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Pharmacokinetics of diaspirin cross-linked haemoglobin in a rat model of hepatic cirrhosis*

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Abstract

The aim of the study was to evaluate the effect of cirrhosis on the disposition of the haemoglobin-based oxygen carrier, diaspirin cross-linked haemoglobin (DCLHb). Cirrhosis was induced in male Sprague-Dawley rats (200–250 g) by inhalational exposure to carbon tetrachloride (CCl_4), over a period of 6 weeks. Pharmacokinetic evaluation was performed after a single intravenous bolus administration of DCLHb (400 mg kg^{-1}). Serum biochemistry, including aspartate transaminase, alkaline phosphatase, bile acids, serum albumin, and serum creatinine, were measured in CCl_4-treated ($n = 6$) and age-matched control ($n = 6$) rats. After 6 weeks, the jugular vein and carotid artery were cannulated for bolus DCLHb administration (400 mg kg^{-1}) and blood sampling, respectively, in both groups of rats. Cirrhosis produced significant ($P < 0.05$) elevations in alkaline phosphatase (497.4 ± 84.8 U L^{-1} vs 241.2 ± 5.1 U L^{-1}), aspartate transaminase (920.5 ± 190.9 U L^{-1} vs 238.2 ± 118.1 U L^{-1}) and bile acids (333.8 ± 77.3 mg dL^{-1} vs 43.8 ± 4.2 mg dL^{-1}) compared with the control group. No significant renal dysfunction was observed as a result of CCl_4 exposure. Plasma DCLHb concentrations declined approximately log-linearly. Systemic clearance of DCLHb was estimated to be 2.2 ± 0.7 mL h^{-1} in the treatment group and was slightly, but not significantly, less in the control group (3.6 ± 1.7 mL h^{-1}). There was also a trend toward a longer elimination half-life in the treatment group (4.7 ± 2.2 h) compared with the control group (3.8 ± 0.8 h), although this difference was not statistically significant. Cirrhosis does not significantly alter the disposition of DCLHb perhaps due to increased extra-hepatic metabolism by the reticulo-endothelial system.

Introduction

Haemoglobin-based oxygen carriers are being developed to replace whole blood (or red blood cells) in the field of transfusion medicine (Winslow 1995; Hess 1996; Williams 1996). The potential applications for these agents include restoration of oxygen delivery, prevention or reversal of hypovolemia and subsequent organ failure, haemodilution in patients undergoing elective surgery, extra corporeal oxygenation during cardiopulmonary bypass, and to improve tissue perfusion in cardiogenic, septic or post-surgical shock.

Diaspirin cross-linked haemoglobin (DCLHb) is a haemoglobin-based oxygen carrier prepared from outdated red blood cells. DCLHb is produced by cross-linking the α-α subunits of the haemoglobin using the diaspirin compound 3,5-dibromosalicyl-bis fumarate (Chatterjee et al 1986). This covalent cross-linking occurs between the *N*-terminal amino acids of the α chains at Lys-α-1 and Lys-α-2. The α-α cross-link stabilizes the native haemoglobin, thereby increasing vascular retention time, decreasing oxygen affinity and preventing the breakdown of haemoglobin to dimers and their subsequent renal elimination.

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It has been shown that DCLHb possesses an excellent oxygen carrying and delivering capacity in rats (Snyder et al 1987). The haemodynamic and cardiovascular effects of DCLHb have also been studied extensively (Gulati & Rebello 1994; Gulati et al 1994). These effects include increases in mean arterial pressure, cardiac output, and blood flow to primary organs. The oxygen delivering capacity coupled with its pharmacological effects have allowed DCLHb to be used as a resuscitative agent in haemorrhagic shock. Recently, Ortiz et al (2000) showed that α-α cross-linked haemoglobin, structurally similar to DCLHb, is an effective resuscitative solution in haemorrhage in cirrhotic rats. The pharmacokinetics of α-α cross-linked haemoglobin have been studied in animal models (Hess et al 1989) and in man (Przybelski et al 1996). In one study, it was found that the plasma half-life for α-α cross-linked haemoglobin was about 4.4 h in rats, whereas that for stroma-free haemoglobin was 0.9 h, and both were shown to have predominantly extra-renal elimination (Hess et al 1989). To date, only one study has evaluated the effect of a pathological condition (sepsis) on the pharmacokinetics of DCLHb (d'Almeida et al 1998). Since the elimination half-life was not affected, it was concluded that the dosage regimen of DCLHb need not be altered in sepsis.

