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Synthesis and Characterization of Drug-Enzyme Conjugates

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Two drug-enzyme conjugates of dexamethasone-subtilisin and dexamethasone-cellulase have been synthesized and characterized to study their drug-protein incorporation ratio, immunoreactivity, enzyme activity and stability and these studies proved that a variety of drug enzyme conjugates could also be synthesized and characterized.

Key words: Enzyme linked immunosorbent assay (ELISA), Flow injection immuno analysis (FIIA)

INTRODUCTION

Steroidal agents or corticosteroids are most commonly used for treatment of local inflammatory disorders(Barry *et al.*, 1990). They are also used in systemic inflammatory disorders which require potent therapy for control. Naturally occurring steroid compounds produced in the adrenal cortex have both anti inflammatory (glucocorticoid) and salt retaining (mineralocorticoid) properties. Synthetic steroids such as prednisone and prednisolone have both glucocorticoid and mineralocorticoid effects but are used primarily for their glucocorticoid properties.

Dexamethasone is a synthetic glucocorticoid which is widely used in human and veterinary medicine. It is a potent anti inflammatory agent and has become a popular alternative to proper rest to keep horses in training (Stanley SMR *et al.*,1993 and Gaignage *et al.*,1989). We have reported the enzyme linked immunosorbent assay (ELISA) for dexamethasone in equine urine by colorimetric (Hassan S.S *et al.*, 1997), fluorescent(Hassan S.S *et al.*, 1996) and Dot ELISA method(Hassan S.S *et al.*, 1998). Drug-enzyme conjugate of dexamethasone-subtilisn and dexamethasone-cellulase have been synthesized and their characterization with respect to their drug, protein incorporation ratio, immunoreactivity, enzyme activity and stability have also been described by Hassan S.S (Hassan,1998).

The main aim of this work was to investigate the potential use of drug-enzyme conjugates in a sequential flow injection immuno-analysis (FIIA) for dexamethasone using fluoro-labelled substrate bioreactors down stream as a means of signal generation. For this purpose drug enzyme conjugate of dexamethasone -subtilisin (Det-Sub) and dexamethasone-cellulase were to be prepared and characterized. These were to be used in conjunction with fluorescently labelled substrates with in bioreactors constructed in house as described by Tang et al (Tang *et al.*, 1995, Tang *et al.*,1996, Tang *et al.* 1995) who used the system for a rapid and sensitive FIA of proteolytic enzymes and for collagenase. In this system on exposure to the enzyme, some of substrate is digested releasing fluorescent labels in the stream of buffer. This is detected down stream of the bioreactor with a spectrofluorimeter (Fig. 1).

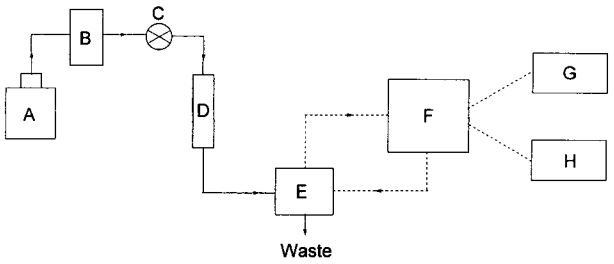

Fig. 1. Schematic diagram of the bioreactor monitoring system: A, buffer reservoir; B, pump; C, injection valve; D, bioreactor column; E, detector; F, computer; G, monitor; and H, printer.

MATERIALS AND METHODS

Apparatus

Perkin-Elmer luminescence spectrometer Model LS50B, the results were continuously logged into and processed by a dedicated PC with Perkin-Elmer FL data Manager software installed. The size exclusion chromatography system was composed of a 9 ml sephadex. G-25M column

