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Quantitative determination of dextran–naproxen ester pro-drugs with varying molecular weights and degrees of substitution in biological media by means of high-performance size exclusion chromatography with fluorescence detection

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Abstract: A high-performance size exclusion chromatographic procedure using a Nucleosil Diol column and fluorescence detection has been developed for the determination of dextran–naproxen ester pro-drugs with varying molecular weights and degrees of substitution in aqueous buffer solutions and biological media in the presence of the parent drug. The effect of several variables on the chromatographic behaviour of the compounds is discussed. Linear standard calibration curves were constructed for all the dextran derivatives incubated in whole blood and urine (human and rabbit), rabbit liver homogenate and human synovial fluid. In whole blood, the detection limit (λ_{ex} = 330 nm, λ_{em} = 360 nm) for a dextran T-70 pro-drug with a degree of substitution (DS) of 10.6 was found to be 2 μg ml^{-1} after applying a 20-μl sample to the column. The assay has been used in stability studies and determination of plasma concentration–time profiles after intravenous administration to rabbits of dextran–naproxen ester pro-drugs.

Keywords: *Dextran naproxen ester pro-drugs; macromolecular pro-drugs; high-performance size exclusion chromatography; detection in biological media.*

Introduction

The potential use of macromolecular pro-drugs as a means of achieving controlled and/or targeted drug delivery has attracted considerable interest in recent years [1–4]. Among the polymeric transport molecules tested, dextrans appear to be one of the most promising carrier candidates due to their excellent physicochemical properties and physiological acceptance [1, 5–8]. Recognizing (i) the multitude of physiological barriers that the carrier has to pass from the site of administration to the receptor [9, 10], and (ii) the task of accomplishing the correct timing of the events leading to optimal drug action, e.g. selective regeneration and suitable maintenance of the active agent at the pharmacological site [3, 11, 12], feasible analytical procedures for both physicochemical characterization and *in vivo* detection of the drug–carrier conjugate are necessary for proper interpretation of biological experiments.

Although molecular weights of dextrans [13–15] and proteins [16–18] have been determined by high-performance size exclusion chromatography [HP(SEC)], using Diol-bonded phase supports, only a few reports have dealt with the application of HP(SEC) procedures to the study of the pharmacokinetics and the stability of macromolecular carrier conjugates [19, 20]. Thus, the present study was undertaken in order to develop methods for the quantitative determination of dextran–naproxen ester pro-drugs in aqueous buffer solutions and in biological media.

Materials and Methods

Materials

The dextran fractions T-10 (M_w 10,300; M_n 4900), T-20 (M_w 20,300; M_n 16,300), T-40 (M_w 41,100; M_n 14,100) and T-70 (M_w 74,300; M_n 36,000) were obtained from Pharmacia (Sweden). M_w and M_n refer to the weight and the number average molecular weights of the dextrans, respectively. Naproxen [(+)-2-(6-methoxy-2-naphthyl) propionic acid] was purchased from Sigma (St. Louis, USA). Naproxen was covalently linked to dextran as previously reported [21], and various characteristics of the synthesized dextran pro-drugs, including drug load, distribution of ligands along the dextran chains and water solubility of the pro-drug derivatives, were determined according to earlier studies [22, 23]. The degree of substitution (DS) of the pro-drugs has been expressed as the percentage of mg naproxen released per mg of the pro-drug. The solvents used in the HPLC procedure were of chromatographic grade. Buffer substances and all other chemicals were of an analytical or reagent grade.

Liquid chromatography

The chromatographic system was composed of a Hitachi Model 655A-11 solvent delivery pump, equipped with a variable wavelength Hitachi F1000 fluorescence detector, a Rheodyne Model 7125 injection valve with a 20-μl loop and a Hitachi Model D2000 chromato-integrator. The column, 250 × 8 mm, was packed with spherically shaped Nucleosil Diol 7-OH particles (7 μm; Macherey-Nagel, Düren, FRG). During chromatography, the column was protected by a small pre-column packed with Nucleosil Diol. In order to avoid dissolution of the Nucleosil packing material, a silica saturation column packed with LiChroprep Si 60, 15–25 μm (Merck, Darmstadt, FRG) was placed between the pump and the injection valve. The flow rate was maintained at 1 ml min^{-1}, and the column eluate was monitored at excitation and emission wavelengths of 330 and 360 nm, respectively.

