Journal of Chromatography, 338 (1985) 404—409
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2437

Note

Clinical analysis of steroids

XXXII*. New method for the determination of hapten number of the antigen prepared for the radioimmunoassay of steroidal sulphates using ion chromatography

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(First received July 20th, 1984; revised manuscript received September 19th, 1984)

Measurement of steroidal conjugates in biological fluids has conventionally been done by prior hydrolysis or solvolysis and the subsequent analysis of deconjugated steroids. Recently, the conjugated steroids have been determined without prior hydrolysis, directly by gas chromatography—mass spectrometry, high-performance liquid chromatography or radioimmunoassay.

Because of its simplicity, convenience and ability to deal with many samples, radioimmunoassay is widely used for the measurement of steroidal sulphates. The first stage in establishing a radioimmunoassay of the sulphates is the preparation of antigen by coupling the sulphates with appropriate carrier protein, usually bovine serum albumin (BSA).

Since the antigens prepared should contain a proper number of haptens in order to become immunogenic, it is necessary to determine the hapten number (hapten/protein molar ratio of the antigen) prior to the immunization of animals. Hapten numbers are usually calculated by measuring the spectrometric absorption due to the chromophore originally present in the steroidal skeleton [1]. This procedure is not sufficient in the case of sulphates, because it is not clear whether the hapten is coupled with BSA without cleavage of the ethereal sulphate group during the reaction. In the radioimmunoassay of steroidal sulphates, therefore, it is necessary to determine the sulphate group in the antigen molecule. This paper describes the procedure for the determination of

*For part XXXI, see K. Watanabe and I. Yoshizawa, J. Chromatogr., 337 (1985) 114.

0378-4347/85/$03.30

sulphate group in the antigen prepared for radioimmunoassay of steroidal sulphates. The principle of this new method is based on the ion chromatographic measurement of SO_4^{2-} liberated by the hydrolysis of prepared antigens.

MATERIALS AND METHODS

Materials

Potassium 6-carboxymethoxyimino-3-hydroxyoestra-1,3,5(10)-trien-17β-yl sulphate (I) [2], potassium 6-carboxymethoxyimino-2,3-dihydroxyoestra-1,3,5(10)-trien-17β-yl sulphate (II) [3], potassium 6-carboxymethoxyimino-2-methoxy-3-hydroxyoestra-1,3,5(10)-trien-17β-yl sulphate (III) [4], potassium 6-carboxymethoxyimino-17-oxooestra-1,3,5(10)-trien-3-yl sulphate (IV) [4] and 6-carboxymethoxyiminooestra-1,3,5(10)-trien-3,17β-diol (V) [5] were prepared in this laboratory according to the known methods as described previously (see Fig. 1 for formulae). BSA (fraction V) was obtained from Armour Pharmaceutical Co. (Kankanee, IL, U.S.A.). Isobutyl chloroformate and tri-*n*-butylamine were obtained from Tokyo Seikagaku Kogyo (Tokyo, Japan). Visking seamless cellulose tubes (Union Carbide, IL, U.S.A.) were used after refluxing in 1% sodium carbonate for 30 min followed by washing with distilled SO_4^{2-}-free water. Other reagents used were of analytical grade.

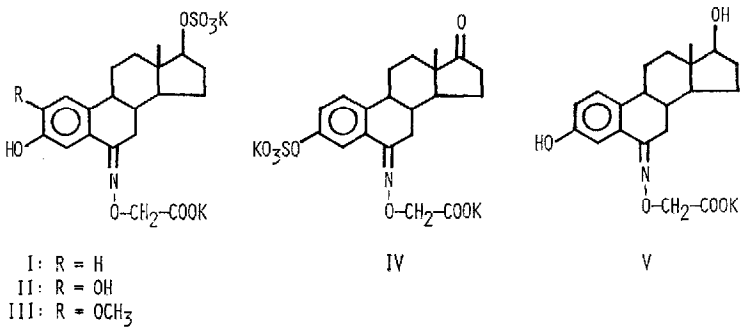

Fig. 1. Chemical formulae of I—V.

Instruments

The ion chromatograph equipped with an electric conductivity detector was a Type 2000i (Dionex, Midland, MI, U.S.A.) with a stainless-steel column (5 cm × 4.6 mm, I.D.) packed with TSK Gel IC-620 SA (Toyo Soda, Tokyo, Japan). The column was maintained at 30°C. A solution (pH 8.5) containing 1.3 m*M* sodium borate and 1.3 m*M* gluconic acid was used as the mobile phase at a flow-rate of 1.2 ml/min and a pressure of 8 kg/cm^2. Ultraviolet (UV) absorption spectra were recorded on a Model 200-20 spectrometer (Hitachi, Tokyo, Japan).

