KINETICS OF HEPATIC ACCUMULATION OF DEXTRANS IN ISOLATED PERFUSED RAT LIVERS

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ABSTRACT:
The role of various processes (uptake, release, metabolism, and excretion) in the hepatic accumulation of dextrans was investigated in isolated perfused rat livers (IPRLs). Single-pass IPRLs were infused with fluorescein-dextran (FD) with a molecular weight (MW) of 70,000 (FD-70) for 15, 30, 45, or 60 min, and inlet and outlet samples and livers were collected. In addition, two groups of livers were infused with FD-70 for 60 min, followed by 30 or 60 min of drug-free perfusion. The concentrations of the macromolecule in the samples were measured by a size exclusion chromatographic method. Similar, but limited, experiments were also conducted for FDs with MWs of 4,000 (FD-4) and 150,000 (FD-150). In addition, the metabolism of all three FDs were investigated using liver homogenates. Because of low hepatic extraction, the concentrations of dextrans in the inlet and outlet perfusates were almost the same during the entire perfusion. However, liver concentrations increased almost linearly during the infusion of FD-70 (0–60 min) and declined slowly thereafter (60–120 min). The apparent hepatic extraction ratio ($E_{app}$) values, estimated directly from the concentrations of FDs in the liver, were MW dependent; $E_{app}$ of FD-4 (0.124% ± 0.015%) was significantly ($p < 0.05$) less than that for FD-70 (0.677% ± 0.193%) or FD-150 (0.711% ± 0.022%). The metabolism and biliary excretion of all FDs were negligible during the perfusion time. The mean residence time of FD-70 in the liver, estimated by nonlinear regression analysis of experimental data, was 248 min. These studies define the role of various processes involved in the slow (but substantial) and MW dependent hepatic accumulation of dextrans.

Dextrans are glucose polymers that have been proposed as drug carriers (1–4). Previous studies (5, 6) using FDs coupled with a specific high-performance size exclusion chromatographic method showed that the in vivo disposition of dextrans is MW- and dose-dependent; low MW dextrans, such as FD-4, were almost entirely excreted in urine with a linear pharmacokinetics (5, 6). On the other hand, the majority of the administered dose of larger MW FDs, such as FD-70 and FD-150, accumulated in the reticuloendothelial system (including the liver) with some degree of nonlinearity at high doses (5, 6).

Despite substantial in vivo accumulation of FD-70 and FD-150 in the liver (up to 70% of the dose) (6), the clearance values of these high MW FDs are much lower than the hepatic blood flow, resulting in estimated $E$ of <1% (5). Therefore, the hepatic accumulation of high MW FDs has been characterized as slow but substantial (5).

The significant accumulation of dextrans in the liver may be advantageous in targeting therapeutic agents into this important organ. Indeed, animal studies have already confirmed the applicability of this concept for targeted delivery of glutathione to the liver (7). Theoretically, the extent of hepatic accumulation of drugs and macromolecules is governed by the relative magnitude of various processes, such as uptake, release, metabolism, and biliary excretion. Therefore, if dextrans are to be used effectively for hepatic drug delivery, a thorough understanding of these processes involved in their hepatic accumulation is necessary.

Hepatic accumulation of dextrans has been the subject of a number of reports dating back to >4 decades ago (8–11). However, most, if not all, of these investigations have used nonspecific methods, such as radiolabeling or fluorometry, that cannot distinguish between the parent macromolecule and its possible degradation products. In addition, the kinetics of the release of dextrans from the liver tissue has not been addressed. Therefore, the objective of this study was to study systematically the role of various processes involved in the hepatic accumulation of the high MW dextran, FD-70, in IPRLs using a specific size-exclusion HPLC method. Moreover, the possible effects of MW on the ex vivo hepatic accumulation of FDs were investigated by including FD-4 and FD-150 in the study.

Materials and Methods

Chemicals. FDs with average MWs of 4,300 (FD-4, lot 63H0121), 69,000 (FD-70, lot 109F0446), and 145,000 (FD-150, lot 32H0451) were purchased from Sigma Chemical Co. (St. Louis, MO). The degree of fluorescein substitution and polydispersity of FDs have been reported before (5). Recently expired human red blood cells were obtained from the Blood Center of Central Iowa (Des Moines, IA) and washed 3 times with Krebs-bicarbonate buffer before use. Bovine serum albumin (Fraction V), sodium taurocholate, and transaminases kit for measurement of SGOT and SGPT were obtained from Sigma. All other reagents were analytical grade and available through commercial sources.

