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Dextran-estrone conjugate: synthesis and in vitro release study

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Abstract

A novel hormone conjugate has been prepared by a coupling reaction between modified estrone and dextran. In order to provide a suitable reactive estrone derivative for coupling with dextran and a spacer between the drug carrier and the hormone, the steroidal sex hormone was succinylated by reaction with succinic anhydride. Subsequently, the carboxylic acid terminal of succinylated estrone was further reacted with thionyl chloride to replace the hydroxy group with chlorine to make a better leaving group. The ester bond was employed as labile linkage between the hormone and the biopolymer carrier backbone so that the coupled estrogen could be released from the conjugate via ester hydrolysis. Structures of the modified estrone and dextran-estrone conjugate were determined by elemental analysis and by FTIR, ^{1}H and ^{13}C NMR spectroscopies. The degree of substitution (D.S.) per anhydroglucose (AHG) unit was 0.33 (11.0 mol% of estrone moieties), as calculated from the ^{1}H NMR spectrum. In vitro hydrolysis of the conjugate in aqueous phosphate buffer/ethanol solutions at pH 8.0 and 7.4 and 37°C released estrone was completed within a few days and followed zero order kinetics. © 1998 Elsevier Science Ltd. All rights reserved.

Keywords: Dextran; Estrogen; Hormone; Drug carrier; Estrone; Hydrolysis

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1. Introduction

The use of natural biopolymers in drug delivery systems has been recognized as an effective means to maintain drug concentrations within therapeutic ranges and to avoid undesirable side effects. Several controlled systems have been developed for prolonging the release of biologically active compounds or drugs from polymeric matrices. These include mechanical and osmotic pumps and diffusion-, swelling-, magnetically- and chemically-controlled systems (Langer, 1990). In the last approach, the drug is covalently coupled to a polymer and released by hydrolytic or enzymatic cleavage of the linking bond. The rate of drug release, therefore, can be controlled by changing the nature of the linking bond, the length of the spacer group, the amount of drug loading, the chemical structure, hydrophilicity/hydrophobicity, level of crystallinity, and the molecular weight of polymer carrier (Harris et al., 1976; Langer & Peppas, 1983).

Estrone (3-hydroxy-1,3,5 (10)-estratrien-17-one) hormones are one of the naturally occurring steroid sex hormones (endogenic biologically active compounds) that promote the growth of the reproductive organs in female. They promote growth and cornification of the vaginal epithelium and stimulate cervical mucous secretion. In estrone steroid therapy, they have been used to treat prostatic cancer and menstrual cycle irregularities (Goth, 1972). Recently, several researchers have suggested therapeutic efficacy of estrogen against Alzheimer's disease. These studies suggested that estrogen helps maintain healthy brain cells and reduces risk of the disease (Sherwin, 1995). However, due to the inherent nature of the minute amounts of hormone released inside human body upon activation, a more precise mode of hormone release via drug carriers is essential. Only a few examples are available where steroids are covalently linked to a polymeric system (Batz et al., 1974; Agarwal & Dhar, 1965). We have previously reported the covalent attachment of estrone onto soluble starch (Won et al., 1997). The starch was modified with bromoacetyl bromide in order to provide the required reactive sites for coupling of estrone. In the present paper, we have extended concept of a polysaccharide-based drug carrier to a dextran-estrone conjugate through the chemical modification of estrone. Dextran has been used as a plasma expander and has been frequently chosen as drug carrier due to its biocompatibility, biodegradability, hydrophilicity and water solubility (Sacco et al., 1989; Ohya et al., 1996; Aranaz et al., 1992).

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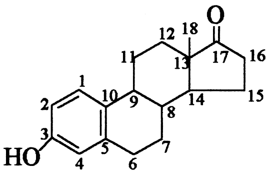
The purpose of this paper is to report the synthesis and characterization of a dextran-estrone conjugate and the release pattern of the estrone from its in vitro degradation.

2. Materials and methods

2.1. Materials

A commercial dextran (produced by *Leuconostoc mesenteroides* B-512) having an average molecular weight of 70,000 was obtained from Sigma Chemical (St. Louis, MO USA). Estrone (from Sigma) was used without further purification. Succinic anhydride, 4-(dimethylamino)pyridine (DMAP), thionyl chloride ($SOCl_2$) and lithium chloride (LiCl) were purchased from Aldrich Chemical (Milwaukee, WI USA). Tetrahydrofuran (THF) was distilled from a purple sodium/benzophenone under nitrogen. Triethylamine (TEA), N-methyl-2-pyrrolidone (NMP) and N,N′-dimethylformamide (DMF) were purified by distillation under reduced pressure over calcium hydride and stored over 4 Å molecular sieves until use. Chloroform and methylene chloride were purified by a conventional method (Riddick et al., 1986). All other solvents and chemicals were obtained from commercial sources and were used without further purification.

