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Effects of storage and homogenization methods on the hepatic recovery of dextrans determined by size-exclusion chromatography

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Abstract

The effects of storage and homogenization methods on the analytical recovery of dextran macromolecules from rat livers were investigated using a high-performance size-exclusion chromatographic (HPSEC) method. Livers were collected from rats dosed with fluorescein-labeled dextrans with molecular weights of 150 or 70 kD. Subsequently, the livers were subjected to different methods to study the effects of the following parameters on the hepatic recovery of dextrans: storage method (freezing the livers before homogenization or freezing the homogenates); contents of the homogenization buffer (addition of 1% Triton X-100); and sample type (HPSEC analysis of the whole homogenate or the supernatant after centrifugation). It is shown that in the absence of Triton in the homogenization buffer, the hepatic recovery of dextrans is substantially affected by all the factors studied. However, in the presence of 1% Triton in the buffer, the hepatic recoveries were maximal and independent of the storage method or sample type. These studies suggest that for optimal recovery of dextran macromolecules from the liver, a sample preparation method capable of disrupting the subcellular membranes should be used.

Keywords: Dextrans; Liver; Analytical recovery; Triton X-100; Freeze–thaw cycle; High-performance size-exclusion chromatography.

1. Introduction

Dextrans are macromolecules consisting of glucose units attached mainly through α-1,6-linkage [1]. Aside from their traditional use as plasma volume expanders, dextrans of varying molecular weights (MWs) have recently been promoted as drug carriers for increasing the longevity of drugs in the systemic circulation [1, 2]. Recent studies [3–5] with fluorescein-labeled dextrans (FDs) have shown that a majority of the administered dose of high MW (70 and 150 kD) dextrans are accumulated in the rat liver. Therefore, the use of high MW dextrans for targeted delivery of therapeutic agents to the liver was suggested [4]. During a recent experiment in this laboratory, however, major disagreements between the hepatic recoveries of FDs depending on the storage methods of livers (frozen or fresh livers) were noticed; the recoveries from the fresh (unfrozen) livers were about four times lower than those from the frozen livers. Therefore, a detailed study

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of the factors affecting the recovery of FDs from the liver was conducted, the results of which are presented here.

2. Experimental

2.1. Chemicals

Triton X-100 and FDs with average MWs of 69 000 (FD-70, lot 109F0446) and 145 000 (FD-150, lot 32H0451) were purchased from Sigma Chemical (St. Louis, MO). The characteristics of the FDs have been reported previously [4].

2.2. Experimental protocol

Adult male Sprague–Dawley rats (230–255 g) were injected with 5 mg kg^{-1} dose of FD-150 or FD-70 ($n = 4$ for each FD) via the tail vein. Five hours after dosing, rats were decapitated following cervical dislocation, and the livers were collected and rinsed in isotonic sodium chloride. The liver from each rat was cut into several pieces and divided randomly into four portions with each portion being subjected to a different handling method. The four handling methods for FD-150 injection were homogenization of fresh or freeze–thawed livers with an isotonic buffer in the presence or absence of 1% Triton X-100. The freeze–thawed livers were subjected to an overnight freeze at −70°C and a subsequent thaw at room temperature. In all four cases, the volume of homogenization buffer (phosphate buffer, pH 7.4) was three times the weight of the tissue. After obtaining an aliquot for analysis, the homogenates from all the four groups were also subjected to freeze–thaw cycles (once or twice). Similar methods were used for FD-70 with two exceptions: (1) the freeze–thaw group in the presence of Triton was replaced by homogenization after a freeze–thaw cycle with freezing at −20°C in the absence of Triton X-'00 in the buffer and (2) subsequent freeze–thaw of the homogenates was not performed. In each case, both the whole homogenates and the resultant supernatants after centrifugation (15 min in a microcentrifuge) were subjected to the analytical method for the determination of FD-150 or FD-70.