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(PD-10), a peristaltic Pump (P-1), a single path monitor and its control Unit (UV-1), a fraction collector (Frac-100) and a chart recorder. These components were all obtained from Pharmacia (Milton Keyes) Buckinghamshire, U.K) Dynatech Immulon 4 micortitre plates (Dynatech Laboratories, West Sussex, U.K) Dynatech MR 7000 Plate reader, Phosphate-buffered saline with 0.1% tween (PBST) pH 7.4 was prepared from 2.15 g of potassium dihydrogen phosphate, 7.4 g of disodium hydrogen phosphate, 36 g of sodium chloride, 5 ml of tween 20 and 0.5 g of sodium azide and was made up to 5 L with distilled water. TRIS buffer 0-1 M was prepared by dissolving 60.57 g of TRIS, 7.35 g of $CaCl_2$. $2H_2O$ and 5 ml of Tween 20 and adjusted to the intended pH (7-10) with 1 M HCl and 1 M NaOH and made up to 5 L.

Reagents

N-succinyl-ala-ala-pro-phe-*p*-nitroanilide, subtilisin carlsberg (Protease type VIII) from Bicillus licheniformis, Lucifer yellow dilithium salt, Tetramethylrhodamine isothiocyanate (TRITC) cellulase (EC 3.2.1.4, enzyme activity 10 units per milli gram) from Pencillium funiculosum cellulose (highly purified), 1-ethyl-(3-dimethylamino)propylcarbodiimide hydrochloride (EDC), Bovine Serum albumin (BSA), ethylenediamine and dexamethasone were purchased from sigma chemical (Poole, Dorset, U.K). All the buffers salts were obtained from BDH and were of analytical reagent grade Sheep antidexamethasone antiserum AD61 was supplied by the Horseracing Forensic Laboratory, Suffolk, U.K). Sheep antiprimaquine antiserum was supplied by Helena Biosciences. Sunderland, U.K, and these were purified by Thiophilic gel (T-gel adsorbent) using the method supplied by Pierce Chemical Co., U.S.A.

Preparation of dexamethasone-subtilisin conjugate

Dexamethasone-subtilisin conjugate was prepared by using in initial 10:1 molar ratio of dexamethasone hemisuccinate (DHS) and subtilisin (Sub). DHS (10 mg), 1-ethyl-(3-dimethylamino)propylcarbodimide hydrochloride (EDC) (4 mg), acetonitrile (2.5 ml) and diethylamine (2 drops) were added to a 50 ml of Quickfit flask and the contents mixed slowly for 25 min using a magnetic stirrer. Subtilisin (56 mg) in 10 ml of distilled water was then added to the flask and the reagents mixed for another 10 min. The reaction mixture was then subjected to size exclusion chromatography (Pharmacia Biotech 1991) to obtain the drug-subtilisin conjugate.

Preparation of dexamethasone-cellulase conjugate

A dexamethasone-cellulase conjugate was prepared by using an initial 10:1 molar ratio of dexamethasone hemisuccinate (DHS) and cellulase. DHS (10 mg), EDC (4 mg) acetonitrile (2.5 ml) and diethylamine (2 drops) were added to a 50 ml of Quick fit flask and the solution slowly stirred using a magnetic stirrer for about 25 min. After that a solution of cellulase (114 mg) in 10 ml of distilled water was added to the flask which was stirred. The reaction mixture was then subjected to size exclusion chromatography to obtain the drug-Cellulase conjugate.

Purification of drug-enzyme conjugates by size exclusion chromatography

A 9 ml Sephadex G-25M column (PD-10) was equilibrated with PBST buffer (pH 7.4) using a flow rate of a 1 ml/minute, a chart speed of 5 mm/minute set at 100 × 2 mv sensitively, and a 280 nm detector (Tang *et al.*, 1995, Tang *et al.*, 1996, Tang *et al.* 1995, Pharmacia Biotech 1991). The reaction mixtures as described above were passed separately through the column in 1 ml aliquots. The eluted fractions were stored at 4°C until used.

Determination of the immunoreactivity of drug-enzyme conjugate

Two separate dexamethasone ELISAs were carried out to assess the immunoreactivity of the dexamethasone-subtilisin and dexamethasone cellulase conjugate obtained were analyzed by colorimetric standard single-reagent ELISA as described (Hassan S. S *et al.*, 1997), using 50 µl aliquots of diluted drug enzyme conjugate in assay buffer which were run as samples. The optical density values for standards of dexamethasone and samples of enzyme conjugates are given in Tables I, II.