The chromatographic conditions were optimized by investigating the effects of mobile phase composition on resolution and analysis time. Conjugates with DS below 6 were chromatographed using a mobile phase consisting of 0.05 M phosphoric acid–acetonitrile (9:1, v/v). Higher substituted dextran–naproxen pro-drugs were chromatographed using 0.05 M phosphoric acid–acetonitrile (7:3, v/v).

Sample preparation

Blood samples were centrifuged for 2 min at 10,000 *g*. Plasma (100–500 μl) was withdrawn and deproteinized by the addition of twice the volume of 20% (w/v) trichloroacetic acid. The mixture was vortexed and centrifuged for 2 min at 10,000 *g*. Aliquots of the supernatant solution were immediately injected onto the column. Rabbit liver homogenate and human synovial fluid samples were treated in a similar manner. In

order to quantitate the dextran conjugates in human and rabbit urine, an alternative procedure was developed. Dowex 50 W-X8 (400 mg) 100/200 mesh (Fluka AG, Switzerland) was added to 1000 μl of urine. After vortexing, the mixture was centrifuged, and aliquots of the clear supernatant solution were injected directly into the chromatograph.

Calibration curves

For each pro-drug studied, calibration curves for the different biological media were constructed spanning a concentration range of the individual compound of 2–120 μg ml^{-1}. Each spiked sample was treated in an identical manner to that described for the samples. In all cases, the relationships between peak area and the concentration of the drug were linear ($r > 0.997$) with zero intercepts.

Results and Discussion

Assay development

Naproxen is slowly released from the dextran pro-drugs at physiological pH ($t_{½}$ = 183 h, to be published elsewhere). Thus the assay was developed with the express purpose of enabling simultaneous determination of the high molecular weight pro-drug and the free drug. As seen from Table 1, the retention time of naproxen decreased with the increasing lipophilic character of the mobile phase. This behaviour is in accordance with the fact that the Nucleosil Diol support exhibits reversed-phase properties [19, 24]. The retention times of the pro-drugs with a DS of 5.6 and 0.23 (derived from the same dextran T-70 sample) were largely unaffected by the alteration of the mobile phase

Table 1
Effect of the composition of the mobile phase on the retention times (min) of naproxen and dextran–naproxen ester pro-drugs with varying average molecular weights and DS

	Mobile phase						
Compound	A	A–B (95:5, v/v)	A–B (90:10, v/v)	A–C (95:5, v/v)	A–C (90:10, v/v)	A–C (80:20, v/v)	A–C (70:30, v/v)
Conjugate T-70* (DS 5.6)	5.1	5.2	5.1	5.1	5.1	5.1	5.4
Conjugate T-70 (DS 0.23)	5.1	5.2	5.1	5.1	5.1	5.1	5.3
Conjugate T-70 (DS 10.6)	7.4	7.2	6.6	6.4	5.9	5.3	5.3
Conjugate T-40 (DS 7.6)	6.2	6.1	6.0	5.9	5.8	5.8	5.9
Conjugate T-20 (DS 8.3)	7.1	6.9	6.9	6.8	6.6	6.6	6.7
Conjugate T-10 (DS 8.9)	8.3	8.2	8.0	7.9	7.8	7.8	7.9
Naproxen	56.0	35.0	28.0	26.0	19.0	13.3	10.7

A, 0.05 M phosphoric acid; B, methanol; C, acetonitrile.
*Naproxen ester conjugates synthesized from the Pharmacia dextran fraction series.

composition. In contrast, the corresponding derivative with a DS of 10.6 eluted much later than expected when a 0.05 M aqueous phosphoric acid mobile phase was used. By increasing the proportion of organic modifier in the eluent, the retention time decreased but remained constant at concentrations of acetonitrile above 20% (v/v). Similar elution patterns were observed for lower molecular weight pro-drugs possessing a relatively high degree of substitution. In the complexation of lipophilic agents to dextrans, it has been shown that the hydrodynamic volume of the derivatives, as expressed by the limiting viscosity number, decreases with the increasing degree of substitution [21, 23]. In size exclusion chromatography this should lead to a progressive retardation of the elution of these conjugates as a function of the DS. Since this is not the case, the higher substituted conjugates were retained longer on the column probably due to hydrophobic interaction between the complexed drug and the column packing material. Similarly, the incorporation of ethylene glycol in an aqueous mobile phase has been reported to suppress the adsorption of lysozyme and chymotrypsinogen onto LiChrosorb Diol, resulting in a marked reduction of the retention volumes of the proteins [25]. Data, in support of the participation of adsorption phenomena in the elution of dextran–naproxen ester derivatives with DS above 6, are presented in Fig. 1.