2.3. High-performance size-exclusion chromatography

A sensitive and specific high-performance size-exclusion chromatographic (HPSEC) method [6] was applied to the quantitation of FD-150 or FD − 70 in the liver samples. Briefly, the method involved addition of 20 μl of a 20% (w/v) solution of trichloroacetic acid to 100 μl of the sample (whole homogenate or the supernatant) for protein precipitation. After vortex mixing (5 s) and centrifuging (3 min in a microcentrifuge) of the samples, 80 μl of the supernatant were added to autosampler inserts containing 20 μl of a 0.3 M solution of NaOH. Subsequently, 5 μl of the samples were injected into an analytical size-exclusion column (Hydropore-5-SEC; Rainin, Woburn, MA). The mobile phase was 0.05 M phosphate buffer (pH 7.0) containing 0.01% (w/v) sodium azide, delivered at a flow rate of 0.5 ml min^{-1}. Dextrans were then detected based on their fluorescein label by means of a fluorescence detector set at excitation and emission wavelengths of 495 and 520 nm, respectively.

2.4. Data analysis

The chromatographic peak areas (mV min) of FD-70 and FD-150 were recorded and mean and standard deviation values were calculated for each of the above recovery methods. The recovery values were analyzed using ANOVA with subsequent post-hoc analysis of the means using Scheffe's F test at a significance level of 0.05. Data are reported as means ± SD.

3. Results

The results of the experiments with FD-150, which were performed first, are reported in Table 1 for the freshly homogenized livers. Consistent with our preliminary study, a strikingly low recovery of FD-150 was found in the supernatant of the freshly homogenized livers in the absence of Triton X-100, compared with the other groups reported in Table 1. On the other hand, the recovery in the presence of 1% Triton X-100 in

Table 1
Analytical recovery (chromatographic peak areas, mV min) of FD-150 from fresh (unfrozen) rat livers using different methods ($n = 4$)

Freeze–thaw of homogenate	No Triton		1% Triton	
	Homogenate	Supernatant	Homogenate	Supernatant
None	15.1 ± 1.4^{a}	4.25 ± 0.77^{b}	$19.7 \pm 1.2^{c,d}$	20.5 ± 1.3^{d}
Once	$17.0 \pm 1.4^{e,f}$	12.6 ± 0.9^{g}	$19.1 \pm 1.2^{c,d,h,i}$	$19.6 \pm 1.2^{c,d,i}$
Twice	$17.6 \pm 1.0^{e,f,h}$	15.2 ± 1.1^{a}	$19.1 \pm 1.2^{c,d,h,i}$	$20.0 \pm 1.6^{c,d}$

Mean values with different superscripts are statistically different from each other.

the homogenization buffer was the highest and independent of the sample type (homogenate versus supernatant) or the number of freeze–thaw cycles after homogenization (Table 1). Additionally, the data in Table 1 indicate that in the absence of Triton X-100, consistently higher recoveries are obtained with the whole homogenattes, compared with the supernatants.

The data in Table 1 also contain information on the effect of freeze–thaw of the homogenate on the recovery FD-150. While freeze–thaw cycle did not have any effect on the recovery FD-150 in the presence of Triton X-100, the opposite was observed when the fresh livers were homogenized with the buffer only (Table 1). Comparing the whole homogenate with the supernatant, the freeze–thaw of the homogenate had a more drastic impact on the recovery when the supernatant was used for analysis (Table 1).

The results of similar experiments conducted on the liver samples undergoing a freeze–thaw cycle before homogenization are reported in Table 2. Similar to the data reported in Table 1 for the freshly homogenized livers, the highest recoveries were observed when the freeze–thawed livers were homogenized with the buffer containing Triton X-100 (Table 2). However, in contrast to the data for the freshly homogenized samples (Table 1), the recoveries in the absence of Triton X-100 were not drastically lower than those in the presence of Triton for the freeze–thawed livers (Table 2). Additionally, subsequent freeze–thaw of the homogenates of the freeze–thawed livers did not result in any substantial increase in the recovery of FD-150 (Table 2) as observed for the freshly homogenized livers (Table 1). However, the recovery data in the presence of Triton for both the fresh (Table 1) and freeze–thawed (Table 2) livers were similar. This inidicates that in the presence of 1% Triton X-100 in the homogenization buffer, the recoveries are independent of the handling method.