Because of its structural similarity to haemoglobin, it is assumed that DCLHb is metabolized in a similar manner to endogenous haemoglobin. Since haemoglobin is metabolized in the reticulo-endothelial system (RES) primarily by liver (Przybelski et al 1996), damage to liver function could potentially influence the pharmacokinetics of DCLHb. Liver cirrhosis is characterized by severe fibrosis and necrosis of the liver tissue, which consequently results in the functional loss of hepatocytes and liver parenchyma. This is accompanied by a reduced metabolic capacity reflected in reduced synthesis of drug metabolizing enzymes and circulating plasma proteins. In addition, severe portal hypertension in cirrhosis contributes significantly to the changing pattern of systemic haemodynamics (hyperdynamic circulation) seen in cirrhosis and consisting of increased cardiac output and reduced systemic vascular resistance, with normal arterial pressure (Bernardi & Trevisani 1997). Hence, functional changes associated with cirrhosis, including reduced metabolic capacity, hypotension, blood flow redistribution, impairment of cardiovascular autoregulation and increased capillary permeability, could affect the distribution and elimination of DCLHb. To our knowledge, there are no reports on the effects of chronic liver damage on the pharmacokinetics of DCLHb. Any alterations in the metabolism of DCLHb due to cirrhosis could hamper the benefits of DCLHb in the treatment of blood loss either due to prolonged cirrhosis or severe traumatic injury. In this study, we examined the pharmacokinetics of DCLHb in a rat model of cirrhosis induced by carbon tetrachloride (CCl_4) inhalation exposure.

Materials and Methods

Animals

Male Sprague-Dawley rats, 200–250 g, were obtained from Harlan Co. (Madison, WI) and were housed and maintained at the Biological Resources Laboratory, University of Illinois at Chicago. The study was approved by the Animal Care Committee of the University of Illinois at Chicago and followed the Guidelines for the Care and Use of Laboratory Animals. All rats received a standard pellet diet throughout the study. Experiments were performed over a period of 6 weeks. The rats were divided into two groups of six rats each: untreated (control group) and CCl_4/phenobarbital-treated (cirrhotic group). They were housed in groups of three under a 12-h light–dark cycle. Rats were weighed twice a week for the duration of the experiment. At the end of the study, rats were killed by decapitation, the livers were excised and examined morphologically for liver injury.

Induction of cirrhosis

A modified version of an extensively studied cirrhotic model (McLean et al 1969) was used. From one week before treatment with CCl_4 until the end of the study, rats in the cirrhotic group were provided with an aqueous phenobarbital solution (0.4 g L^{-1}) as the only drinking source. Rats in the cirrhotic group were transferred to a chamber and exposed to CCl_4 inhalation twice weekly for a period of six weeks. The initial duration of exposure was 1 min and was increased by 1 min each week, to a maximum of 5 min. Exposure was achieved by bubbling air through a 1-L flask containing CCl_4 and was piped directly into the animal chamber. Air was allowed to equilibrate with the volatilized CCl_4 before the rats were placed in the chamber. The flow rate of CCl_4 gas was maintained at 2 L min^{-1} using a 65 × 150-mm flowmeter (Gilmont Instruments, IL).

Estimation of biomarkers

To determine the extent of liver damage, the measurement of various serological biomarkers was performed at baseline and after 6 weeks. Rats were anaesthetized

with ketamine HCl (100 mg kg^{-1}) (Fortdodge Animal Health, IO) and blood (0.5 mL) was withdrawn from the anterior vena cava puncture. Blood was allowed to clot and serum was collected for measurement of alkaline phosphatase (AP), aspartate transaminase (AST), bile acids (BA), albumin (ALB), blood urea nitrogen (BUN), and serum creatinine (CR) concentrations, using commercially available diagnostic kits. All assays were performed on the same day using standardized automated procedures performed in the Clinical Chemistry Laboratory in the Biological Resources Laboratory.