Equal amounts of pro-drugs (135 μg ml^{-1}) with varying degrees of substitution, but derived from the same dextran T-70 fraction, have been chromatographed under identical conditions, employing mobile phases containing from 10 to 30% (v/v) acetonitrile. In the plots of peak area versus DS of the compounds, deviation from linearity was observed with the 10 and 20% (v/v) acetonitrile eluents, which indicates that pro-drugs with relatively high DS were retained on the column in varying degrees. Thus, proper quantitation of highly substituted dextran derivatives necessitated the use of a mobile phase containing 30% (v/v) acetonitrile.

The effective removal of compounds from the biological media, which could contaminate the analytical columns or invalidate the assay of the content of dextran

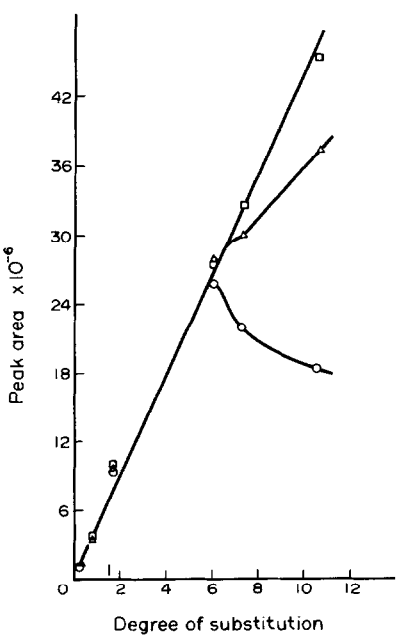

Figure 1
Peak areas obtained for dextran T-70–naproxen ester conjugates with varying degrees of substitution. Equal amounts of the derivatives (135 μg ml^{-1}) were eluted with mobile phases consisting of 0.05 M phosphoric acid–acetonitrile: ○, 9:1 (v/v); △, 8:2 (v/v); □, 7:3 (v/v). λ_{ex} = 330 nm and λ_{em} = 360 nm.

Table 2
Proportions of volumes (plasma to deproteinizing agent) tested for precipitation efficacy

Deproteinizing agent	Range of volume precipitant added per volume of plasma
Trichloroacetic acid (10%, w/v)	0.2–2.0
Trichloroacetic acid (20%, w/v)	0.2–2.0
Perchloric acid (6%, w/v)	1.0–3.0
Methanol	0.5–3.0
Ethanol	0.5–2.0
Acetonitrile	0.5–2.0
Sulphosalicylic acid (solid)	30 mg–200 mg

conjugate in the samples, posed considerable difficulties. Several precipitation systems are listed in Table 2.

Other metal salt solutions have been tested for precipitation efficacy [26]. Most of the agents failed to remove interfering components, as made evident by the uninterpretable chromatograms. Others (methanol, ethanol and acetonitrile) caused partial precipitation of the dextran conjugates. Although 6–14% (w/v) trichloroacetic acid solutions have been used successfully to prepare blood samples for analysis of other drugs [27–29], the samples in the present study required a 20% (w/v) solution of the acid (two volumes per volume of the individual medium). Furthermore, a change of the analytical wavelengths to λ_{ex} = 330 nm and λ_{em} = 360 nm was necessary in order to afford sufficient specificity of the assay. In comparison with the wavelengths for the optimal detector response for simple aqueous samples (λ_{ex} = 270 nm and λ_{em} = 380 nm), this alteration of the wavelengths resulted in an almost 5-fold reduction of the sensitivity of the assay.

The stability of the dextran–naproxen ester derivatives was assessed by the incubation of a T-40 conjugate (DS = 6.4) in the precipitant solution at ambient temperature. At appropriate intervals, samples were withdrawn and analysed for intact dextran conjugate by the HP(SEC) procedure, using the mobile phase consisting of 30% acetonitrile. First-order hydrolysis kinetics of the pro-drug was established as evidenced from the linear plot of log residual intact conjugate against time (Fig. 2). From the slope of the plot, the degradation rate constant was calculated to be 0.12 h^{-1}. By using this value, the time for 2% degradation of the conjugate was determined to be 10 min. Thus, chromatography has to take place within 5 min after deproteinization of the samples in order to obtain reproducible quantitative data. In contrast to the instability in the precipitant solution, the conjugate was relatively stable in biological media, with hydrolysis half-lives of approximately 180 h.

