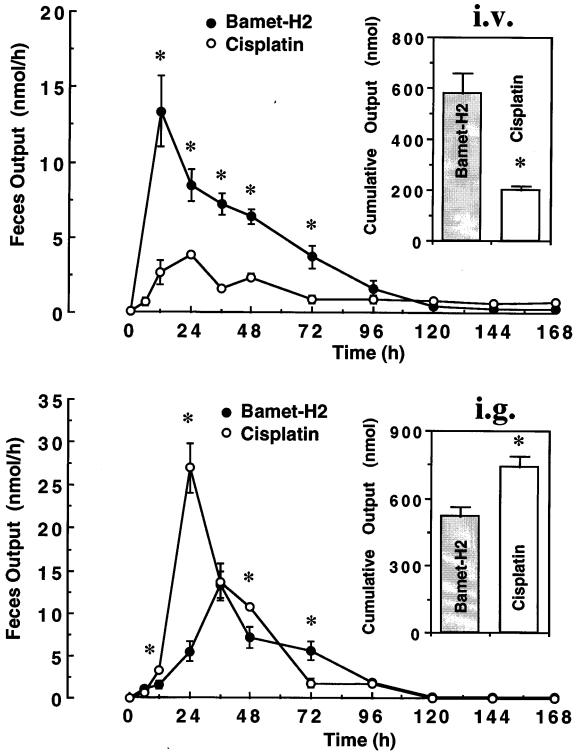

Fig. 3. Drug excretion into feces following intravenous (i.v., upper panel) or intragastric (i.g., lower panel) administration of 1 μmol Bamet-H2 (●, $n = 5$) or cisplatin (○, $n = 5$). Using metabolic cages, feces were collected at the indicated times. Samples were weighed and digested before platinum measurement was carried out by flameless atomic absorption spectroscopy. The insets are cumulative output into feces. Values are means ± S.E. * $p < 0.05$ as comparing Bamet-H2 to cisplatin by Student t-test.

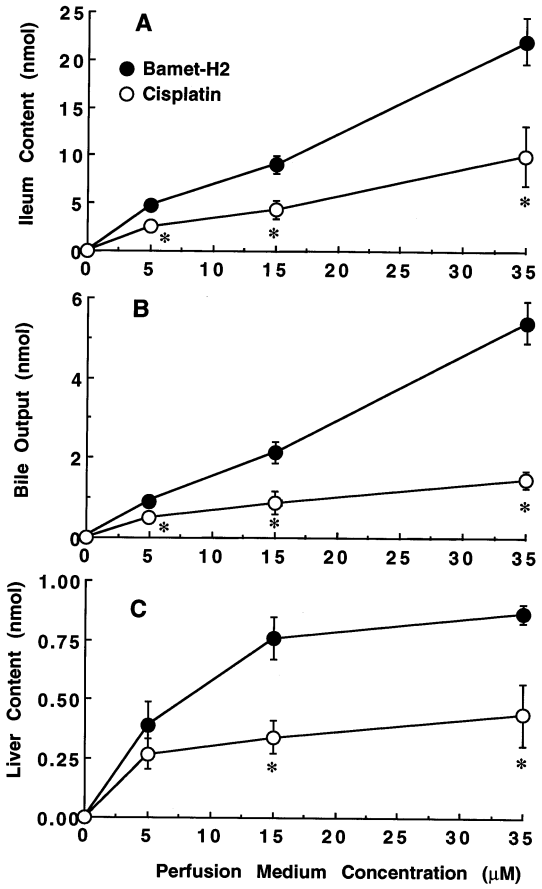

Fig. 4. Relationship between dose of administered drug and the amount of drug retained by the ileum (A), excreted into bile (B) and retained by the liver (C) after 60 min intraluminal perfusion of the rat ileum with 10 ml recirculating Krebs–Henseleit solution containing the indicated initial concentration of Bamet-H2 (●, $n = 5$) or cisplatin (○, $n = 5$). Values are means ± S.E. * $p < 0.05$ on comparing Bamet-H2 to cisplatin by Student's t-test.