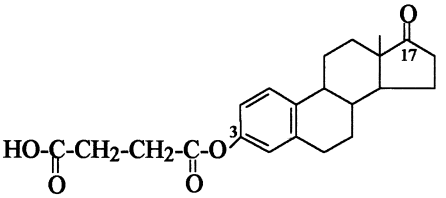


Estrone (1)

2.2. Chemical modification of estrone

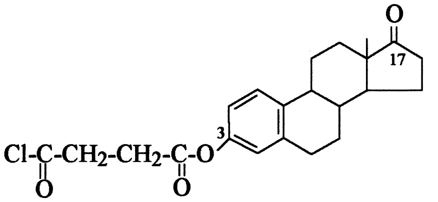
In order to provide a suitable reactive derivative of estrone for its subsequent coupling with dextran, estrone was succinylated by reaction with succinic anhydride. The carboxylic acid of succinoylated estrone then reacted with thionyl chloride to replace the hydroxy group of the carboxylic acid with chlorine to make a better leaving group. Since estrone receptor affinity is related with the 17-keto region, this modification of the 3-hydroxyl should not affect biological activity. Also, the estrone may be expected to be biologically active, even in the dextran conjugate form, because the functionally active center is intact.

2.2.1. Estrone-3-hemisuccinate (2).
Estrone (1.00 g, 3.7 mmol) was dissolved in 25 mL of THF, then succinic anhydride (0.74 g, 7.4 mmol), TEA (1.02 mL, 7.4 mmol) and DMAP (0.45 g, 3.7 mmol) were added and the reaction mixture was stirred forr 24 h at 60°C. The solvent was removed under reduced pressure and the resulting residue was dissolved in a minimum volume of methanol/methylene chloride (1 : 9; v/v). The crude product was then purified by flash chromatography (Still et al., 1978) using 10% methanol/methylene chloride as eluent. Appropriate fractions were collected, concentrated, and dried in vacuo. Yield was 0.87 g (63.5%). It was recrystallized from chloroform.



Estrone-3-hemisuccinate (2)

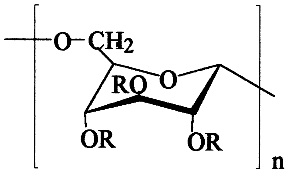
2.2.2. Estrone-3-hemisuccinic acid chloride (3).
Thionyl chloride (0.79 mL, 10.8 mmol) was added dropwise to a solution of estrone-3-hemisuccinate (**2**) (0.2 g, 0.54 mmol) dissolved in 20 mL of dry chloroform ($CHCl_3$), and the resulting mixture was refluxed for 2 h. The solution was cooled and the solvent and excess thionyl chloride were completely removed at room temperature in vacuo followed by repeated evaporation with benzene. The resulting acid chloride dissolved in dry chloroform was filtered under nitrogen and used without further purification. Yield was 0.16 g (76.2%).



Estrone-3-hemisuccinic acid chloride (3)

2.3. Dextran-estrone hormone conjugate (4)

Dextran (0.162 g, 3 mmol OH) in LiCl/DMF solvent system (9 wt%, 10 mL) was placed into a three-necked flask equipped with a nitrogen inlet and outlet, heating mantle, thermometer and magnetic stirrer. The mixture was heated to 80° until the dextran was completely dissolved. The solution was then cooled to room temperature. To initiate the coupling reaction, (**3**) (0.4 g, 1 mmol) and TEA (0.32 ml, 2.3 mmol) were added to the solution. After stirring at 40°C for 6 h, the reaction mixture was poured into an excess of cold isopropanol to precipitate the product. The resulting dextran-estrone conjugate (**4**) was purified by reprecipitation, using DMF as solvent and cold isopropanol as precipitant, and then dried in vacuo at 30°C for 48 h in the presence of phosphorus pentoxide.



Dextran-Estrone Conjugate (4)

$R = H \text{ or } (-\underset{\substack{\| \\ O}}{C}CH_2CH_2\underset{\substack{\| \\ O}}{C}O-)\text{Estrone}$

where ( ) is a spacer

2.4. Characterization

The resulting dextran-estrone conjugate was characterized by FTIR, NMR and elemental analysis. The FTIR spectrum of the dextran-estrone conjugate was recorded on a Mattson Galaxy Series 5000 spectrophotometer with a resolution of 2 cm^{-1} by use of a dispersion in Nujol cast between two KBr plates. All other samples were cast on KBr discs from chloroform solution. Elemental analyses (EA) were carried out on a Carlo Erba 1106 EA instrument. ^1H NMR spectra were obtained from a Varian Unity 500 spectrometer operating at 400 MHz. ^{13}C NMR spectra were obtained from a 100 MHz Varian VXR 400 s spectrometer. All of the chemical shifts are reported in parts per million (ppm) with tetramethylsilane as an internal standard for both ^1H and ^{13}C NMR spectra. The sample tube size was 5 mm with a sample concentration of 10 mg/mL in DMSO-d_6. A Cary 5 spectrophotometere with 1 cm quartz cuvettes was used to obtain UV spectra at a scanning rate of 60 nm/min.