Table I. Colorimetric determination of dexamethasone and dexamethasone subtilisin conjugates by rapid ELISA

For Standard Curve				For Dex-subtilisin fraction			
Conc. of Dex (M)	OD (n=6)	SD	CV %	Dilution of fraction	OD (n=6)	SD	CV %
Blank (zero)	0.969	0.045	4.64	$1:10^3$	0.359	0.019	5.29
1×10^{-9}	0.840	0.027	3.21	$1:10^4$	0.552	0.011	1.99
5×10^{-9}	0.710	0.025	3.52	-	-	-	-
1×10^{-8}	0.607	0.006	0.98	-	-	-	-
5×10^{-8}	0.395	0.009	2.27	-	-	-	-
1×10^{-7}	0.372	0.012	3.22	-	-	-	-

Table II. Colorimetric determination of dexamethasone and dexamethasone cellulase conjugates by rapid ELISA

	For Standard Curve			For Dex-cellulase fraction			
Conc. of Dex (M)	OD (n=6)	SD	CV %	Dilution of fraction	OD (n=6)	SD	CV %
Blank (zero)	0.955	0.050	5.23	$1:10^4$	0.391	0.010	2.25
1×10^{-9}	0.881	0.057	6.46	$1:10^5$	0.829	0.016	1.93
5×10^{-9}	0.793	0.029	3.65	-	-	-	-
1×10^{-8}	0.520	0.013	2.50	-	-	-	-
5×10^{-8}	0.301	0.016	5.31	-	-	-	-
1×10^{-7}	0.236	0.014	5.93	-	-	-	-

Assessment of enzyme activity for the dexamethasone-subtilisin conjugate using a lucifer yellow/rhodamine labelled bovine serum albumin label

A rapid homogeneous assay for determining the proteolytic activity of subtilisin was recently described (Tang *et al.*,1996). The method employs a bovine serum albumin substrate labelled with two fluorescent dyes with fluorescence energy transfer (FET) characteristics. The doubly labelled substrate was prepared by chemically coupling bovine serum albumin with Lucifer yellow (L.Y) and rhodamine (Rh) dyes following the literature method (Tang *et al.*,1996). The fluorescence emission from the Lucifer labels was initially quenched due to the FET of L.Y fluorescence to the adjacent Rh labels. Upon the addition of subtilisin to the labelled substrate solution, increased fluorescence is observed as the enzyme hydrolyzes the substrate generating small fragments in which only one fluorophore is present. Hence enhancement of the LYV fluorescence is observed as the FET effect is reduced.

Synthesis of FET-substrate (BSA-LY-Rh)

The literature method (Tang *et al.*, 1996) was followed for the preparation of doubly labelled fluoro substrate. The reaction mixture (75 mg BSA, 6.5 mg LY and 5 mg TRITC) was purified by the size exclusion chromatography system with the PD-10 column (9 ml) pre-equilibrated and eluted with PBST buffer. The fractions collected from the first peak were pooled together (total volume 20 ml) and diluted 1:40 in PBST for the use in assay of subtilisin at a working conc. of 27 $\mu g \cdot ml^{-1}$ of protein.

Assessment of enzyme activity

The method followed that reported in the literature (Tang *et al.*,1996). Subtilisin solution (10 µl) equivalent to 25-300 ng was added in turn into a fluorescence microcell, followed by the BSA-LY-Rh solution to make the final volume of 600 µl and the fluorescence reading was immediately started. The excitation and emission wavelengths selected were 436 and 535 nm, both the slit widths of 8 nm using Perkin-Elmer luminescence spectrometer, (model LS 50 B, Perkin Elmer). The concentration of subtilisin standards were used to established a standard curve. The corresponding fluorescence intensity for the dex-sub (10 µl) sample was also obtained, from which the activity of the subtilisin was calculated. The results are given in Table III and Fig. 2.