Preparation of Isolated Livers. Adult male Sprague-Dawley rats (290–330 g) were used as liver donors. The liver isolation and cannulation methods

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were similar to those reported before (12, 13). In addition, the common bile duct was cannulated using PE-10 tubing. Isolated livers were perfused using a commercial perfusion apparatus (MX Peruser II; MX International, Aurora, CO).

The perfusate was a Krebs-Henseleit bicarbonate buffer (pH 7.4) containing 1.2 g/liter glucose, 2% (w/v) albumin, 10% (v/v) washed red blood cells, and 75 mg/liter sodium taurocholate. Livers were perfused, in a single-pass manner, with 15 ml/min of the perfusate.

The viability of the liver was confirmed through stable levels of transaminases (SGOT and SGPT) in the outlet perfusate at the beginning and end of perfusion, relatively high (0.9–1.7 ml/hr) and constant bile flow over the entire perfusion period, wet liver weights of ≤4% of body weight at the end of perfusion, and overall macroscopic appearance of the liver.

**Dextran Infusion and Sample Collection of IPRLs.** The study protocol is summarized in scheme 1. Before the infusion of FDs, the livers were perfused with dextran-free perfusate for ~15 min. FDs were dissolved in distilled water (40 mg/ml) and delivered to the liver at a constant rate of ~720 µg/min, resulting in an inlet concentration of ~50 µg/ml. To determine the time course of FD-70 in the liver during the infusion of the macromolecule, four groups (N = 3) of livers were infused for 15, 30, 45, or 60 min. Additional groups of livers (N = 3) were also infused with FD-70 for 60 min; thereafter, the livers were perfused with dextran-free perfusate for an additional 30 or 60 min to determine the time course of FD-70 in the liver in the absence of FD-70 input.

At the end of the perfusion, livers were collected for measurement of tissue concentrations of FD-70. Before storage, livers were washed with drug-free perfusate for 5 min to remove the macromolecule from the liver vasculature. In addition, the inlet and outlet perfusate samples were taken at various times during the perfusion, and red blood cell-free perfusates (plasma) were obtained after centrifugation. Bile samples were also collected in preweighed microcentrifuge tubes in 15-min (for 15- and 45-min groups) or 30-min (for all the other groups) intervals. Samples were stored at −60°C until analysis for their content of FD-70.

To determine the effects of MW on the kinetics of hepatic accumulation of FDs, additional experiments were also conducted for FD-4 and FD-150. However, for each of these FDs, only two groups of livers (N = 3) were used. In one group, livers were infused with FD for 60 min, whereas in the second group, a 60-min infusion of FD was followed by 60 min of dextran-free perfusion (scheme 1). Sample collection and storage for FD-4 and FD-150 were similar to those for FD-70.

**In Vitro Metabolism Studies.** Livers were obtained from male Sprague-Dawley rats and homogenized in an equal volume of 0.2 M acetate buffer (pH 4.8) containing 1% Triton-X (15) to allow for lysis of subcellular organelles and release of lysosomal enzymes into the media. The homogenate was centrifuged in a microcentrifuge for 10 min, and the supernatant was collected. Equal volumes of the supernatant and stock solutions of FD-4, FD-70, or FD-150 in acetate buffer were mixed resulting in FD concentrations of ~10 µg/ml (N = 3). Three one hundred microliter aliquots of each sample were transferred into microcentrifuge tubes for measurement of their FD contents after 0, 24, and 48 hr incubation at 37°C in a shaking waterbath. Controls were prepared similarly with one difference; the liver supernatant was replaced with the acetate buffer solution containing Triton. At the previously described intervals, control and test samples were taken and immediately frozen at −60°C until analysis.

**Analysis of FDs.** Samples were analyzed using a size exclusion chromatographic method (16) after minor modifications (15).

