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ANALYTICAL CHEMICAL STUDIES ON STEROIDS

LII. STUDIES ON STEROID CONJUGATES. VII. GAS CHROMATOGRAPHY OF STEROID N-ACETYLGLUCOSAMINIDES

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SUMMARY

The direct analysis of steroid N-acetylglucosaminides by gas chromatography was accomplished successfully on OV-17 and OV-1 columns by using the trimethylsilyl derivatives. The structure of the trimethylsilylation product was confirmed by combined gas chromatography–mass spectrometry. A simple procedure for the extraction of the N-acetylglucosamine conjugate from the aqueous phase was also investigated with 17α-estradiol 17-N-acetylglucosaminide. The conjugate was recovered almost quantitatively when it was adsorbed on Amberlite XAD-2 resin and then eluted with methanol.

INTRODUCTION

Recently considerable attention has been drawn to the problems associated with the physiological significance of steroid conjugates. Besides the well known glucuronide and sulphate formation, conjugation with N-acetylglucosamine was found to be an important biotransformation in steroid metabolism by LAYNE *et al.*[1] who first isolated 17α-estradiol N-acetylglucosaminide from rabbit urine. The occurrence of the N-acetylglucosaminides of pregnene-3β,20α-diol, 15α-hydroxylated estrone and estradiol in human urine and bile has also been reported[2,3]. The direct analysis of steroid glucuronides by gas chromatography (GC) was accomplished with success by converting them to their methyl ester–trimethylsilyl (TMS) ether[4–6] or acetate–methyl ester derivatives[7]. However, studies on the GC of the steroid N-acetylglucosamine conjugates have not hitherto been reported. This investigation was therefore carried out to determine the feasibility of using GC for the separation, identification and quantitation of steroid N-acetylglucosaminides.

EXPERIMENTAL

Materials

17α-Estradiol 17-N-acetylglucosaminide (3-hydroxyestra-1,3,5(10)-trien-17α-

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yl-2′-acetamido-2′-deoxy-β-D-glucopyranoside) (I) was synthesized in this laboratory[8] and the others (II–XI) were donated by Dr. D. K. FUKUSHIMA.

Preparation of derivatives

The TMS derivatives were prepared by the method of SWEELEY *et al.*[9]. To a solution of the sample (1 mg) in tetrahydrofuran (0.5 ml) were successively added pyridine (0.1 ml), hexamethyldisilazane (0.1 ml) and trimethylchlorosilane (0.1 ml), and the mixture was allowed to stand at room temperature for 15 min. After evaporation of the solvent with the aid of a nitrogen gas stream, the residue was dissolved in tetrahydrofuran and injected into the gas chromatograph.

Gas chromatography

The apparatus used for this work was a Shimadzu Model GC-5AIFE Gas Chromatograph equipped with a hydrogen flame-ionization detector and a U-shaped stainless-steel column (1.3 m × 3 mm I.D.). The column was packed with 2 % OV-17 (phenylmethylsiloxane polymer) or 1.5 % OV-1 (methylsiloxane polymer) on a support of Chromosorb W (60–80 mesh). The temperature of the detector and flash heater was kept at 320°, and that of the column at 300°. The pressure and flow rate of the nitrogen carrier gas were 2.6 kg/cm² and 100 ml/min, respectively. The relative retention time of each compound was measured using the TMS ether of digitoxigenin as a reference compound.

Extraction and determination of steroid N-acetylglucosaminide

The test solutions were prepared by dissolving 200, 300 or 500 μg of 17α-estradiol 17-N-acetylglucosaminide in 20 ml of water. The aqueous solution was percolated through a column (8.5 × 1.0 cm I.D.) packed with 5 ml of Amberlite XAD-2 (Rohm & Haas Co., Philadelphia, U.S.A.) and then washed with 20 ml of distilled water. The conjugate was eluted with methanol and the eluant was collected in 5-ml portions. The methanol was evaporated *in vacuo*. To this residue was added a tetrahydrofuran solution containing a known amount of digitoxigenin (100–300 μg), and the mixture was evaporated with the aid of a nitrogen gas stream. The residue thus obtained was submitted to trimethylsilylation followed by GC determination as described above.

RESULTS AND DISCUSSION

In this study, eleven synthetic N-acetylglucosaminides were employed as model compounds. Trimethylsilylation of the hydroxyl groups in both the sugar and steroid moieties was readily achieved by reaction with hexamethyldisilazane and trimethylchlorosilane in pyridine according to the method of SWEELEY *et al.*[9]. The use of OV-17 or OV-1, which have high thermal stability, as the stationary phase proved to be effective for the GC separation of the TMS derivatives of the steroid N-acetylglucosaminides. All the compounds showed a single peak of the theoretical shape indicating the excellent GC properties of the reaction product. The structure of a typical derivative, the TMS ether of 17α-estradiol 17-N-acetylglucosaminide, was studied by combined GC–mass spectrometry*. In the mass spectra, a molecular

* A Shimadzu Model LKB-9000 Instrument was used.

ion peak was observed at $m/e = 763$ (M) although the relative intensity was low. A prominent peak was present at $m/e = 748$ (M-15) for the loss of a CH_3 moiety from one of the trimethylsilyloxy groups[10]. These data indicated that the structure remained intact during the GC separation. The retention values of these TMS derivatives relative to digitoxigenin TMS ether are presented in Table I.