administered) but that of cisplatin was greatly enhanced (74.0% of dose administered). "In situ" perfusions of rat ileum with a solution containing cisplatin or Bamet-H2 were carried out in order to elucidate whether the small intestine was able to carry out efficient absorption of these compounds. A dose-dependent amount of drug was found in ileum, liver and bile at the end of the perfusion period (Fig. 4). No significant differences in liver or ileum weight among experimental groups were found (data not shown). Portal blood collection during ileum perfusion confirmed that intestinal

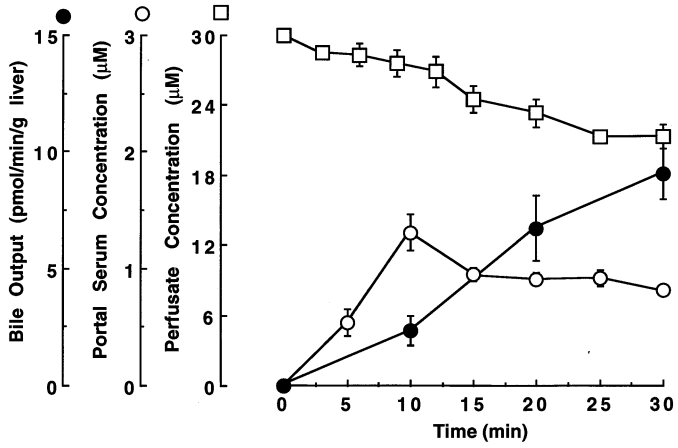

Fig. 5. Time course of Bamet-H2 perfusate concetration (□), portal serum concentration (○) and bile output (●) during 30 min intraluminal perfusion of the rat ileum with 10 ml recirculating Krebs–Henseleit solution containing 30 μM Bamet-H2 at min 0. Values are means ± S.E. from four rat ileum preparations.

Bamet-H2 uptake results in an increase in portal serum Bamet-H2 concentrations (Fig. 5). Moreover, a markedly higher amount of the drug was found in ileum, liver and bile after perfusion with media containing Bamet-H2 as compared with experiments where cisplatin instead of Bamet-H2 was added to perfusion media (Fig. 4).

4. Discussion

Using several "in vitro" experimental models Bamet-H2 has been previously reported to be efficiently taken up by the rat liver and secreted into bile (Marin et al., 1997). The present study shows that Bamet-H2 follows transient enterohepatic circulation because intestinal absorption of this compound also occurs. Bamet-H2 uptake by the rat ileum is markedly more efficient than that of cisplatin, although it does not reach the high efficiency of endogenous major bile acids (Hofmann, 1994). Therefore, after a single i.v. injection or i.g. administration Bamet-H2 does not remain sequestered within the enterohepatic circulation for long. Indeed, animals that received the drug eliminated, mainly in the feces, an important part of the dose administered in just few days.

It has been reported that Bamet-H2 does not undergo major biotransformation during its hepatic transfer from blood to bile (Marin et al., 1997). However, because in the present work fecal Bamet-H2 elimination was calculated from platinum output into feces, the existence of Bamet-H2 biotransformation by intestinal bacteria, as happens with natural bile acids (Hylemon, 1985), and, hence, a modification in the absorption rate of the resulting products, cannot be ruled out.

The presence of Bamet-H2 and cisplatin in ileal tissue after intraluminal perfusion of the ileum with these drugs may be due to the existence of both uptake and extracellular or intracellular binding to the ileal mucosa. However, trans-epithelial ileum transport, subsequent release to portal blood, liver uptake and secretion by the hepatocytes did occur. Thus, the finding of low serum platinum concentrations following i.g. administration of cisplatin to conscious rats together with the low amount of this drug present in ileum, bile and liver after intraluminal ileum perfusion are consistent with the well-known poor intestinal absorption of cisplatin which occurs via simple diffusion (Binks and Dobrota, 1990). By contrast, the findings of high serum concentrations after i.g. administration of Bamet-H2 to conscious rats, high ileum and liver content, and high biliary output observed during ileum perfusion with this compound support the hypothesis that the intestinal absorption of Bamet-H2 is markedly more efficient than that of cisplatin.

