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Short communication

Evaluation and separation of steroid–bovine serum albumin conjugates by high-performance liquid chromatography

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Abstract

The conventional methods for characterization of steroid immunogens are based on the determination of the total amount of hapten bound to the protein carrier either by the UV spectroscopy or titration of unsubstituted amino groups. These methods do not allow more detailed insight into the immunogen composition. HPLC of the immunogen combined with UV detection is a relatively rapid and convenient method enabling determination of the hapten content in each fraction and, eventually, separation of individual fractions differing in the hapten content or purification of crude product.

Keywords: Steroids; Bovine serum albumin

1. Introduction

The conjugates of steroid carboxy derivatives with proteins are commonly used as immunogens for production of the antisera employed in various steroid hormone immunoassays. Conventional methods for evaluation of the immunogen quality use the titration of unsubstitued amino groups with trinitrobenzenesulfonic acid (TNBS) or UV spectroscopy of the unfractioned immunogen [1]. These approaches, however, do not provide us with a more detailed insight into the immunogen composition, which is usually a statistical mixture of conjugates differing in the number of substituted amino groups and in their position. The situation may be further complicated by a cross-linking of the conjugate due to reactive amino and carboxy groups of the carrier protein.

In this paper we demonstrate that HPLC is not only able to separate the conjugate from the starting compounds, but also can be used for separation of the main fractions of the immunogen.

It may be of practical importance when preparing monoclonal antibodies to recognize the certain structural determinant of the hapten, where the more precise knowledge of the immunogen structure is needed.

2. Experimental

2.1. Chemicals

Bovine serum albumin (BSA) fraction V, fatty acid free, was purchased from Bioveta (Ivanovice, Czech Republic). 19-Nortestosterone, 17α-methyltestosterone, testosterone-3-O-(carboxymethyl)oxime

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(CMO), cortisol-3-CMO, 17α-hydroxyprogesterone-3-CMO, estradiol-6-CMO and androstenedione-11α-hemisuccinate (HS) were purchased from Steraloids (Wilton, NH, USA). 19-Nortestosterone-3-CMO and 17α-methyltestosterone-3-CMO were synthesized according to Erlanger et al. [2] as modified by Hampl and Stárka [3]. Progesterone-19-HS and progesterone-11β-HS were prepared according to Kasal [4]. All the steroid carboxy derivatives were conjugated with BSA by a standard procedure [5], dehydroepiandrosterone-7-CMO-BSA was prepared according to Rosenfelt et al. [6]. Acetonitrile and water, HPLC grade were from Merck (Darmstadt, Germany), trifluoroacetic acid (TFA) was from Sigma (St. Louis, MO, USA), all other chemicals were from Lachema (Brno, Czech Republic).

2.2. HPLC

Isocratic elution on reversed-phase wide pore column (ET 250/4 300-5 C_8 Macherey–Nägel) was performed at 35°C in acetonitrile–water with 0.1% TFA at different solvent strengths (acetonitrile 45%, 60% and 75%, with flow-rates 1.3, 1.6 and 2 ml/min, respectively), using the Shimadzu LC-6A pump, column oven LCO 100 and UV detector LCD 2082 from ECOM, Prague (Czech Republic). The UV detection at 280 nm and 254 nm was used.

The samples of immunogens and BSA were dissolved in acetonitrile–water mixture (1:1) to give the concentration 4 mg/ml. The steroid carboxy-derivatives were dissolved in the same mixture but with the acetonitrile water ratio either 1:1 or 1:4 with regard to their solubility, to give the final concentration 250 μg/ml. 40 μl of these solutions was applied to the column.

The percentage of eluted sample was calculated from the response obtained from the chromatogram, compared with that where the column was replaced by an empty capillary tube.