**Analysis of Data.** For all three FDs, E_{app} was calculated by dividing the amount of FDs found in the liver at time t by the total input from 0–t for the duration of FD infusion. Although not accounting for other processes that may be involved in the hepatic accumulation of FDs (e.g. release from cells into vasculature and liver metabolism/excretion), E_{app} is a useful parameter for comparison of the net hepatic accumulation of different MWs of FDs. In addition, for FD-70, data were fit to a one-compartment liver model with first-order uptake and release processes responsible for the transfer of FD-70 to and from the liver tissue, respectively. The hepatic blood was not modeled as a separate compartment because the concentrations of dextrans in the inlet and outlet perfusates were almost the same, resulting in a quasi steady-state condition for the hepatic blood. The following differential equation along with the experimental data for the concentration of FD-70 in the tissue (C_{liver}) and inlet plasma (C_{in}) were incorporated into the program WinNonlin and the uptake clearance (CL) and intrahepatic turnover rate constant (k_{out}) were estimated.

\[
\frac{dC_{liver}}{dt} = \frac{C_l \cdot C_{in}}{V_{liver}} - k_{out} \cdot C_{liver}
\]

where V_{liver} represents the volume of liver tissue estimated by subtraction of the volume of hepatic blood from the liver weight. The hepatic blood volume fraction used in our calculation (0.2) was based on data reported (17) for IPRLs with similar flow rates used in our studies. The intrahepatic mean residence time was estimated from 1/h_{out}. For C_{in} values, the plasma, rather than whole blood, data were used because FDs do not penetrate red blood cells to a significant degree (16).

The amount of FDs excreted in bile was estimated by multiplying the volume of bile by the concentration of FDs in the sample.

For metabolism studies, the concentrations at each time point after the incubation were expressed as the percentages of the initial concentrations added to the media. In addition, to account for possible nonenzymatic degradation of FDs, the percentage values were corrected by the mean values of the control samples (no liver homogenate) incubated for the same length of time as tests.

The differences among FDs in their hepatic metabolism and accumulation parameters were analyzed using ANOVA with post-hoc analysis of means by Scheffe’s F test at a significance level of 0.05. Data are presented as mean ± SD.

**Results**

**IPRL.** In fig. 1, the amount of FD-70 recovered in the livers infused with the macromolecule for 15, 30, 45, or 60 min is plotted against the total amount of FDs injected to the livers during the infusion time. Also depicted in fig. 1 are similar data for FD-4 and FD-150 after 60 min of macromolecule infusion. Hepatic accumulations of FD-70 and FD-150 were comparable and substantially higher than that of FD-4 (fig. 1). The E_{app} values for FD-70 at different times after infusion are...
presented in table 1. The values were low and time-independent during the 60-min period studied. Therefore, for FD-4 and FD-150, only one infusion time (60 min) was studied, and the $E_{\text{app}}$ values were compared with that of FD-70 for the same infusion time (table 1). Although the $E_{\text{app}}$ values of FD-70 and FD-150 were not different, $E_{\text{app}}$ of FD-4 was substantially lower than the values for both FD-70 and FD-150 (table 1).

Figure 2 depicts the inlet, outlet, and liver tissue concentrations of FD-70 both during the 60-min infusion of FD-70 and also at 30 and 60 min after the infusion of the macromolecule is stopped. The fit of the hepatic model with uptake and release processes is also demonstrated in fig. 2. Due to very low hepatic extraction, the concentrations of FD-70 in the outlet samples were not significantly different from those in the inlet samples. Therefore, based on the outlet concentrations of FD-70, one may erroneously conclude that the steady-state condition is achieved in $\leq 5$ min (fig. 2). However, the concentration of FD-70 in the liver tissue increased almost linearly over the infusion time, indicating lack of steady state. Moreover, the decline in the liver tissue concentration of FD-70 in the absence of macromolecule input was slow (fig. 2). The estimated values of $C_l$ and $k_{\text{out}}$ from the hepatic model were $0.0681 \text{ ml/min}$ and $0.00404 \text{ min}^{-1}$, with coefficient of variation of $8.4\%$ and $41\%$, respectively. The hepatic mean residence time of FD-70 was 248 min.

For FD-4 and FD-150, the inlet and outlet concentrations were similar to those for FD-70 (data not shown), and the liver concentrations at 60 and 120 min, respectively, were $4.34 \pm 0.24$ and $3.62 \pm 0.32 \mu g/g$ (FD-4) and $22.4 \pm 0.8$ and $12.8 \pm 2.4 \mu g/g$ (FD-150).