Moreover, low intestinal absorption of cisplatin was probably responsible for the high amount of drug eliminated into feces when the compound was given i.g. as compared with the amount of fecal platinum found after i.v. administration. In the latter case most of the platinum found in feces predominantly derived from biliary secretion. By contrast, a high amount of Bamet-H2 was secreted into bile after i.v. injection and was hence found in feces. The amount of fecal platinum was not modified after i.g. administration of Bamet-H2, except that the peak of drug elimination into feces was delayed 24 h.

The existence of cholephilic properties (high liver uptake and efficient secretion into bile), the enhanced intestinal absorption and the low relevance of Bamet-H2 excretion by the kidney probably account for the maintenance of serum concentrations at higher level and for longer time during the first hours following i.v. administration of Bamet-H2 as compared to cisplatin. By contrast, after this initial period, a more efficient elimination of Bamet-H2 was observed.

It has been previously reported by others (Goel et al., 1990) that immediately after intravenous cisplatin administration the major proportion of the drug is found in the ultrafiltrable plasma fraction. However, as the free drug is eliminated, non-ultrafiltrable platinum-containing macromolecules with longer half-life begin to predominate. Our results are fully consistent with this type of dynamics for both cisplatin and Bamet-H2 in serum, although slower but more efficient elimination of Bamet-H2 from the body probably accounted for the reduction in the amount of ultrafiltrable forms of this drug 7 days after administration.

The cholephilic characteristics of Bamet-H2 are probably endowed by the presence of two bile acid moieties in the molecular structure. Endogenous bile acids, which are typical cholephilic molecules, are poorly extracted by the kidney (Alme et al., 1977). The existence of specific carrier systems at the sinusoidal and canalicular membrane of the hepatocyte is responsible for the very efficient bile acid uptake from sinusoidal blood and later secretion into bile. Moreover, via both carrier-mediated processes and simple diffusion endogenous bile acids are efficiently recovered by the intestine, mainly in the ileum, and released into the portal blood, hence returning to the liver and closing the so-called enterohepatic circulation. Regarding the mechanisms underlying this similarity between bile acids and Bamet-H2, it should be pointed out that the both sodium-dependent and sodium-independent mechanisms have been reported to be involved in Bamet-H2 uptake by rat hepatocytes (Marin et al., 1997). At least sodium-independent mechanisms are also expressed in hepatocellular carcinoma (Kullak-Ublick et al., 1997), which suggests that cytostatic agents coupled to bile acids can be directed toward these tumours.

Both the liver (Villanueva et al., 1997) and the kidney play an important role in cisplatin clearance from blood (DeConti et al., 1973; Gromley et al., 1979). This accounts for the high concentrations of this drug observed in both organs. The powerful detoxification machinery present in hepatocytes probably protects them in part from the high amounts of this drug crossing these cells from blood to bile after i.v. administration. However, high cisplatin concentrations in kidney tissue are probably responsible for the most notable side effect of this drug: nephrotoxicity. Cisplatin induces histologically observable tubular injury (Gonzalez-Vitale et al., 1977) resulting in reduced proximal tubular salt and water reabsorption, magnesium depletion, normoglycaemic glycosuria, proteinuria, albuminuria, amino aciduria and acute enzymuria (Daugaard et al., 1988a). The severity of clinical manifestations ranges from reversible asymptomatic increases in the serum concentrations of urea and creatinine to frank acute renal failure (Daugaard et al., 1988b). The low ability of the kidney to excrete Bamet-H2 reduces drug concentrations in this organ and is hence probably the reason for lower risk of nephrotoxicity reported for this compound (Marin et al., 1996).

In sum, our results indicate that the presence of two cholylglycinate moieties in Bamet-H2

molecule endows this complex with interesting organotropic characteristics typical of bile acids, at the same time maintaining an important part of the typical cytostatic activity of platinum(II)-containing drugs (Criado et al., 1997). Several potential benefits of this new drug can be suggested: lower exposure of the kidney, re-exposure of tumors located in the enterohepatic circuit, maintenance of higher serum levels early after administration, ability of the body to efficiently eliminate the drug into feces at mid-term and the possibility of oral administration.

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