2.5. In vitro release studies

Disks (diameter 12 mm) of the dextran-estrone conjugates were prepared by slow evaporation of DMF solutions of the conjugates into films and compressing a known amount of these films (100 mg) at high pressure in a laboratory press. Four dextran-estrone disks were then placed in stoppered test tubes and 10 mL of aqueous phosphate buffer and ethanol mixture (pH 7.4 or 8.0) was added to each test tube. The incorporation of ethanol into phosphate buffer was to improve the solubility of estrone. The tubes were then placed in an incubator at 37°C. Aliquots (1 mL) were withdrawn from the incubated test tubes at regular intervals up to 40 h. The removed aliquots were replaced by fresh solution in each tube to maintain a constant volume in each tube. The amount of estrone released via the heterogeneous hydrolysis of the conjugate was determined by UV spectroscopy. The concentration of estrone released at different hydrolysis time was estimated from the calibration curves by measuring absorbances at 256 nm ($\varepsilon_{256} = 2860.6\ L{\cdot}mol^{-1}{\cdot}cm^{-1}$).

3. Results and discussion

3.1. Chemical modification of estrone hormone

There are two main purposes for the chemical modification of estrone. One is to increase its reactivity for the subsequent coupling reaction with a polymeric carrier, the other is to introduce spacers that would facilitate enzyme access for the hydrolysis of linking bonds. For the preparation of the dextran-estrone conjugate, the estrone was first converted to a suitable reactive derivative. Since estrone contains only one reactive functional group (3-position hydroxyl), it provides an ideal reactive site for chemical modification of estrone without any side reactions.

The reaction of estrone with succinic anhydride was used for the introduction of a carboxylic acid functional group. Generally, succinylation of compounds containing hydroxyl groups requires drastic conditions, such as high reaction temperatures and long reaction times. Pyridine has commonly been used as acylation catalyst. However, Zalipsky et al. (1983) found that the reaction can be carried out at room temperature and for a shorter reaction time by using 4-dimethylamino pyridine (DMAP) as a catalyst. The succinylated estrone was isolated by flash chromatography in order to remove all unreacted succinic anhydride.

The structure of the resulting succinylated estrone (**2**) was confirmed by data from FTIR and ^{13}C NMR spectroscopies. These results are summarized in Table 1. The FTIR spectrum (Fig. 1) of succinoylated estrone exhibits very broad O–H stretching of carboxylic acid in the region of 3300–2500 cm^{-1}. The characteristic ester carbonyl stretching of

Table 1
Chemical characterizations of modified estrone

Techniques	Estrone-3-hemisuccinate (**2**)	Estrone-3-hemisuccinic acid chloride (**3**)
	$C_{22}H_{26}O_5$	$C_{22}H_{25}O_4Cl$
Elemental Analysis (EA)	Calc. C 71.33% H 7.07%	Calc. C67.95% H 6.48% Cl 9.12%
	Found C 71.19% H 6.59%	Found C 67.82% H 6.35% Cl 9.09%
FTIR (cm^{-1})	3300–2500 (OH carboxylic acid)	1792 (C=O acid chloride)
	1732 (C=O ester)	1758 (C=O ester)
		1737 (C=O 17-keto)
^{13}C NMR	219.73 (C=O 17-keto)	219.60 (C=O 17-keto)
(DMSO-d_6)	173.34 (C=O ester)	202.47 (C=O acid chloride)
	171.23 (C=O carboxylic acid)	171.23 (C=O ester)