Relatively small amounts of FDs ($< 1.5 \mu g$ or $< 0.003\%$ of the total input) were excreted in the bile during the entire perfusion period, with the levels of FDs in some bile samples being below the analytical detection limit. The biliary excretion of FD-4 ($1.31 \pm 0.18 \mu g$ after 60 min of infusion at a rate of $\sim 720 \mu g/min$) was the highest among the threeMWs studied.

**Metabolism Studies.** The percentage of FDs remaining in the supernatants of liver homogenates at 24 and 48 hr after the incubation of samples are depicted in fig. 3. For FD-4, no significant metabolism was observed during the incubation period. However, for FD-70 and FD-150, a small, but statistically significant, degree of metabolism was observed (fig. 3). The first order rate constants for metabolism of FD-4, FD-70, and FD-150 in this system were $0.00955$, $0.0646$, and $0.0715 \text{ day}^{-1}$, respectively.

**Discussion**

For very low $E$ drugs or macromolecules, the available analytical methods are not capable of distinguishing between the concentrations of the drug in the inlet and outlet samples of IPRLS. Accordingly, Nishida et al. (10) did not detect any measurable hepatic extraction for neutral and anionic-radiolabeled dextrans (MW of 70,000) based on the inlet and outlet samples of IPRLS. This is in agreement with similar concentrations of FDs observed in the inlet and outlet samples of our present studies with fluorescein-labeled neutral dextrans (fig. 2). A more appropriate method for estimation of $E$ of low $E$ drugs is from the clearance value obtained in a recirculating IPRL. However, preliminary studies in our laboratory indicated negligible reductions in the concentrations of FDs in the reservoir of recirculating IPRLS during the relatively short period of time that IPRLS are viable. Alternatively, $E_{\text{app}}$ of FDs were estimated here by direct measurements of the macromolecules in the liver tissues at various times after their infusion into the liver (table 1).

In the presence of significant hepatic metabolism and/or free diffusion of the drug out of the liver cells, estimation of $E$ from the liver concentration of drugs may result in a substantial underestimation. However, previous (18) and present (fig. 3) data suggest that the metabolism of dextrans during the period of perfusion ($\leq 2$ hr) is not substantial; in fact, initial attempts to detect any measurable metabolism of FDs during short incubation times, similar to those used for liver perfusion, was not successful. In addition, a continuous rise in the hepatic tissue concentration of FD-70 associated with steady-state
outlet concentration for FD-70 (fig. 2) suggests that the macromolecule inside the tissue is not easily redistributed into the blood. This postulate is supported by a relatively slow decline in the hepatic tissue concentration of FD-70 when the inlet concentrations are virtually 0 (fig. 2) and by the large mean residence time (248 min) of the macromolecule in the liver. Therefore, the \( E_{ap} \) values reported in table 1 should be close to the actual \( E \) values in IPRLs.

After intravenous administration, up to 80% of the dose of FD-4 is recovered relatively rapidly in urine, with minimal accumulation of the dose in the liver (6). On the other hand, high MW FDs (such as FD-70 and FD-150) are accumulated in the liver (up to 70% of the dose), with minimal excretion of the dose (<2%) in urine (5, 6). Theoretically, the different degree of hepatic accumulation of FD-4 and FD-70 or FD-150, observed \textit{in vivo}, could be due entirely to a well-documented MW-dependent renal clearance of dextrans (5, 6, 16, 19); dextrans with MWs of higher than 40,000 are excluded almost entirely from renal excretion. However, data presented in table 1 indicate that, even in the absence of a competing renal excretion pathway, the degree of accumulation of FD-4 in IPRLs is much less than that of FD-70 or FD-150.

The significantly lower hepatic accumulation of FD-4 (table 1 and fig. 1) cannot be explained by differences in the metabolic (fig. 3) or excretory processes between FD-4 and higher MW FDs. Therefore, likely mechanisms are lower uptake and/or higher release processes for FD-4, compared with FD-70 or FD-150. The relatively small decline in the liver concentrations of FD-4 after 60 min of perfusion with drug-free perfusate (see Results) points to a lower uptake rate constant as the main mechanism for the lower hepatic accumulation of FD-4. However, this postulate should be treated with caution because cellular uptake and release of dextrans may involve several complex mechanisms, including surface adsorption (10). Nevertheless, consistent with \textit{in vivo} data (5), the current IPRL studies indicate that low MW (such as 4,000 kDa) dextrans, are not suitable for hepatic targeting of therapeutic agents.