Table III. Study of enzyme activity for subtilisin and dex-subtilisin by homogeneous fluorescence method

Conc. of Subtilisin (ng/10 µl)	Fluorescence Units	Fluorescence Units for dex-subtilisin as unknown (10 µl)
25	15	22
50	19	-
100	26	-
150	33	-
200	41	-
250	46	-
300	50	-

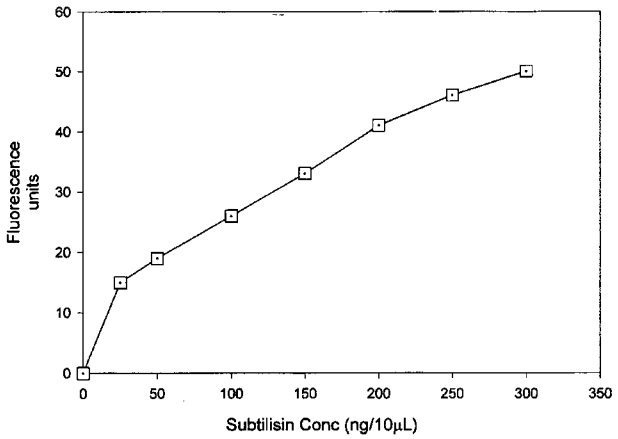

Fig. 2. Standard curve for subtilisin by homogenous fluorescence method

Stability studies of the dex-subtilisin conjugate by the homogeneous fluorescence excitation transfer (FET) method

A fresh lot of dex-subtilisin conjugate was made as described previously and purified by size exclusion chromatography. The fractions were collected and labelled as FR1 to FR4. These fractions were divided into two portions, one portion was stored in a refrigerator at 4°C and the other portion in a freezer at -20°C. Each sample was tested by taking a sample equivalent to 200 ng on the zero day, the day on which the sample was prepared, and every week the samples were tested as described in Section-VII. The total time of analysis for each sample was 10 min. A sample of standard subtilisin (200 ng) was also tested as a control. The results for stability profile are shown in Fig. 3.

Study of enzyme activity for subtilisin and dex-subtilisin by the homogeneous colorimetric method

Different doubling dilutions of standard subtilisin (100 ng - 6.3 ng·ml^{-1}) were made in 0.1 M Tris buffered with 0.1% Tween at pH 8.5. These were used to obtain a standard curve for the assay. In the same buffer, the dilution's for dex-subtilisin of 1:16000 and 1:32000 were made. The starting concentration of subtilisin used to prepare these dilution's was 5.5 mg in 8 ml of total volume. It was obtained as fraction of dex-subtilisin eluted through size exclusion chromatography. Aliquots (100 µl) of each standard subtilisin dilution 6.3-100 ng·ml^{-1} were loaded in the columns 1-3 (A-E) of a mircotitre plate in the descending order and similarly 100 µl aliquots of the dex-subtilisin dilutions were added to columns 4-5. After that 50 µl aliquots of 0.1 m M *N*-succinyl-ala-ala-Pro-Phe-*p*-nitroanilide substrate in 0.1 M Tris buffer were added to each well of the microtitire plate containing enzyme. As a blank, 50 µl portions of PNA substrate with 100 µl of the Tris buffer were dispensed in row F of column 1-3. OD values were measured after incubation for 15 min. when pronounced yellow colour developed at 405 nm. The results are shown in Table IV.

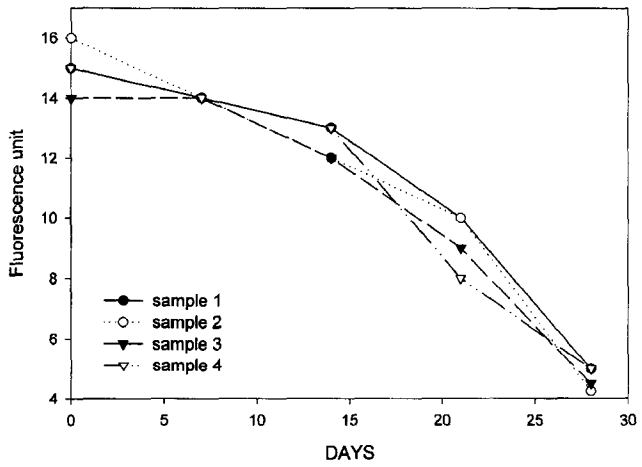

Fig. 3. Stability study of dexamethasone-subtilisin conjugate by homogeneous fluorescence method.