Animal surgery

After 6 weeks, the rats were anaesthetized by an intraperitoneal injection of ketamine HCl (100 mg kg^{-1}) and xylazine HCl (5 mg kg^{-1}) (Phoenix Scientific Inc, St Joseph, MO). A midline incision was made around the neck region. The right jugular vein and left carotid artery were cannulated using PE50 tubing and the skin was sutured after the surgery using suture clips. All surgical procedures were performed under aseptic conditions. Animals were allowed to recover from the surgical anaesthesia and were fed overnight with a normal diet. Water or aqueous phenobarbital solution was freely available.

Pharmacokinetics of DCLHb

Control and cirrhotic rats received a single intravenous bolus dose of DCLHb (400 mg kg^{-1}) through the catheter placed in the jugular vein. Blood samples (0.5 mL) were collected from the catheter placed in the carotid artery before and at 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 12, and 24 h after dose administration. An equal volume of normal saline (0.9% NaCl) was injected after each withdrawal. Samples were immediately centrifuged (3000 rev min^{-1} for 15 min) and refrigerated at 4°C before analysis.

Plasma was assayed for haemoglobin content according to the method described by d'Almeida et al (1998). Briefly, free plasma haemoglobin was measured using a commercially available kit (Sigma Chemical Co., St Louis, MO). The colorimetric determinations were performed at a wavelength of 600 nm using a Spectronic Genesys-5 UV-VIS spectrophotometer (Milton Roy Instruments, Rochester, NJ). The assay is linear up to a haemoglobin concentration of 50 mg dL^{-1} and the lowest detectable concentration is 5 mg dL^{-1}. Haemoglobin content attributable to DCLHb plasma concentrations was corrected for endogenous haemoglobin concentrations. Pharmacokinetic parameter estimates were determined using both compartmental and non-compartmental methods as implemented in WinNonlin Pro (Pharsight Corp., Mt. View, CA). In the non-compartmental analysis, the area under the curve ($AUC_{0-\infty}$) was estimated using the linear trapezoidal rule to the last measurable concentration (C_{final}) and extrapolated to infinity by dividing C_{final} by the negative value of the terminal slope (k) of the log-linear plasma concentration–time curve. The following parameters were also calculated: mean residence time (MRT_{iv}) was calculated as k^{-1}; systemic clearance (CL) was calculated as the ratio of dose to $AUC_{0-\infty}$; the apparent volume of distribution (Vd_{ss}) was calculated as the product of CL and MRT_{iv}; and plasma half-life ($t_{\frac{1}{2}}$) was calculated as the product of ln2 and MRT_{iv}. Mono and bi-exponential functions were fitted to the plasma DCLHb concentration–time data using iterative nonlinear regression. Adequacy of the model was assessed by examining residual plots and comparison of the Akaike Information Criterion (AIC) for nested models (one compartment and two compartment models) (Yamaoka et al 1978).

Statistical analysis

Liver function biomarker data are expressed as mean ± s.e.m. Differences in biomarkers between the control and cirrhotic groups at baseline and at 6 weeks were analysed by the Student's *t*-test (paired analysis for within groups comparisons and unpaired analysis for between groups comparisons). Pharmacokinetic parameter estimates obtained from the individual analysis are expressed as mean ± s.d. Parameter estimates obtained using compartmental methods were compared with those obtained using the non-compartmental analysis using the Student's paired *t*-test. Differences in pharmacokinetic parameter estimates between groups were evaluated using the Student's unpaired *t*-test. In all analyses, $P < 0.05$ was taken as the level of significance.

Results

The average bodyweight of the cirrhotic group and the control group was similar at baseline (246.9 ± 1.3 and 243.8 ± 3.1 g, respectively). After 6 weeks the average bodyweight of the cirrhotic group was less than ($P = 0.05$) that of the control group (372.8 ± 5.7 and 416.7 ± 19.6 g, respectively). Morphologically, the livers of rats in the cirrhotic group were yellowish in colour, inelastic, nodular and shrunken, indicating a fibrotic liver as a result of increased collagen production.