2.3. Calculation of hapten/BSA ratio

The hapten/BSA was calculated from the chromatograms at 280 nm and 254 nm by solving the following two equations:

$$R_{\mathrm{BSA,\ 280\ nm}} n_{\mathrm{BSA}} + R_{\mathrm{hap,\ 280\ nm}} n_{\mathrm{hap}} = R_{\mathrm{280\ nm}}$$

$$R_{\mathrm{BSA,\ 254\ nm}} n_{\mathrm{BSA}} + R_{\mathrm{hap,\ 254\ nm}} n_{\mathrm{hap}} = R_{\mathrm{254\ nm}}$$

where $R_{\mathrm{BSA,\ 280\ nm}}$, $R_{\mathrm{hap,\ 280\ nm}}$ are the areas of chromatogram in cm^2 representing the response of equimolar amounts (1 nmol each) of the starting BSA and steroid carboxyderivative, respectively, at 280 nm. Analogically $R_{\mathrm{BSA,\ 254\ nm}}$, $R_{\mathrm{hap,\ 254\ nm}}$ are the areas corresponding to the response of 1 nmol of the starting BSA and steroid carboxyderivative at 254 nm. $R_{\mathrm{280\ nm}}$, and $R_{\mathrm{254\ nm}}$ are the areas representing the response of hapten–protein conjugate at 280 nm and 254 nm, respectively, and n_{BSA}, n_{hap} represent the substance amount of BSA and hapten, respectively in nmol.

3. Results and discussion

The hapten/protein ratios of 11 steroid–BSA conjugates determined by HPLC with UV detection at two wavelengths (280 nm and 254 nm), and those obtained by classical methods, along with the yields of conjugate elution by 10 min, are shown in Table 1. In order to achieve a better separation of individual fractions of the immunogen, chromatography was carried out at three solvent strengths of acetonitrile (75, 60 and 45%, v/v, respectively). Generally, with decreasing acetonitrile content the amount of the conjugate eluted from the column within 10 min decreased considerably, regardless the extent of amino group substitution of the carrier protein.

The separation of the conjugates from the starting components is illustrated on Figs. 1–3. Fig. 1A and Fig. 1B show the chromatograms of two conjugates prepared from two steroid carboxymethyloximes of α,β-unsaturated ketones, differing in the position through which the steroid derivative was attached to BSA (3 and 7). In both instances the conditions were chosen so to achieve a complete elution. In the case of testosterone-3-CMO conjugate (Fig. 1A), at least two peaks were found, apparently differing in the hapten content, as evidenced by different ratio of absorbancies of each fraction at 254 and 280 nm, reflecting those of the hapten and of BSA. As demonstrated in Fig. 1B and in Figs. 2 and 3, this was not the case of the other conjugates, where this ratio in individual fractions remained constant. In

Table 1
Hapten/BSA ratios in 11 steroid–BSA conjugates as determined from HPLC with UV detection at three solvent strengths and by classical methods together with the yields of conjugate elution by 10 min

Conjugate	HPLC with UV detection						UV spectra or amino-groups titration hapten/protein ratio±S.D.
	CH_3CN 75%, water, TFA 0.1%		CH_3CN 60%, water, TFA 0.1%		CH_3CN 45%, water, TFA 0.1%		
	hapten/protein ratio±S.D.	% eluted	hapten/protein ratio±S.D.	% eluted	hapten/protein ratio±S.D.	% eluted	
Testosterone-3-CMO-BSA	22.8±2.1	100	21.6±2.2	30.3	27.6±4.1	26.8	27.1±2.6
Cortisol-3-CMO-BSA	38.1±1.8	100	33.9±1.6	68.90	40.3±1.9	15.1	30.4±3.0
17α-OH-progesterone-3-CMO-BSA	27.4±1.2	100	25.7±2.0	16.1	26.6±1.9	0.59	28.5±2.4
Estradiol-6-CMO-BSA (batch 1)	34.8±0.7	100	36.4±1.1	100	36.8±1.5	39.6	33.4±3.9
Dehydroepiandrosterone-7-CMO-BSA	22.8±1.9	100	22.6±2.3	72.5	23.4±2.1	13.9	24.5±1.9
17α-Methyltestosterone-3-CMO-BSA	8.69±0.9	100	9.14±1.2	27.4	10.5±1.6	0.42	9.2±0.7
Progesterone-19-HS-BSA	12.1±0.5	100	11.6±0.8	44.1	13.6±1.7	2.38	8.4±1.5
19-Nortestosteron-3-CMO-BSA	18.7±1.3	100	16.8±1.5	88.2	20.4±1.6	2.74	21.1±2.2
Estradiol-6-CMO-BSA (batch 2)	12.9±0.4	100	13.1±1.0	31.4	13.7±2.0	9.55	11.3±1.7
Androstenedione-11α-OH-HS-BSA	3.23±0.5	100	3.63±0.2	100	3.01±0.5	5.32	5.8±0.9
Progesterone-11β-HS-BSA	10.3±0.8	100	8.84±0.4	33.4	11.4±1.2	1.93	10.1±1.2