Immobilization of BSA-Lucifer yellow on to cellulose

BSA and Lucifer yellow solutions were prepared separately by dissolving 100 mg of BSA in 10 ml of coupling buffer (0.1 M sodium bicarbonate pH 8.5) and 4.1 mg of Lucifer yellow in 4 ml of labelling buffer. (0.5 M carbonate and bicarbonate buffer pH 9.5), cellulose (1.0 g) was weighed in a beaker and washed with distilled water and 2 M sodium carbonate (activation buffer) respectively. The cellulose was then activated in activation buffer (15 ml) using CNBr-coupling chemistry, following standard methods (Hemanson *et al.*, 1992). The activated cellulose was washed with distilled water and with 0.1 M sodium bicarbonate (coupling buffer pH 8.5). After that the entire BSA solution was added to the activated cellulose and the reagents mixed overnight slowly using a mixing invertor. The BSA-cellulose product was then washed with distilled water and treated with Lucifer yellow solution as a single batch. Mixing was continued for 2-3 h. The resulting cellulose-BSA-LY product was then washed with distilled water and coupling buffer. Finally 5 ml of 1 M ethanolamine (pH 9.0) in coupling buffer was added and mixed for 2 h. The solid support was thoroughly washed with distilled water and stored at 4°C in phosphate buffered saline (PBS pH 7.5) until used.

Flow injection fluorescence measurement of subtilisin and dex-subtilisin using a mini-bioreactor with immobilized BSA-Lucifer yellow on cellulose

A rapid assay method for protease enzymes including subtilisin has been reported by Tang *et al.* (Tang *et al.*,1996)

Table IV. Study of enzyme activity for subtilisin and dex-subtilisin by homogeneous colorimetric method (n=3)

Conc. (ng/ml)	OD	SD	CV %	Dex-sub Dilutions	OD	SD	CV %
100	0.828	0.0080	0.96	1:16000	0.419	0.0016	0.3
50	0.572	0.0020	0.35	1:32000	0.265	0.0004	0.1
25	0.334	0.0008	0.24	-	-	-	-
12.5	0.213	0.0010	0.46	-	-	-	-
6.3	0.145	0.0008	0.55	-	-	-	-
Substrate	0.075	0.0020	2.66	-	-	-	-

Table V. Study of enzyme activity for subtilisin through flow injection analysis (n=3)

Conc. of subtilisin (ng/20 µl)	Fluorescence Intensity	SD	CV %	Fluorescence Intensity for Dex-subtilisin
25	2.2	0.06	2.7	14.05
50	5.85	0.10	1.7	-
100	11.70	0.0	0.0	-
200	20.45	0.25	1.22	-

in which a variety of fluoro-substrates have been immobilized onto surface of glass beads and cellulose. In this study BAS-Lucifer yellow was immoblized onto cellulose to assess the enzyme activity. Protease enzymes such as subtilisin digest the fluoro-substrate and release fluorescent fragments which are detected down stream by a fluorimeter.

The BSA-Lucifer yellow cellulose solid phase reagent prepared previously was packed into a mini bioreactor (1 ml plastic syringe) up to 0.6 ml which was then incorporated into a flow injection fluorescence measurement system. The excitation and emission wavelength of the fluorescence detector were set at 430 and 540 nm respectively both with a slit width of 8 nm. Assays of subtilisin enzyme were conducted at a flow rate of 0.4 ml min^{-1} using 0.1 M disodium hydrogen phosphate and dihydrogen sodium phosphate buffered with 0.1% Tween at pH 8.6.