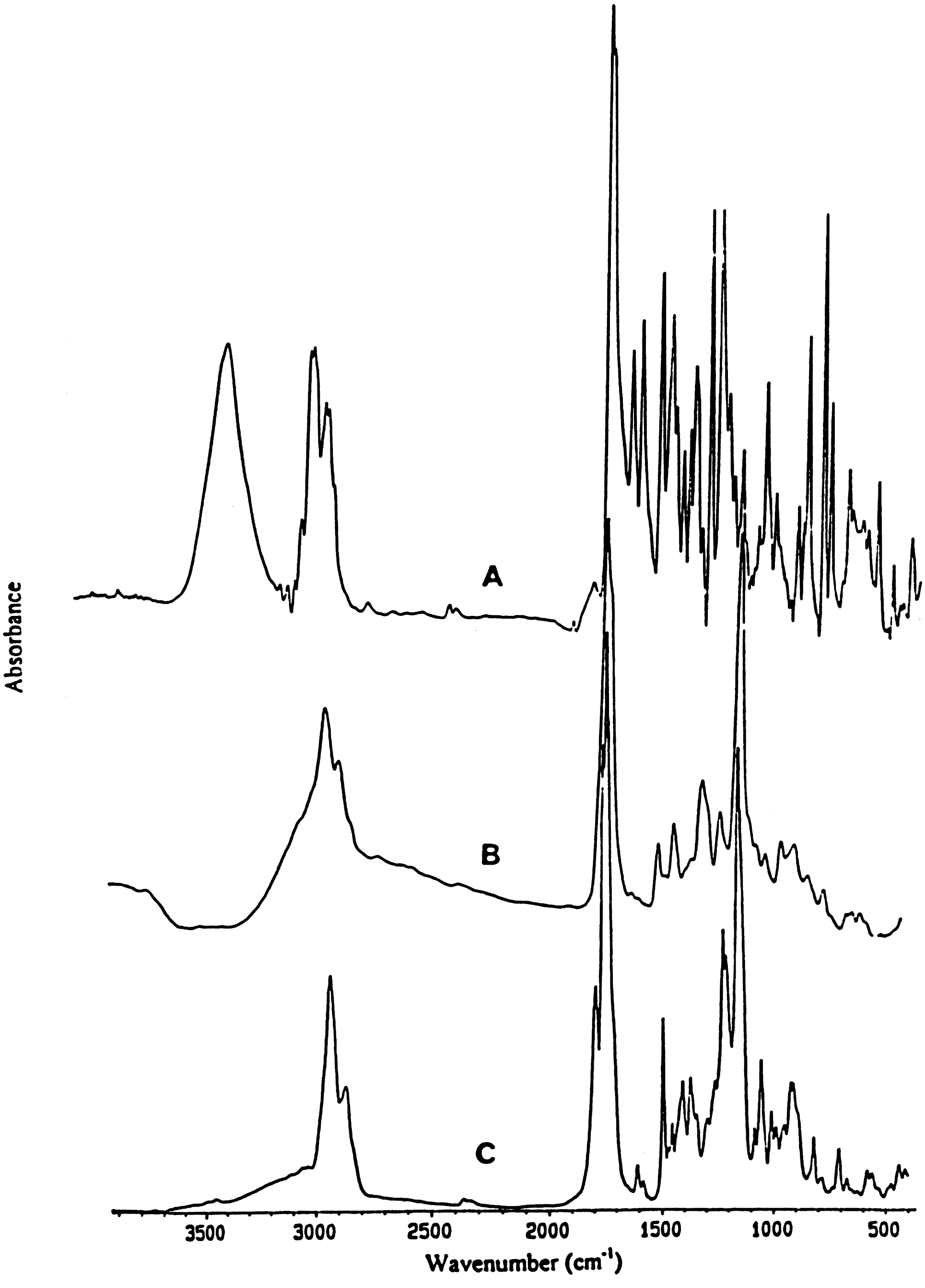


Fig. 1. FTIR spectra of estrone and modified estrone. (A) estrone (**1**); (B) estrone-3-hemisuccinate (**2**); (C) estrone-3-hemisuccinic acid chloride (**3**).

the succinylated estrone absorbs at 1732 cm^{-1}. The C=O absorptions of carboxylic acid and 17-keto of estrone appear as a broad band due to their overlapping with the 3-position ester group. The ^{13}C NMR spectrum shows the characteristic chemical shifts at $\delta = 219.73$ corresponding to the carbonyl carbon atom of the 17-keto group. The peaks $\delta = 171.23$ and 173.34 can be assigned to ester and carboxylic acid carbonyl carbon atoms, respectively.

The carboxylic acid terminal group of estrone-3-hemisuccinate (**2**) was converted to the acid chloride prior to the coupling reaction with dextran in order to increase its reactivity toward dextran. The preparation of acid chlorides from carboxylic acids with excess $SOCl_2$ is a very simple and convenient procedure. Since both byproducts, HCl and SO_2, are gaseous, they were readily removed and any excess thionyl chloride was distilled off. The chlorination reaction is confirmed by the appearance of an acid chloride carbonyl absorbance (1792 cm^{-1}); i.r. peaks (Fig. 1) at 1758 and 1737 cm^{-1} are attributed to the carbonyl bands of the ester and 17-keto, respectively. The ^{13}C NMR spectrum exhibited peaks at $\delta = 219.60$ (17-ketone carbon), 202.47 (carbonyl