Preparation of antigens

Hapten compounds (I—V) were coupled with BSA by the mixed anhydride method under the usual conditions [1]. For hapten I, three kinds of antigen (antigen-I_1, antigen-I_2 and antigen-I_3) were prepared in the following manner. Isobutylchloroformate (3.0 mg for antigen-I_1, 6.0 mg for antigen-I_2 and 9.0 mg

for antigen-I_3) was added to a solution (2.0, 4.0 and 6.0 ml) containing hapten (8.0, 15.0 and 25.0 mg) and tri-*n*-butylamine (5.0, 10.0 and 15.0 mg) in a mixture of dioxane and dimethylformamide (1:2). After the mixtures were stirred under cooling for 1 h, BSA (80 mg) dissolved in 60% aqueous dioxane (6.0 ml) was added to each mixture. The whole was stirred under cooling for 5 h followed by additional stirring at room temperature for 6 h. The solution was kept neutral by occasional addition of a few drops of 1 *M* potassium hydroxide. The resulting solution was dialysed against SO_4^{2-}-free water at 4°C for 72 h. The steroid—protein conjugates were obtained by lyophilization as white powder; 77 mg, 83 mg and 87 mg for antigen-I_1, antigen-I_2 and antigen-I_3, respectively.

Antigens of other haptens (II—V) were prepared in the same way as described in the preparation of antigen-I_2 except for the neutralization by 1 *M* potassium hydroxide for hapten II, where 0.05 *M* potassium carbonate was used. Lyophilized antigens of II—V were obtained as white powder in the amount of 67—88 mg. The antigens obtained from haptens II, III, IV and V, are referred to hereafter as antigen-II, antigen-III, antigen-IV and antigen-V, respectively.

Determination of hapten number

The steroid/protein molar ratio of the antigens prepared was determined spectrometrically in methanol at a wavelength 270 nm using the previous method [1].

Determination of SO_4^{2-}

An exact amount (ca. 3.0 mg) of the hapten—BSA conjugates was dissolved in 2 *M* hydrochloric acid (3.0 ml), and the solution was refluxed for 2 h. After being cooled to room temperature, the solution was neutralized by addition of 2 *M* potassium hydroxide, followed by addition of a known amount of disodium hydrogen phosphate as an internal standard. The whole was diluted with SO_4^{2-}-free water to 10 ml, and centrifuged at 1500 *g* for 10 min to remove the precipitate, followed by extraction with ethyl acetate (3 × 2 ml). The aqueous layer was centrifuged again at 1500 *g*, and injected into the chromatograph with a microlitre syringe. Ten experiments were carried out on each antigen.

Standard curve

To obtain a standard curve, standard solutions of 1.0, 2.0, 3.0, 5.0, 10.0, 20.0, 30.0 and 50.0 ppm of SO_4^{2-} (sodium sulphate) containing 10 ppm of HPO_4^{2-} in each were prepared. Each mixture was injected into the chromatograph. The peak areas were determined by the half-band width method.

RESULTS AND DISCUSSION

In order to establish radioimmunoassay methods for oestradiol 17-sulphate, 2-hydroxyoestradiol 17-sulphate and 2-methoxyoestradiol 17-sulphate, the antigens of these sulphates were prepared by coupling with BSA by the mixed anhydride method. By difference of the hapten contents in the antigen, three

kinds of antigens for hapten I were prepared: antigen-I_1, antigen-I_2 and antigen-I_3. Similarly, antigen-II and antigen-III were prepared by coupling haptens II and III, respectively, with BSA.

The hapten numbers in these antigens were easily obtained from the absorbance at 270 nm due to the benzophenone oxime chromophore of the steroidal skeleton (UV method). However, it is uncertain whether these antigens contain theoretical numbers of ethereal sulphate groups because of the possibility of partial solvolysis of sulphate during the reaction. In order to confirm this, we developed a method to determine the sulphate group present in the antigen molecule. For this purpose, the antigens were hydrolysed to give the SO_4^{2-}, which was determined by ion chromatography.

Initially, the separation of anions was investigated by using several kinds of solvents as mobile phase. When a solution (pH 8.5) of 1.3 m*M* sodium borate and 1.3 m*M* gluconic acid was used, the anions concerned were separated satisfactorily as shown in Fig. 2.

Secondly, the quantification of SO_4^{2-} was investigated under the above conditions. The calibration curve was constructed by plotting the peak area of SO_4^{2-} to that of the internal standard against the amount of the former, and a satisfactory linearity was observed in the range of 1—50 ppm of SO_4^{2-}.