The standard curve for measuring the subtilisin was obtained by injecting standard subtilisin solutions ranging from 25 ng to 200 ng/20 µl per injection into the bioreactor. Each injection was repeated three times and the resulting fluorescent signals were recorded. In a similar way, the dex-subtilisin sample was injected (20 µl) and the concentration of dex-subtilisin was calculated from the standard curve. The results are shown in Table V and Fig. 4.

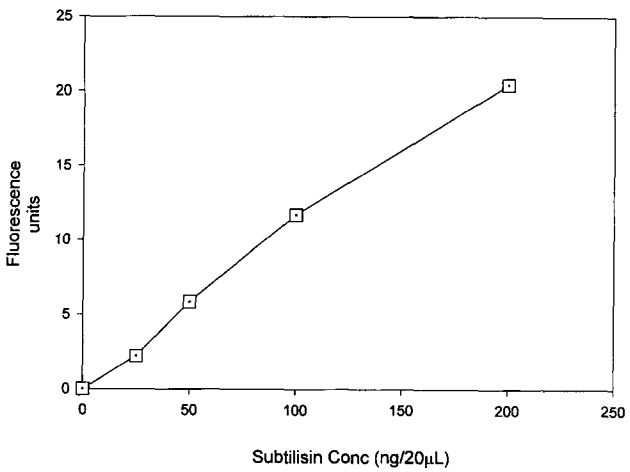

Fig. 4. Standard curve for subtilisin activity by Folw injection analysis.

DISCUSSION

The results in Tables I, II indicates that both the conjugates (Dex-subtilisin and Dex-cellulase) are immu-noreactive to the dexamethasone specific antibodies. The incorporation ratios for dex-subtilisin and dex-cellulase were interpolated from the standard curves using OD values of the respective conjugates and were 6.1 for dex-subtilisin and 9.1 for dex-cellulase.

The results in Table III also indicates that the linear region for standard concentration curve for subtilisin was between 25-200 ng per 10 µl as shown in Fig. 2. The concentration of subtilisin in the dex-subtilisin was also interpolated from the standard curve as 62 ng per 10 µl indicating that the proteolytic activity of dex-subtilisin towards the doubly labelled substrate was retained in the conjugate. This activity is lower than the equivalent mass of unreacted subtilisin corresponding to a 62% reduction in signal.

The stability profile for dex-subtilisin stored at 4°C and -20°C over 28 days was assessed. The enzyme activity was determined using the homogeneous fluorescence assay. The results in Fig. 3 indicate a half life of about 21 days for the samples stored under these conditions. The sample stored at -20°C were found to be the best storage conditions on the basis of the enzyme activity of dex-subtilisin.

The results in table 4 indicate that the linear region of standard curve is between 10-50 ng·ml^{-1} of subtilisin. The concentration of subtilisin in dex-subtilisin fractions were extrapolated from the standard curve as 0.22 mg·ml^{-1}. The relative activity of dex-subtilisin deactivate was 78% of the subtilisin starting product whereas in FET assay was 62%. The difference in the %age signal was due to the use of the substrates and the end point detection method. It is concluded that both the methods are suitable for the determination of proteolytic activity of subtilisin and dex-subtilisin.

The enzyme activity for subtilisin and dex-subtilisin were determined with a BSA Lucifer yellow cellulose bioreactor. The linear region of this system was between 25 ng-200 ng of subtilisin/20 µl of injection (Table V, Fig. 4). The concentration of subtilisin from dex-subtilisin was interpolated from the standard curve and was found to be equivalent to 128 ng/20 µl of the injection. It is interesting to note that the enzyme activity for subtilisin in the complex form (Dex-subtilisin) retain its activity and

both the methods *i.e* homogeneous colorimetric method and homogeneous fluorescence method are suitable for the determination of enzyme activity in the complex form.

It is concluded from the above discussion that the drug enzyme conjugate retain their immuno-reactivity as well as enzyme activity in the complex form which indicates that these studies could be useful for further synthesis of a variety of a drug labelled conjugates.

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