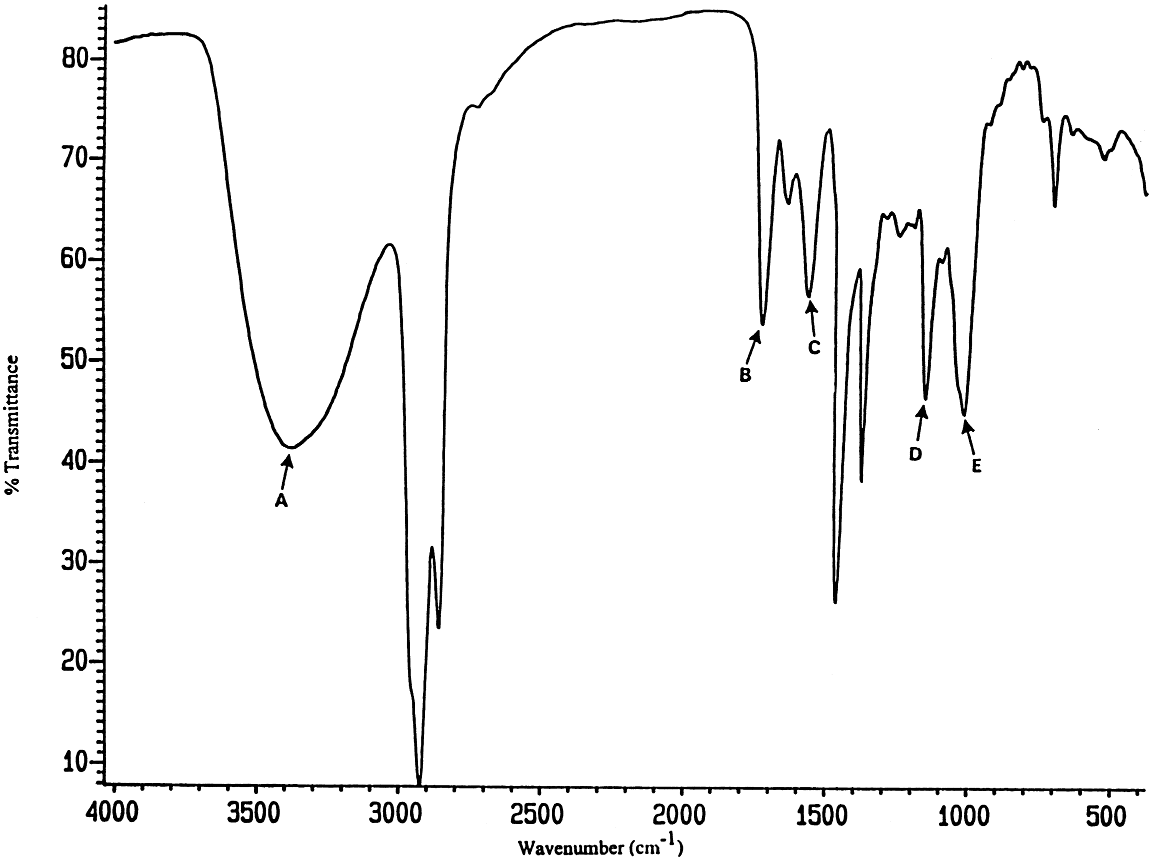


Fig. 2. FTIR spectrum of dextran-estrone conjugate (Nujol). (A) unreacted OH stretch; (B) combined ester and keto C=O stretch; (C) aromatic ring C=C stretch; (D) asymmetric C–O–C stretch; (E) symmetric C–O–C stretch.

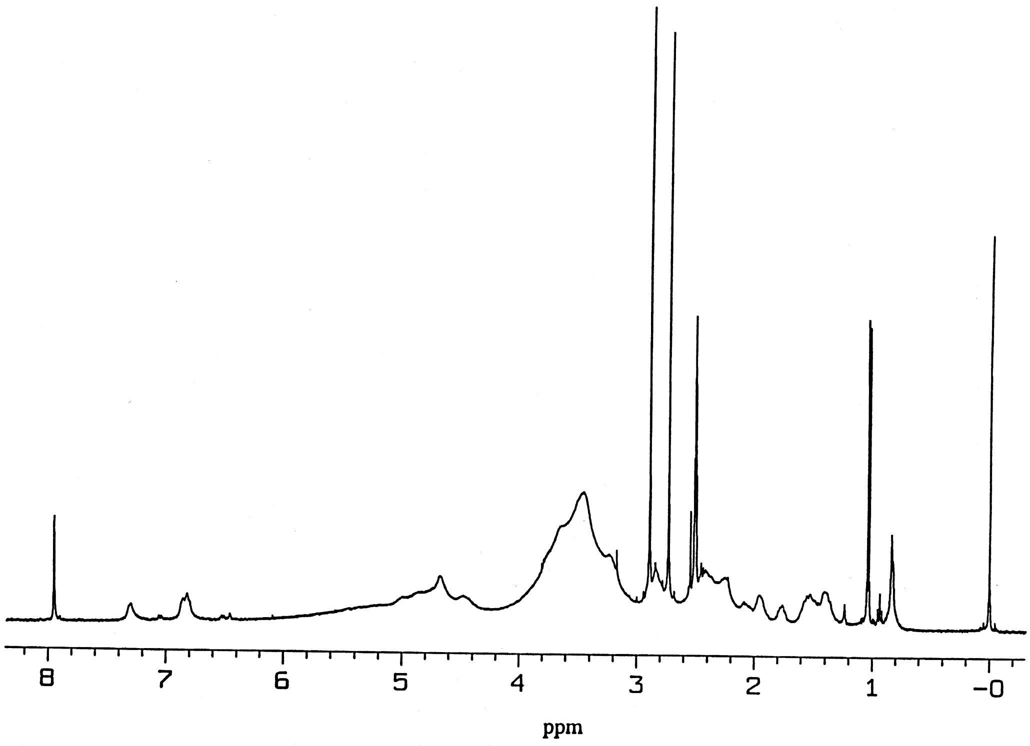


Fig. 3. ^{1}H NMR (400 MHz) spectrum of dextran-estrone conjugate (D.S. = 0.33) in DMSO-d_6.

carbon of acid chloride) and 171.23 (carbonyl carbon of ester group).