Abbreviations: TFA = trifluoroacetic acid, CMO = carboxymethyloxime, BSA = bovine serum albumin, HS = hemisuccinate.

these cases the different mobility of the conjugate fractions may be explained by different cross-linking of the protein in the immunogen, rather than by different hapten/protein ratio. In Figs. 2 and 3 the retention factors instead of retention times are used, reflecting more clearly the effect of the solvent strength and taking into account the different flow-rates. The effect of the former is clearly demonstrated by the chromatograms of the same conjugate (DHEA-7-CMO-BSA) at two solvent strengths:

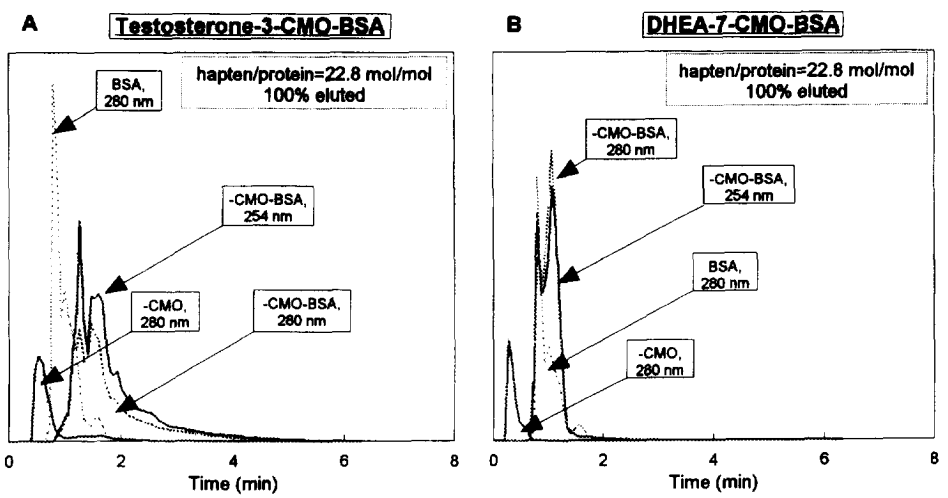

Fig. 1. HPLC of two conjugates of steroid carboxy derivatives with BSA and of the starting components (steroid carboxymethyloximes and BSA). The chromatograms demonstrate heterogeneity of the conjugates due to different hapten/BSA ratio; (A) testosterone-3-CMO-BSA) or cross-linking of the carrier protein; (B) dehydroepiandrosterone-7-CMO). Chromatographic conditions: column, Nucleosil ET, 250/4, 300-5, C_8 (Macherey–Nägel); mobile phase, acetonitrile–water (75:25, v/v) with 0.1% TFA; flow-rate, 2 ml/min; column temperature, 35°C; detection, UV at 254 and 280 nm, respectively. The chromatographic mobilities are expressed as retention time.