In addition to FD-150, previous studies [3–5] indicated a high accumulation of FD-70 in the liver. Therefore, to investigate the MW dependence of the hepatic recovery of FDs, additional experiments were conducted for FD-70, the results of which are reported in Table 3. Similar to the data for FD-150, the maximum underestimation of the hepatic concentration of FD-70 was observed when the supernatant of the freshly homogenized liver (without Triton X-100) was subjected to the HPSEC method. These results (Table 3) are both qualitatively and quantitatively similar to those obtained for FD-150 (Tables 1 and 2). Additionally, the data in Table 3 suggest that the freezing temperature (-20 versus -70°C) does not have a significant impact on the recover results.

4. Discussion

Theoretically, a finding of a low recovery of dextrans from the fresh livers, compared with frozen livers, could be attributed to metabolism (depolymerization) of dextrans during the homogenization process and/or a lack of release of dextrans from the subcellular components of the liver cells in the fresh livers. Although dextranases have been reported to be present in the liver of mammals [1,7,8], the hepatic metabolism of dextrans

Table 2
Analytical recovery (chromatographic peak areas, mV min) of FD-150 from the freeze–thawed rat livers using different methods ($n = 4$)

Freeze–thaw of homogenate	No Triton		1% Triton	
	Homogenate	Supernatant	Homogenate	Supernatant
None	$17.8 \pm 1.1^{f,h,i}$	$16.0 \pm 1.1^{a,e}$	$19.5 \pm 1.5^{c,d,i}$	$19.7 \pm 1.5^{c,d}$
Once	$18.5 \pm 0.9^{e,f,h}$	$18.8 \pm 1.3^{e,d,h,i}$	$18.6 \pm 1.2^{e,f,h,i}$	$19.4 \pm 1.4^{e,d,i}$

Mean values with different superscripts are statistically different from each other.

appears to be a very slow process [8]. Consistent with this, addition of a known amount of FD-150 to dextran-free livers and subsequent homogenization of the livers in a preliminary study did not result in any difference between the fresh and frozen livers in their recovery FD-150. Therefore, the differences between the fresh and frozen livers appeared to be due to the effects of freeze–thaw cycle on the disruption of subcellular membranes and release of dextran into the homogenizaation medium. The results of further experiments (Table 1–3) were consistent with this postulate as addition of 1% Triton X-100 resulted in maximal recovery of dextrans from the liver tissue. Triton X-100 is known to be a strong membrane disrupter, as evidenced by its ability to solubilize all membrane constituents of *Micrococcus lysodeikticus* to a maximal degree at a concentration similar to that used in this study [9].

The data presented here are also in agreement with a previous report [10] indicating that ca. 50% of the radiolabeled dextran with an MW of 70 kD present in the liver tissue was associated with intracellular vesicles. Similarly, in the presence of 1% Triton X-100, almost all of the radioactivity was released into the supernatant [10]. Therefore, it is logical to assume that the freezing-induced increase in the recovery of FD-70 and FD-150 from the livers, as observed in these experiments, is due to the effects of the freeze–thaw process on the membrane disruption.

In the absence of Triton X-100 in the buffer, the analytical recoveries of FD-150 and FD-70 were higher when the whole homogenate, as opposed to the supernatant, was used for analysis (Tables 1–3). This is probably due to the fact that the sample preparation procedure for the HPSEC analysis [4,6] included addition of trichloroacetic acid to the samples for protein precipitation. Trichleoroacetic acid is expected to disrupt intracellular membranes and release dextrans from its intracellular accumulation sites. Nevertheless, in the absence of triton X-100 in the buffer, the recoveries from the whole homogenate samples, although higher than their respective supernatants, were not complete (Tables 1–3).