3.2. Dextran-estrone conjugate

The dextran-estrone conjugate (**4**) was prepared by reacting the modified estrone (**3**) with dextran. In general, the reactions between acid chlorides and polymers containing hydroxyl groups in the presence of pyridine as a hydrogen chloride acceptor and catalyst are efficient methods for the esterification of polymers (Arranz et al., 1980). Thus, the succinyl chloride modification is a valid approach for the coupling of estrone to the biopolymer. The type of linkage between the drug and the polymeric carrier plays an important role in the rate of drug release. The relative rates of hydrolysis of various labile bonds at pH 7.4 is carbonates (–OCOO–) > esters (–COO–) > urethanes (–NHCOO–) > amide (–CONH–). Carbonates (Khur & Galin, 1985) and amides (Negishi et al., 1987) have been used for linking drugs to polymers. In this study, an ester linkage was designed to couple estrone to dextran.

The FTIR spectrum of the dextran-estrone conjugate (**4**), taken in a nujol mull, is shown in Fig. 2. The band at 1736 cm^{-1} corresponds to the combined 2 esters in the spacer and one ketone from the estrone. It also shows new absorption at 1573 cm^{-1} associated with the C=C stretching band of aromatic ring system in estrone. The unreacted residual OH groups of dextran show their stretching vibration at around 3392 cm^{-1}.

The ^{1}H NMR spectrum of the same conjugate (Fig. 3) shows a band at $\delta = 1.04$, which corresponds to the 18-methyl protons in the estrone steroid system, and complex multiplets in the region $\delta = 1.2$ to 2.9 due to the protons of aliphatic cyclic rings of estrone hormone. The peaks at $\delta = 6.82$, 7.30 and 7.95 can be assigned to aromatic protons. There are also broad bands between $\delta = 3.00$ and 5.52 which are due to protons linked to the dextran backbone carbon atoms.

The ^{13}C NMR spectrum, Fig. 4, shows the characteristic chemical shifts at $\delta = 162.34$ corresponding to the carbonyl carbon atom of ester groups. The signals between $\delta = 62.04$ and 98.28 are due to sugar carbon atoms. A peak at $\delta = 220$ can be assigned to keto carbonyl, and the carbons (methyl, methylene and methine) of the estrone residue exhibit bands from $\delta = 13.52$ to 49.61.

The dextran molecule contains three primary hydroxyl groups per anhydroglucose (AHG) residue in the polymer chain. Each of these can react with the acid chloride of estrone-3-hemisuccinate (**3**) in the same manner; however, their relative reactivities should vary considerably. Arranz et al. (1987) reported that the relative reactivity of hydroxyl groups of dextran followed the order: $C_2 > C_4 > C_3$ based