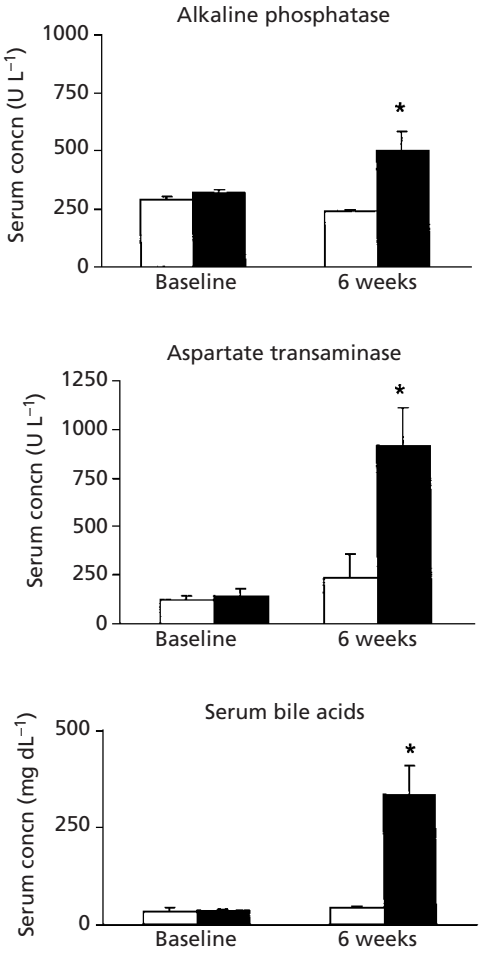

Figure 1 Serum concentrations of alkaline phosphatase, aspartate transaminase and bile acids at baseline and 6 weeks after exposure to CCl_4/phenobarbital in control (□) and cirrhotic (■) rats. Data are mean ± s.e.m., n = 6. *$P < 0.05$ compared with control.

Effect on serological biomarkers

At baseline, aspartate transaminase (124.5 ± 19.5 U L^{-1} vs 146.0 ± 35.1 U L^{-1}), bile acids (32.4 ± 9.6 mg dL^{-1} vs 30.0 ± 3.4 mg dL^{-1}), alkaline phosphatase (286.3 ± 15.3 U L^{-1} vs 318.0 ± 12.4 U L^{-1}) and serum albumin (3.3 ± 0.1 g dL^{-1} vs 3.4 ± 0.1 g dL^{-1}) concentrations were not significantly different between control and cirrhotic groups, respectively. In the control group, the concentrations of aspartate transaminase, bile acids, and alkaline phosphatase after 6 weeks were not significantly different from those at baseline. Aspartate transaminase concentrations were significantly increased in the cirrhotic group (920.5 ± 190.9 U L^{-1}) after 6 weeks compared with the control group (238.2 ± 118.1 U L^{-1}). Similarly, alkaline phosphatase concentrations (497.4 ± 84.8 U L^{-1} vs 241.2 ± 5.1 U L^{-1}) and bile acids concentrations (333.8 ± 77.3 mg dL^{-1} vs 43.8 ± 4.2 mg dL^{-1}) were significantly increased in the cirrhotic group after 6 weeks compared with the control group (Figure 1). Serum albumin concentrations after 6 weeks were not significantly different between control (4.4 ± 0.1 g dL^{-1}) and cirrhotic (3.9 ± 0.3 g dL^{-1}) groups. Serum creatinine concentrations were similar in both control (0.53 ± 0.03 mg dL^{-1}) and cirrhotic (0.54 ± 0.03 mg dL^{-1}) groups at baseline and did not significantly change after 6 weeks (0.58 ± 0.02 mg dL^{-1} and 0.46 ± 0.04 mg dL^{-1} for control and cirrhotic groups, respectively). Blood urea nitrogen concentrations were similar in control (19.0 ± 1.3 mg dL^{-1}) and cirrhotic (19.7 ± 1.2 mg dL^{-1}) groups at baseline and did not change significantly after 6 weeks (20.1 ± 0.9 mg dL^{-1} and 19.8 ± 1.9 mg dL^{-1} for control and cirrhotic groups, respectively).