In addition to MW, electrical charge plays a significant role in the hepatic uptake of dextrans (10, 20); despite nondetectable \( E \) for neutral and anionic dextran 70,000, significant extraction of cationic dextrans were easily measurable from the outlet and inlet data of IPRLs (10). The higher hepatic accumulation of positively charged dextrans has been attributed to a nonspecific adsorptive endocytosis of the charged molecules (10).

Theoretically, labeling dextrans with fluorescein, which assumes negative charge in physiological pH, may alter their hepatic kinetics. However, a label effect, if any, is expected to be minimal because the degree of fluorescein substitution of FDs used herein is low (0.006 mol of fluorescein/mol of glucose). In addition, as previously described, the hepatic disposition of neutral and anionic dextrans are reportedly similar (10).

In general, dextrans are believed to enter the hepatic cells by fluid-phase endocytosis (9, 21). By adjusting the estimated hepatic clearance of FD-70 (0.0681 ml/min) for the average body weight of rats in our study (306 ± 10 g), an uptake clearance of 13 ml/hr/kg may be calculated. This value in IPRLs is very close to the in vivo hepatic clearance of FD-150 after an intravenous dose of 1 mg/kg of FD-150 (6). However, both values are substantially higher than the hepatic clearance of the fluid-phase endocytosis marker polyvinylpyrrolidone in rats (0.33 ml/hr/kg) (22). Therefore, other mechanism(s) (11) may be involved in the hepatic accumulation of dextrans.

The \( k_{out} \) value for FD-70 in IPRLs (0.0040 min \(^{-1}\)) is much larger than the apparent rate constant for the decline in the liver concentrations of FD-70 after its \textit{in vivo} administration (5). This is because after the \textit{in vivo} administration, a majority, if not all, of the exocytosed FD-70 is expected to reenter the liver in subsequent circulations, because no other major elimination pathway (\textit{e.g.} renal clearance) exists for this high MW FD. Therefore, estimation of \( k_{out} \) from the \textit{in vivo} data may be misleading.

One of the characteristics of an ideal carrier is its susceptibility to biodegradation and/or excretion. Although it is desirable for a liver-specific carrier to accumulate in the liver, it is necessary that the carrier be eliminated from the body within a reasonable time. Because the high MW dextrans are not excreted by the kidneys, their removal from the body will depend on their metabolism and biliary excretion.

It has long been recognized (14) that dextrans are metabolized by exo- and endodextranases present in various organs, such as liver, producing lower MW fractions and glucose. Interestingly, our \textit{in vitro} metabolism studies indicated a hepatic metabolism dependent on the MW of dextrans (fig. 2); the high MW dextrans, which are not subject to renal excretion, were metabolized at a faster rate. Nevertheless, the metabolism of FDs by the liver homogenates seemed to be slow (fig. 3). The rate of \textit{in vivo} metabolism of dextrans, however, is expected to be higher because the \textit{in vivo} concentration of the enzyme(s) are expected to be at least 4-fold higher than those in our liver homogenates. In addition, the dextranase enzyme is sensitive to alterations in pH and temperature (14), thus making it difficult to reproduce the optimum \textit{in vivo} conditions outside the body.

The concentrations of FDs in the bile were the lowest at earlier sampling times and in most cases reached their maximum ~30–60 min after the infusion of FDs (data not shown). In all of the experiments, however, the ratio of bile:perfusionate concentration of FDs were <0.03. These data are in agreement with those reported by Lake et al. (21) demonstrating a lag time of ~12 min in the appearance of dextran with a MW of 70,000 in the bile of IPRLs and a steady-state bile:plasma concentration ratio of 0.05 in the presence of taurocholate in the perfusate. Although small, the biliary excretion of dextrans, coupled with their hepatic metabolism, is expected to reduce gradually hepatic concentrations of dextrans after their \textit{in vivo} administration.

In conclusion, IPRLs and a specific analytical method were used to study the kinetics of hepatic accumulation of dextrans of various MWs. It was shown that hepatic uptake and release are major processes involved in the accumulation of dextrans in the liver. In addition, slow metabolic degradation and biliary excretion processes would contribute to a gradual removal of these macromolecules from the liver. These data may be used for a more rational design of dextran-drug conjugates.

References