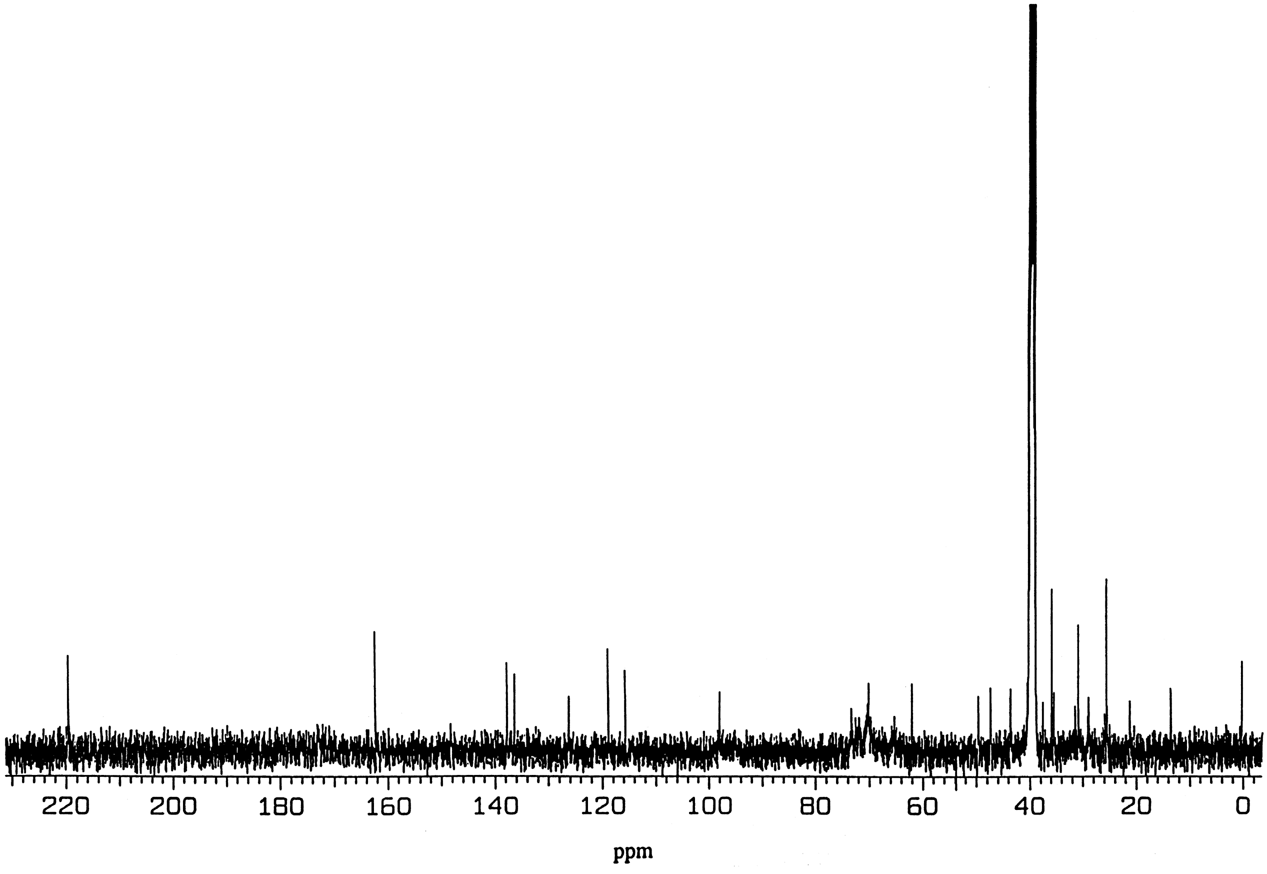


Fig. 4. ^{13}C NMR (100 MHz) spectrum of dextran-estrone conjugate (D.S. = 0.33) in DMSO-d_6.

Table 2
Solubilities of modified estrones, dextran-estrone conjugate and unmodified dextran

	Samples[a]			
Solvents[b]	EHS (**2**)	EHCl (**3**)	DEC (**4**)	Unmodified dextran
DMF	++	++	+	+[c]
NMP	++	++	±	–
DMSO	++	++	++	+[d]
DMAc	++	++	±	–
$CHCl_3$	±	++	–	–
THF	–	–	–	–
ether	–	–	–	–

Solubility: ++, soluble at room temperature; +, soluble in hot solvent; ±, partially soluble; –, insoluble

[a] EHF, estrone-3-hemisuccinate; EHCl, estrone-3-hemisuccinic acid chloride; DEC, dextran-estrone conjugate

[b] DMF, N,N-dimethylformamide; NMP, N-methyl-pyrrolidone; DMSO, dimethyl sulfoxide; DMAc, N,N-dimethylacetamide; THF, tetrahydrofuran

[c] DMF/LiCl (9 wt%)

[d] DMSO/Pyridine

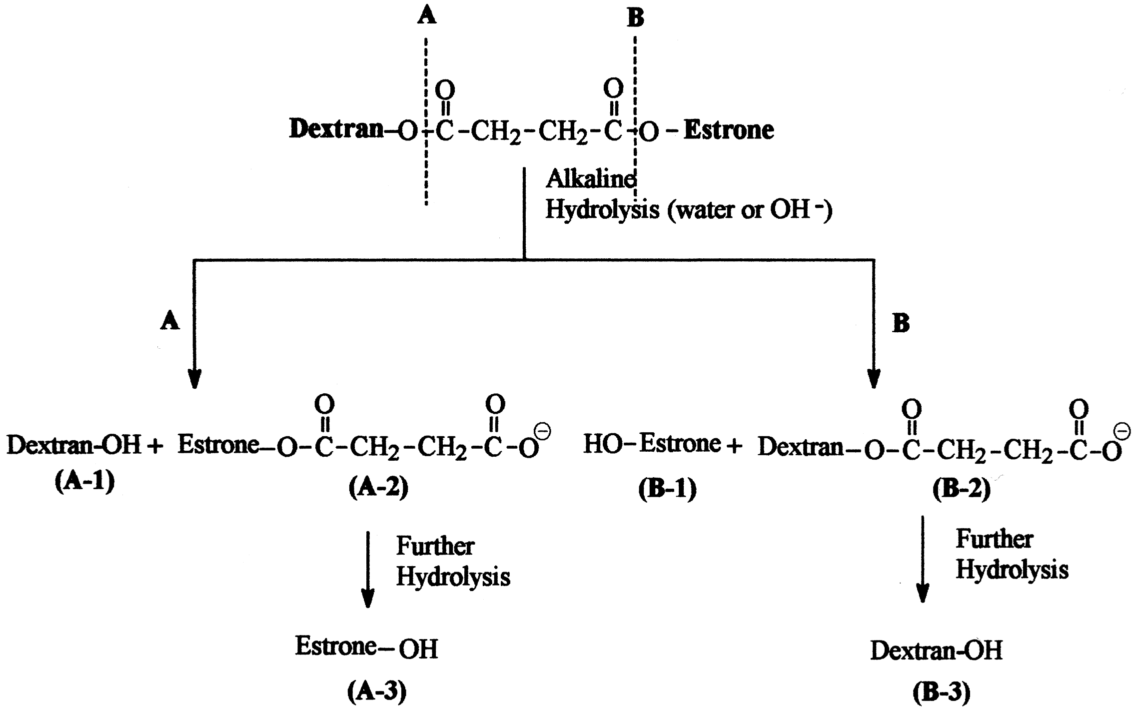
on the analysis of the ^{13}C NMR spectra of the ring carbons in the AHG units.