None of the precipitation systems tested was able to remove the urinary components which interfered with the detection of the dextran derivatives. Urine samples have been satisfactorily prepared by passing the solution through a combination of cation and anion ion-exchange columns prior to the interference refractometric detection of dextran [27]. Consequently, the feasibility of employing ion-exchange materials was evaluated by the HP(SEC) procedure with fluorescence detection (330 and 360 nm) for urine samples treated separately with a strong anion resin (Dowex 1-X8), a strong cation exchanger (Dowex 50W-X8) and a mixed bed type (Amberlite MB-3). An addition of 400 mg Dowes 50W-X8 per ml of urine, almost completely removed the interfering peaks (Fig. 3). No further improvement was accomplished by treating the urine with combinations of the three ion-exchange types.

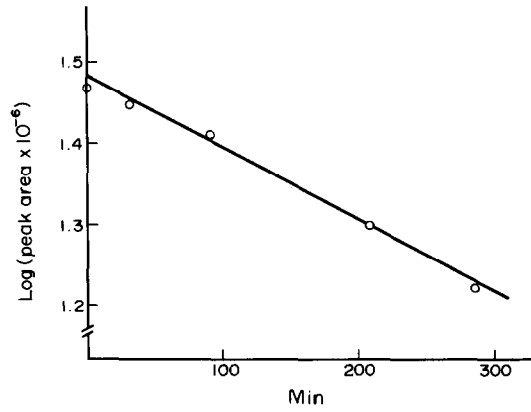

Figure 2
First-order plot for degradation of a dextran T-40–naproxen ester conjugate (DS 6.4) in 20% (w/v) trichloroacetic acid at ambient temperature.

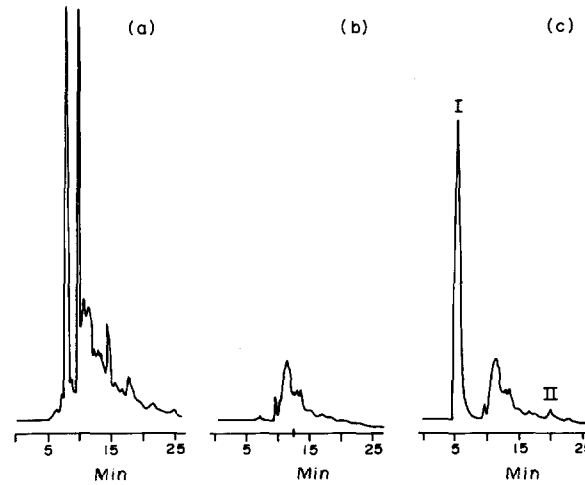

Figure 3
Chromatograms of human blank urine samples, treated with 20% (w/v) trichloroacetic acid (1:2, v/v) (a); after addition of 400 mg Dowex 50W-X8 per ml urine (b); after addition of a dextran T-70 conjugate (DS 5.6; 110 $\mu g\ ml^{-1}$) and 400 mg of the anion-exchanger (c). Mobile phase: 0.05 M phosphoric acid–acetonitrile (9:1, v/v). λ_{ex} = 330 nm and λ_{em} = 360 nm. I, Dextran conjugate; II, parent naproxen.

Assay validation

Linear standard calibration curves, based on peak area measurements, were obtained for all the dextran derivatives incubated in the different media. Although the dextran T-70 pro-drug (DS 5.6) was eluted close to the exclusion volume, identical linear plots were found after incubation of the compound in water and 20% (w/v) trichloroacetic acid with the slope (α) calculated to be 0.175 (peak area $\times$ 10^{-6} μg^{-1} ml). A corresponding α-value of 0.0430 was found in human plasma. Taking into account the dilution of the samples, due to the addition of the deproteinizing agent, a corrected value of the slope of 0.133 was derived. By a comparison of this value with that of the slope obtained for solutions treated with 20% (w/v) trichloroacetic acid, an absolute recovery 76% for the dextran T-70 derivative from plasma was calculated. Similarly, the recovery for the other conjugates ranged between 72 and 83%. Calibration curves have been constructed for

conjugates with varying molecular weight and DS in human plasma. Linear correlation between the slopes of the lines and the DS of the pro-drugs was observed, indicating that conjugated naproxen was responsible exclusively for the fluorescence response. The plots of peak area versus concentration obtained by spiking rabbit plasma, rabbit liver homogenate and human synovial fluid with the individual compounds, were in close agreement with that observed after incubation of the derivatives in plasma. In both rabbit and human whole blood, however, the α-value of the calibration curves was 0.0778, indicating that the pro-drugs were concentrated in the supernatant solution after centrifugation of the blood samples.