The data in Tables 1–3 indicate that the maximum understimation of the hepatic concentration of FDs (77–79% for FD-150 and 80–81% for FD-70) would occur when the supernatant of the freshly homogenized livers is subjected to the HPSEC assay. Additionally, all the other handling methods not utilizing Triton X-100 would result in some degree of underestimation of the hepatic concentration of FDs. In previous studies on dextrans, the liver samples were stored at −70°C (FD-150 and FD-70) [4] or −20°C (FD-70) [3,5] before the homogenization in a buffer in the absence of Triton X-100. Subsequently, the supernatant of the homogenate was subjected to the HPSEC method. According to the data in Tables 2 and 3, the recoveries for these methods were 14–22% (FD-150) or 12–19% (FD-70) less than the maximal recoveries obtained in the presence of Triton X-100. Therefore, the hepatic amounts or concentrations of FDs reported in previous studies [3–5] should be considered as 14–22% and 12–19% underestimations of the actual values for FD-150 and FD-70, respectively.

In addition to the liver, high concentrations of higher MW FDs are reportedly found in the spleen [4]. Therefore, in these studies with FD-70,

Table 3
Analytical recoveries (chromatographic peak areas, mV min) of FD-70 from fresh (unfrozen) and freeze-thawed rat livers using different methods ($n = 4$)

	Fresh livers		Freeze–thawed livers	
Sample	No Triton	1% Triton	−70°C[c]	−20°C[c]
Homogenate	14.2 ± 0.7^{a}	20.3 ± 1.7^{b}	17.8 ± 1.3^{c}	17.7 ± 1.6^{c}
Supernatant	3.95 ± 0.54^{d}	21.1 ± 1.5^{b}	17.0 ± 1.2^{c}	17.9 ± 1.7^{c}

Mean values with different superscripts are statistically different from each other.
[c] Freezing temperature. Additionally, Triton X-100 was not used for these groups.

spleen samples were also collected. However, because of its small size, the spleen from each rat was divided into only two parts and pooled samples were used in the following experiments. One portion of the samples was subjected to the method used in the previous study (freeze–thaw of the samples, homogenization withouth Triton X-100 and analysis of the supernatant), while the other portion was homogenized in the presence of 1% Triton. Thr recovery of FD-70 in the absence of Triton (2.20 mV min) was 25% less than that obtained in the presence of Triton (2.92 mV min). These data suggest that the underestimation of hepatic recovery of FDs in the absence of Triton X-100 can probably be extrapolated to other tissues.

5. Conclusions

The data presented here indicate that the anallytical recovery of dextran macromolecules from the liver, and possibly other tissues, is significantly affected by the method used for the storage and homogenization of the tissue. Addition of Triton X-100 to the homogenization medium would result in maximal release of dextrans from their intracellular storage sites. Subsequently, the tissue recovery of dextrans becomes independent of the storage method and the type of sample (whole homogenate or the supernatant) used for the analysis. Therefore, in studies of the tissue accumulation of dextrans, addition of Triton X-100 to the homogenization medium is suggested for optimal and consistent recovery of these macromolecules.

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References

[1] C. Larsen, Adv. Drug Deliv. Rev. 3 (1989) 103–154.
[2] H. Sezaki and M. Hashida, Crit. Rev. Ther. Drug Carrier Syst. 1 (1984) 1–38.
[3] R. Mehvar and J.M. Reynolds, J. Pharm. Sci. 83 (1994) 1020–1025.
[4] R. Mehvar, M.A. Robinson and J.M. Reynolds, J. Pharm. Sci. 83 (1994) 1495–1499.
[5] R. Mehvar and J.M. Reynolds, J. Pharmacol. Exp. Ther 264 (1993) 662–669.
[6] R. Mehvar and T.L. Shepard, J. Pharm. Sci. 81 (1992) 908–912.
[7] M.A. Dubick, B.A. Ryan, J.J. Summary and C.E. Wade, Drug Dev. Res. 25 (1992) 29–38.
8] E. Schacht, R. Vercautetren and S. Vanskeenkiste, J. Bioactive Compat. Polym., 3 (1988) 72–80.
[9] M.L.P. Colling and M.R.J. Salton, Biochim. Biophys. Acta, 553 (1979) 40–53.
[10] J.R. Lake, V. Licko, R.W. Van Dyke and B.F. Scharschmidt, J Clin. Invest., 76 (1985) 676–684.