The degree of substitution (D.S.) was estimated from the ^{1}H NMR spectrum by taking a ratio of the normalized, integrated intensities of the sum of the bands at $\delta = 6.82$, 7.30 and 7.95 ppm (corresponding to the aromatic ring protons of estrone) to the normalized, integrated intensities of the dextran backbone protons (the signals between $\delta = 3.00$ and 5.52 ppm). In this study, the D.S. of 0.33 (11.0 mol% of estrone moieties) was obtained when a 1 : 3 molar ratio of acid chloride of estrone-3-hemisuccinate (**3**) to the hydroxyl groups of dextran was used.

Solubility data for the unmodified and estrone-conjugated dextran are given in Table 2. The estrone-conjugated dextran was soluble at room temperature in such polar, aprotic solvents as DMF, DMSO and NMP, whereas the unmodified dextran was dissolved in water, DMF/LiCl and DMSO/pyridine solvents at high temperature (80°C).

3.3. *In vitrio release studies*

In order to evaluate estrone release from the dextran-estrone conjugate (**4**) through a hydrolytic process, the conjugate (11.0 mol% of estrone moieties) was hydrolyzed heterogeneously in aqueous phosphate buffer and ethanol mixtures of either pH 7.4 or 8.0. The hydrolyses of the two ester linkages located in the spacer were presumed to occur according to the reactions shown in Scheme 1. However, the hydrolysis of the enolic ester portion of the dextran-estrone conjugate (route B) may be faster than that of the aliphatic ester portion (route A) due to the higher sensitivity of the enolic ester toward water. If enough time is permitted for the two intermediate esters (A-2 and B-2) to be continuously hydrolyzed, the eventual products of hydrolysis would be estrone and dextran, regardless of A or B route of hydrolysis. The buffer medium obtained from 48 h hydrolysis of the dextran-estrone conjugate (**4**) was extracted with benzene and examined by FTIR. There was no characteristic FTIR ester peak in the medium, an indication of the possible lack of ester spacer group found in A-2. Therefore, the estrone-succinic acid ester derivative (A-2)



Scheme 1.

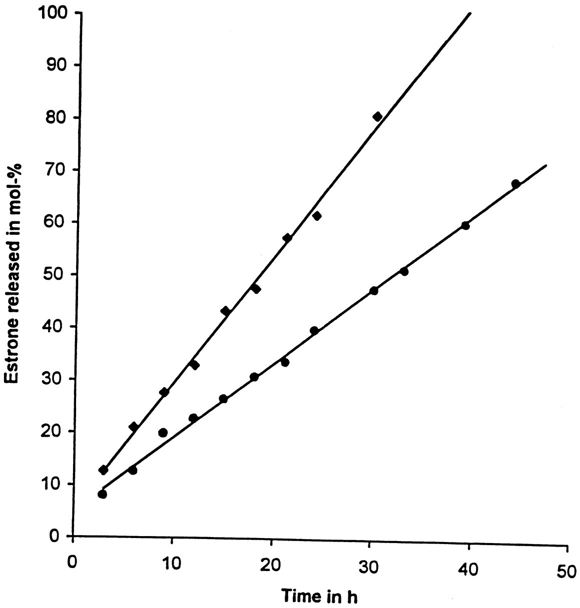


Fig. 5. Heterogeneous hydrolysis of dextran-estrone conjugate (11.0 mol% of estrone) at 37°C; pH 8.0 (◆) and 7.4 (●).

has been further hydrolyzed and the parent estrone(A-3) or (B-1) was the end product. However, FTIR data are not sufficient to provide a definite answer to the question that whether A-2 exists in the buffer media because the carbonyl IR band in the ester spacer could be masked by the ketone band of the estrone. Thus, other chemical means must be used to draw a definite conclusion of the presence of A-2 in the buffer medium. Fig. 5 illustrates the release of the conjugate at pH 8.0 and 7.4 at 37°C. A linear release of estrone vs time (Fig. 5) indicates that the release rate was zero order for up to 45 h. A faster release rate was observed at pH 8.0, and the total release of the estrone was achieved within a few days. In general, the hydrolysis rates of simple organic esters increase with the pH level of the medium. Also, Chu (1981 & 1982) reported that linear, aliphatic biodegradable polyesters, such as pol(glycolic acid) and poly(glycolide-co-L-lactide), hydrolytically degraded more in a highly alkaline buffer solutions than in slightly alkaline or acidic buffer solutions. The rate of release may also be controlled by the amount of ethanol (acting as a weak nucleophile for the cleavage of ester bonds), drug loading in the conjugate (i.e. degree of substitution), and hydrophilicity of the dextran. Because of the relative hydrophobic nature of estrone, a low amount of the conjugated estrone moieties (11.0 mol%) would make the dextran-estrone conjugate more hydrophilic than a high loaded conjugate.

4. Acknowledgements

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