3β-Hydroxy-5α-androstan-17β-yl-N-acetylglucosaminide (V) showed a longer retention time than the 3α-epimer (III) with OV-17 and OV-1 liquid phases. Similar behaviour was observed with two epimeric 17-oxo-5α-androstan-3-yl derivatives (VIII and IX), which exhibited better separation, probably owing to the presence of the bulky group at C-3. These results are consistent with the previous finding[6]

TABLE I

RELATIVE RETENTION TIMES OF TMS DERIVATIVES OF STEROID N-ACETYLGLUCOSAMINIDES

Conditions: stainless-steel column (1.3 m × 3 mm I.D.); packing 1.5% OV-1 or 2% OV-17 on Chromosorb W (60–80 mesh); nitrogen flow rate, 100 ml/min; column temperature, 300°; detector temperature, 320°; flash heater temperature, 320°.

Compound[a]	*Relative retention time*	
	OV-17	*OV-1*
3-Hydroxyestra-1,3,5(10)-trien-17α-yl-β-NAG (I)	1.71	2.67
3-Oxoandrost-4-en-17β-yl-β-NAG (II)	3.13	3.62
3α-Hydroxy-5α-androstan-17β-yl-β-NAG (III)	1.29	2.70
3α-Hydroxy-5β-androstan-17β-yl-β-NAG (IV)	1.23	2.57
3β-Hydroxy-5α-androstan-17β-yl-β-NAG (V)	1.74	3.42
3β-Hydroxy-5β-androstan-17β-yl-β-NAG (VI)	1.29	2.64
17-Oxoandrost-5-en-3β-yl-β-NAG (VII)	2.60	3.00
17-Oxo-5α-androstan-3α-yl-β-NAG (VIII)	1.64	2.17
17-Oxo-5α-androstan-3β-yl-β-NAG (IX)	2.64	3.17
17-Oxo-5β-androstan-3α-yl-β-NAG (X)	1.72	2.62
17β-Hydroxy-5β-androstan-3α-yl-β-NAG (XI)	1.64	2.59
Digitoxigenin	1.00 (4.30 min)	1.00 (2.36 min)

[a] β-NAG = 2'-acetamido-2'-deoxy-β-D-glucopyranoside.

TABLE II

RECOVERY OF 17α-ESTRADIOL 17-N-ACETYLGLUCOSAMINIDE FROM THE AQUEOUS PHASE FOLLOWING GAS CHROMATOGRAPHY ON AN AMBERLITE XAD-2 COLUMN[a]

Study	*Added Amount (μg)*	*Methanol eluant*			
		Fraction 1	*Fraction 2*	*Fraction 3*	*Total*
1	200	122.2 (61.1)	63.6 (31.8)	10.0 (5.0)	195.8 (97.0)
2	200	116.4 (58.2)	61.8 (30.9)	12.8 (6.4)	191.0 (95.5)
3	300	180.9 (63.3)	84.6 (28.2)	13.5 (4.5)	288.0 (96.0)
4	300	180.9 (60.3)	90.0 (30.0)	18.0 (6.0)	288.9 (96.3)
5	500	316.5 (63.3)	156.0 (31.2)	12.0 (2.4)	484.5 (96.9)
6	500	293.0 (58.6)	168.0 (33.6)	10.0 (2.0)	471.0 (94.2)

[a] The methanol eluant was collected in 5-ml portions and the amount of 17α-estradiol 17-N-acetylglucosaminide was determined by GC using a 2% OV-17 column. The figures in parentheses represent the recovery rate (%).

that the TMS ether of 3β-hydroxy-5α-androstan-17-one is eluted before that of the 3α-epimer on either of these two columns. In the 5β-steroids, however, the configuration of the C-3 substituent was not significantly reflected in the retention time (IV and VI).

In order to apply the present method to biological materials, a simplified procedure for the separation of steroid conjugates from the aqueous phase was then investigated. It has recently been demonstrated[11] that Amberlite XAD-2, a neutral crosslinked styrene polymer, adsorbs steroid conjugates quantitatively from urine, and these in turn can be readily eluted from the resin with an organic solvent. An aqueous solution containing a known amount of 17α-estradiol 17-N-acetylglucosaminide was percolated through a column of the resin. After washing with distilled water, the resin was eluted with methanol and each 5-ml fraction was submitted to GC. The calibration curve for the determination was constructed by plotting the ratio of the peak area of the sample to that of the internal standard (digitoxigenin) against the weight ratio of these two compounds, and good linearity was observed. Typical results are given in Table II. The data indicate that methanol rapidly eluted the steroid N-acetylglucosaminide and *ca.* 60 % was recovered in the first portion of solvent. It should be noted that quantitative recovery of the desired N-acetylglucosaminide without any structural alteration was attained by a simple procedure involving elution with a small volume of methanol after washing with water.

It is hoped that this separation technique will be applicable to studies on endocrinological and biochemical problems associated with steroid N-acetylglucosaminides.

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