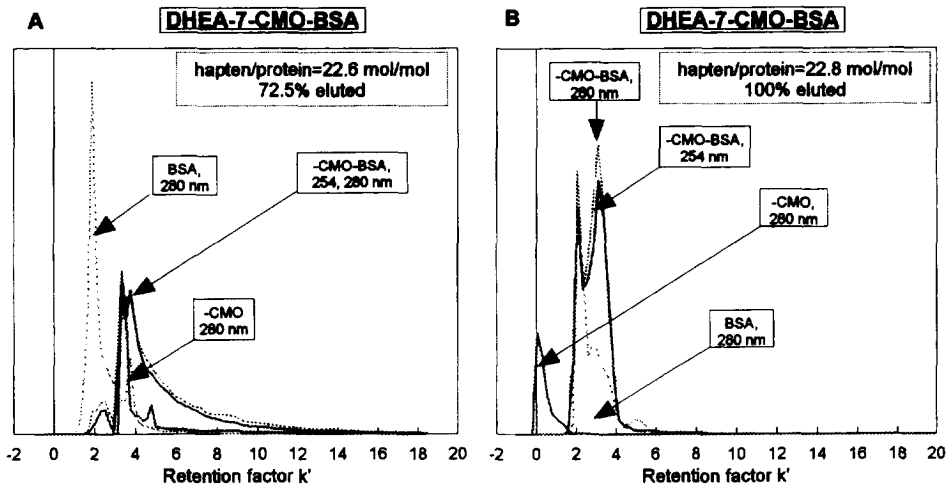

Fig. 2. HPLC of the conjugates of steroid carboxyderivatives with BSA and of the starting components (steroid carboxymethyloximes and BSA). Retention factors instead of retention times are given. Chromatographic conditions as in Fig. 1 with two exceptions: in (A) a mobile phase with a lower solvent strength (60% acetonitrile) and the flow-rate 1.6 ml/min were used. The chromatograms A and B illustrate the effect of solvent strength on the mobility of all the compounds including separation of individual conjugate fractions.

whilst at lower acetonitrile content (60%, Fig. 2A) a good separation of the conjugate from BSA but not from the hapten was achieved, the opposite situation was observed at 75% acetonitrile (Fig. 2B). Generally, the shift of the retention factor depending on the solvent strength decreased in the order: steroid carboxy derivatives > their conjugates with BSA ≫ BSA.

Fig. 3 shows the chromatograms of the conjugates prepared from the same derivative (estradiol-6-CMO), but strongly differing in the hapten/BSA ratio and, accordingly, in their chromatographic

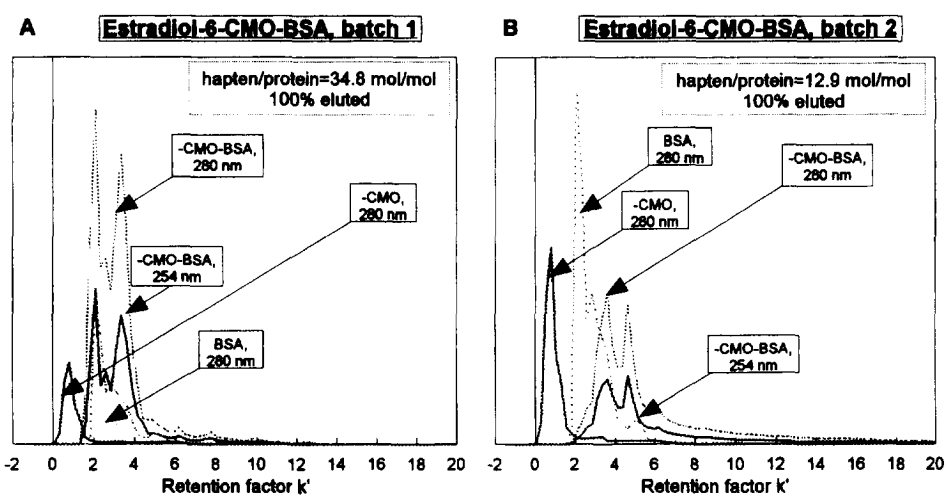

Fig. 3. HPLC of two batches of conjugate prepared from the same hapten (estradiol-6-CMO) illustrating the chromatographic behaviour of the conjugates differing in the hapten/BSA ratio and in the cross-linking of the carrier protein. Molar hapten/BSA ratio (A) 34.8, (B) 12.9. Chromatographic conditions as in Fig. 1.

mobility. It may be seen again that their mobilities were apparently influenced by the cross-linking of the carrier protein due to the reaction conditions during conjugate preparation rather than by the different hapten content.

4. Conclusions

HPLC on the reverse C_8 phase appeared to be a simple and rapid tool not only for separation of steroid-hapten conjugates from the starting components, but also for a better characterization of the heterogeneity of the conjugate, caused by different steroid/hapten ratio and cross-linking of the protein in individual fractions. This approach may be used not only in the field of steroids, but also for other hapten–carrier protein conjugates used for immunization of animals, in order to raise antisera for use in immunoassays.

Acknowledgments

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