Pharmacokinetics of DCLHb

Peak plasma haemoglobin concentrations after DCLHb administration (840.5 ± 147.8 mg dL^{-1} and 1140.5 ± 300.6 mg dL^{-1} for control and cirrhotic groups, respectively) were approximately 300–500-times higher than baseline endogenous haemoglobin concentrations. Baseline concentrations of plasma haemoglobin measured just before DCLHb administration were 26.1 ± 25.6 mg dL^{-1} and 17.3 ± 8.0 mg dL^{-1} for the control and cirrhotic groups, respectively. At 24 h after drug administration, the plasma haemoglobin concentrations were 30.0 ± 10.2 mg dL^{-1} and 47.8 ± 27.6 mg dL^{-1} for control and cirrhotic groups, respectively. Since 24-h DCLHb concentrations in control and cirrhotic groups were not significantly different from baseline values, we assumed constant baseline haemoglobin concentrations. Therefore, plasma DCLHb concentrations were corrected for baseline haemoglobin concentrations in subsequent data analyses.

After DCLHb administration, the baseline-adjusted total plasma haemoglobin concentrations declined approximately log-linearly. Figure 2 shows the mean plasma concentration–time profile of DCLHb in the control and cirrhotic groups.

In a compartmental analysis, the selection of the best model was based on the evaluation of AIC and residual plots. Both one- and two-compartment models showed similar residual plots, but in general the fit of the one-compartment model to concentration–time data in both control and cirrhotic groups produced a lower AIC. Thus, the disposition of DCLHb is well described by a one-compartment model. Pharmacokinetic parameter estimates obtained from the one-compartmental method and non-compartmental analyses are compared in

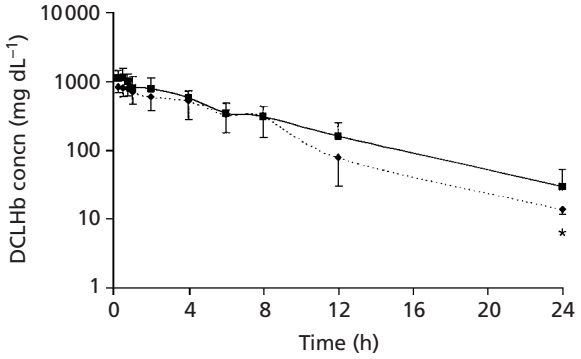

Figure 2 Plasma concentration–time profile of DCLHb (400 mg kg^{-1}) in control (◆) and cirrhotic (■) rats. Each point represents mean ± s.d., n = 6 (except *n = 4).

Table 1. Both methods provide similar pharmacokinetic parameter estimates in both experimental groups.

Systemic clearance of DCLHb in the cirrhotic group was slightly, but not significantly, less than that in the control group. Although it was not statistically significant, there was a trend towards a longer elimination half-life in the cirrhotic group compared with the control group. In addition, Vd_{ss} was similar in control and cirrhotic groups, as was MRT_{iv}.

Discussion

Hepatic cirrhosis was induced in rats using the CCl_4 inhalation procedure and the pharmacokinetics of DCLHb evaluated. This model of cirrhosis induction is well established and is characterized by diffuse hepatocellular damage that produces functional changes, including elevated serum liver enzymes, comparable with that in man (McLean et al 1969; Recknagel et al 1989).

In this study there was a significant elevation of aspartate transaminase, alkaline phosphatase and serum bile acids. Renal function markers including serum creatinine and blood urea nitrogen did not increase, suggesting that renal dysfunction did not occur in this model of cirrhosis. Also there was no evidence of hypoalbuminaemia, an indicator of the presence of peripheral oedema. This was confirmed by the absence of ascites in the cirrhotic group. Hence, the CCl_4 inhalation exposure procedure produced a pre-ascitic stage of hepatic cirrhosis without renal dysfunction.

Studies suggest that metabolism of DCLHb is similar to that of native haemoglobin (Przybelski et al 1996). The metabolism of native haemoglobin A_o takes place in the RES of liver and spleen. The bulk of senescent red cells are engulfed by macrophages of the RES, particularly liver and spleen. Approximately 10–20% of senescent red cells break up within the blood stream, where the liberated haemoglobin is bound to a specific carrier protein, haptoglobin. Some plasma haemoglobin is also cleaved into globin and haeme; the latter binds to another carrier protein, haemopexin. Both complexes are cleared from the circulation by the liver and catabolized to bilirubin by hepatic parenchymal cells.