Ten 500-μl aliquots of 20% (w/v) trichloroacetic acid or blank plasma were spiked with 80 μg of a dextran T-40 pro-drug (DS 5.1) and analysed by the described procedure. The relative standard deviations, used as a measure of precision, were 2.8 and 2.0%, respectively. The corresponding values derived from peak height measurements were 3.3 and 3.9%.

Since the detector response was proportional to the amount of naproxen linked to the polymer backbone, the detection limit varied with the degree of substitution of the dextran derivatives. For the dextran T-70 pro-drug (DS 10.6) 2 μg per ml of whole blood could be quantified reproducibly. The limit of detection can probably be reduced if a greater volume of analyte solution is applied to the column.

Applications

The applicability of the assay procedures was demonstrated by pharmacokinetic and stability studies of dextran–naproxen ester pro-drugs. T-40 (DS 4.6) and T-70 (DS 6.9) derivatives were administered parenterally to male rabbits (approximately 3.5 kg). The administration of the compounds (1 ml of a 10% (w/v) aqueous solution of the individual pro-drug) was made intravenously into an auricular vein, and samples were taken by vein puncture of the other ear. After the administration of the pro-drug, blood samples were taken at appropriate intervals, and the plasma fractions were assayed for intact dextran pro-drug by the fluorescence HP(SEC) method. From the plasma concentration–time profiles (Fig. 4), plasma half-lives for the T-40 and the T-70 dextran derivatives of 58 and 90 min, respectively, were calculated. Further pharmacokinetic studies will be presented in detail elsewhere.

First-order rate constants for alkaline hydrolysis of dextran–naproxen ester pro-drugs were determined after incubation of a T-70 derivative (DS 5.6) in 0.05 M buffer solutions

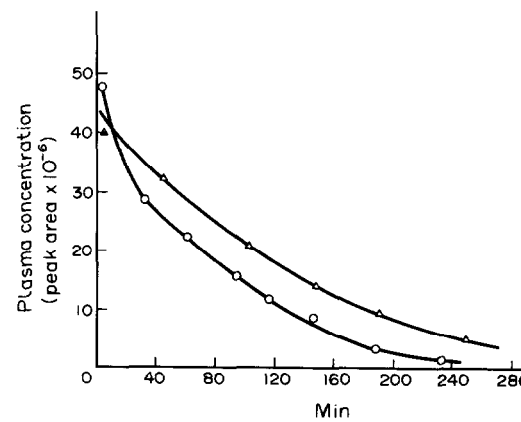

Figure 4
Plasma concentration–time profiles for a dextran T-40 derivative (DS 4.6; ○) and a dextran T-70 derivative (DS 6.9; △) after intravenous administration of 1 ml of a 10% (w/v) aqueous solution to male rabbits (~3.5 kg).

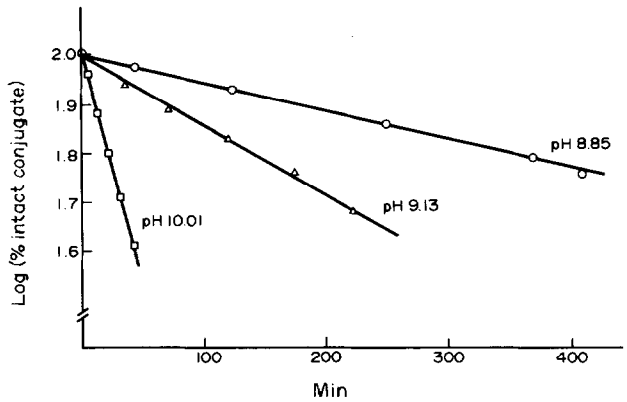

Figure 5
First-order plots for regeneration of naproxen from a T-70 conjugate (DS 5.6) in 0.05 M borate buffers and a 0.05 M carbonate buffer (37°C and an ionic strength of 0.5).

at 37°C. The rate constants were calculated from the slopes of a semilogarithmic plot of the remaining intact conjugate against time (Fig. 5). The following values were obtained: pH 8.83 ($k = 8.04 \times 10^{-2}$ h^{-1}); pH 9.13 ($k = 1.97 \times 10^{-1}$ h^{-1}); and pH 10.01 ($k = 1.28$ h^{-1}).

With macromolecular pro-drugs it is of importance to determine the pharmacokinetic fate of the carrier molecule, *per se*, administered by various routes in realistic doses. In this connection, dextran–naproxen ester derivatives cannot function optimally as a tracer molecule, due to the lack of sensitivity of the assay and the relative instability of the conjugates under physiological conditions. To this end we are presently investigating the possibility of using FITC (fluoresceinyl) dextrans as tracer molecules.

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