Application of the present method to oestradiol 17-sulphate—BSA conjugate was then carried out. The steroid numbers of antigen-I_1, antigen-I_2 and antigen-I_3 were determined spectrometrically and compared with the SO_4^{2-} values of each antigen. Oestradiol—BSA conjugate (antigen-V) was used as blank. The results obtained for these antigens are shown in Table I, from which it can be seen that, at any content of hapten residue in the antigens, there is no substantial difference between the UV method and the ion chromatography procedure. This means that no cleavage of the sulphate group at C-17 of the

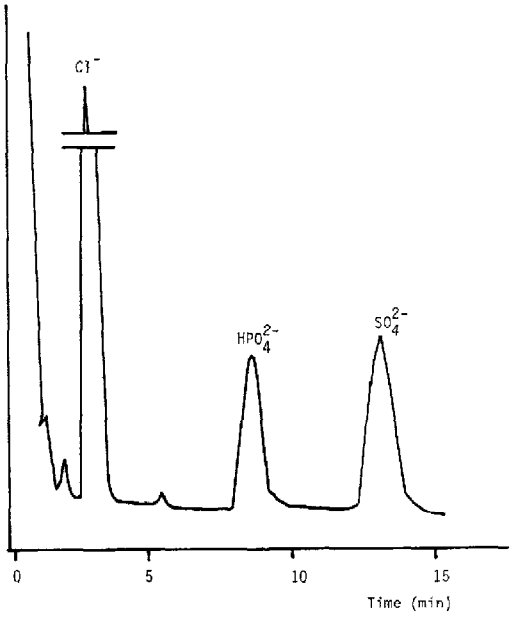

Fig. 2. Ion chromatogram showing the separation of the anions Cl^-, HPO_4^{2-} and SO_4^{2-}.

TABLE I

COMPARISON OF HAPTEN NUMBERS DETERMINED BY THE UV METHOD AND AMOUNTS OF SULPHATE ION (SO_4^{2-}) DETERMINED BY ION CHROMATOGRAPHY FOR THREE KINDS OF OESTRADIOL 17-SULPHATE–BSA CONJUGATES

Antigen	Hapten number	Molar ratio of SO_4^{2-} to BSA		Coefficient of variation (%)
		Mean	S.D. (n = 10)	
Antigen-I_1	10.7	11.3	0.23	2.04
Antigen-I_2	18.9	19.8	0.33	1.67
Antigen-I_3	30.2	31.4	0.42	1.34
Antigen-V	22.6	0.21	0.005	2.38

TABLE II

COMPARISON OF HAPTEN NUMBERS AND AMOUNTS OF SULPHATE ION (SO_4^{2-}) FOR VARIOUS KINDS OF ANTIGENS

Antigen	Hapten number	Molar ratio of SO_4^{2-} to BSA		Coefficient of variation (%)
		Mean	S.D. (n = 10)	
Antigen-I_2	18.9	19.8	0.33	1.67
Antigen-II	20.2	21.4	0.34	1.59
Antigen-III	19.4	19.8	0.29	1.46
Antigen-IV	22.6	7.8	0.43	5.51
Antigen-V	22.6	0.21	0.005	2.38

hapten residue occurred during the coupling reaction. Although a trace amount of SO_4^{2-} was detected in the case of antigen-V, this may be due to contamination during the course of experiments. The reproducibility is considered to be satisfactory as shown in Table I, where the C.V. values are between 1.3% and 2.0% except for antigen-V.

Similar experiments were carried out on four kinds of antigens. The results are summarized in Table II, and imply that the ethereal sulphates of the alcoholic hydroxyl group are not hydrolysed in the conditions of the reaction; thus the SO_4^{2-} values agree with the steroid numbers obtained by the UV method. In contrast, oestrone sulphate–BSA conjugate (antigen-IV) showed a big difference between these two values. Because the blank value is negligible, the result shows that a majority of the phenolic sulphate is solvolysed in the course of coupling with BSA. The mixed anhydride method is thus not suitable for preparing antigens of phenolic sulphate. This has been already demonstrated by Nambara et al. [6], who succeeded in obtaining oestrone sulphate–BSA conjugate by the activated ester method instead of the mixed anhydride method.

The detection limit of SO_4^{2-} by the present method using electric conductivity detector was 0.4 ppm (signal-to-noise ratio = 4.0 at full scale). Provided that an antigen contains twenty sulphate groups in the molecule, the amount of antigen required for the hydrolysis is calculated theoretically as about 0.2 mg (on the basis of a molecular weight of BSA of 66 210 [7]). Thus, the sensitivity of the present method is considered to be satisfactory.

In order to develop a radioimmunoassay for sulphoconjugated steroids, measurement of the sulphate group of the antigen is necessary. Many investigators have used antigens for immunization of animals without prior confirmation of the complete retention of the sulphate group, and only checked the molar amount of steroid nucleus by the UV method. The antibodies they obtained were, fortunately, specific for the steroidal sulphates, but they were not specific for the deconjugated haptens. This means simply that the sulphate group of the hapten residue was not hydrolysed inadvertently.

In planning a radioimmunoassay for endogenous steroidal sulphates, the present method may be applicable to confirmation of the characteristics of the antigen because of its simplicity, reproducibility and sensitivity. This procedure may also be useful in the radioimmunoassay of sulphoconjugated foreign compounds.

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