Similar metabolic pathways are involved in the elimination of cross-linked and polymerized haemoglobins. Although tissue distribution studies have shown that the highest distribution is associated with kidneys, liver, and spleen (Keipert et al 1992, 1994), it has been reported that the renal elimination of DCLHb is limited (< 1%) (Hess et al 1989). Taken together, these data suggest that the major metabolic pathway for cross-linked haemoglobin is by catabolism in the RES system of liver and spleen.

In this study, it is hypothesized that metabolism, and therefore the elimination, of DCLHb is impaired in cirrhosis as a result of pathological damage to a major organ required for DCLHb elimination. However, we

Table 1 Pharmacokinetic parameter estimates by compartmental and non-compartmental analysis.

Estimates	Control group		Cirrhotic group	
	One-comptmental	Non-comptmental	One-comptmental	Non-comptmental
$AUC_{0-\infty}$ (mg h mL^{-1})	58.5 ± 20.9	53.4 ± 17.7	67.5 ± 20.6	70.7 ± 20.0
CL (mL h^{-1})	3.4 ± 1.6	3.6 ± 1.7	2.5 ± 1.0	2.2 ± 0.7
Vd_{ss} (mL)	20.0 ± 5.1	17.5 ± 5.3	14.2 ± 5.3	14.6 ± 5.2
$t_{\frac{1}{2}}$ (h)	4.6 ± 1.2	3.8 ± 0.8	4.4 ± 2.2	4.7 ± 2.2
MRT_{iv} (h)	6.6 ± 1.8	4.6 ± 0.5	6.3 ± 3.1	5.7 ± 0.8

Data are mean ± s.d.

found that the pharmacokinetics of DCLHb in the cirrhotic group was not significantly different from that in rats with normal liver function, although there was a trend towards a slower clearance and a longer elimination half-life in the cirrhotic group.

A possible explanation for the absence of a significant reduction in the elimination of DCLHb in cirrhotic rats could be the result of increased metabolism of haemoglobin by the extra-hepatic RES system and increased presentation of the drug to an alternate metabolizing system, in particular spleen, due to increased blood flow to organs other than liver.

In studies done to assess the phagocytic and metabolic activity of the RES, it was shown that RES metabolic activity and phagocytic activity are severely impaired in cirrhosis (Cooksley et al 1973; Lahnborg et al 1981). This is because the RES in liver accounts for 80–85% of the total RES. However, Lahnborg et al (1981) reported that destruction of reticulo-endothelial cells in the liver appears to be compensated by an increased activity in spleen, skeleton and lungs.

It is also possible that an increased blood flow could result in presentation of more drug to the extra-hepatic RES metabolizing system. We have estimated the liver, spleen, gastrointestinal tract and portal blood flows using a radioactive microsphere technique. Although not statistically significant, we found that, after 6 weeks, there was a trend towards an increase (approx. 20%) in blood flow to the spleen in cirrhotic rats compared with the control group (unpublished results). Studies in man have shown that splenic blood flow increases in cirrhosis (Williams et al 1968; Gitlin et al 1970). In addition, increased splenic blood volume is seen in cirrhosis (Merkel et al 1985; Oguro et al 1993). Although, to our knowledge, there is no established work to support altered extra-hepatic metabolism in cirrhosis, it is possible that increased functional activity of extra-hepatic RES and increased presentation of DCLHb to this system may compensate for reduced liver metabolism of DCLHb in our study. Future studies should therefore focus on the role of spleen in the metabolism of DCLHb in cirrhosis and tissue distribution of DCLHb in cirrhosis.

In summary, we evaluated a hepatic cirrhotic model in rats and examined the pharmacokinetics of DCLHb in this model. A reproducible pre-ascitic stage of hepatic cirrhosis was produced without renal dysfunction. The pharmacokinetics of DCLHb appear to be well described by a one-compartment open model in both experimental groups and we found no change in the pharmacokinetic parameter estimates due to the presence of cirrhosis.

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