Journal of Chromatography, 126 (1976) 641–650

CHROM. 9360

STRUCTURE–GAS CHROMATOGRAPHIC ELECTRON CAPTURE SENSITIVITY RELATIONSHIPS OF SOME SUBSTITUTED 17α-ACETOXYPROGESTERONES

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SUMMARY

Structure–electron capture sensitivity relationships were established for underivatized 17α-acetoxyprogesterones. While progesterone was very insensitive, 17α-acetoxyprogesterone had a response of 4.8×10^2 C/mole. Methyl groups in the A or B ring of 17α-acetoxyprogesterone had no effect. A keto group at C-6 was 25 times more sensitive (1.2×10^4 C/mole). A double bond at C-6,7 enhanced the sensitivity sevenfold (3.5×10^3 C/mole), but double bonds at C-1,2 or C-9,11 had only slight effect. Substitution at C-16 was important. A methyl group at C-16 had two and three times the sensitivity in the 3-keto Δ^4 and 3-keto $\Delta^{4,6}$ series (1.1×10^5 and 1.1×10^4 C/mole), respectively. A methylene group at C-16, in contrast, showed a six- and twofold greater sensitivity over the C-16 methyl in the two series (7×10^3 and 2.2×10^4 C/mole), respectively. The most sensitive compound was 6-dehydro-6-methyl-16-methylene-17α-acetoxyprogesterone (melengestrol acetate). Its sensitivity was 2.2×10^4 C/mole, comparable to the most sensitive halo esters of steroid alcohols reported in the literature. Its electron capture coefficient was $3–7.6 \times 10^{10}$ l/mole. The coefficient was independent of the detector temperature, indicating low activation energy for electron absorption.

INTRODUCTION

It is well known that relatively few organic compounds respond to detection by an electron capture detector (ECD). Steroids generally are not considered to have any strong electron attachment properties. Lovelock *et al.*[1] determined the electron absorption coefficients of some underivatized steroid hormones using a tritium detector in conjunction with a cross-section ionization detector. Unsaturated keto steroids were shown to have some affinity for electrons and the most sensitive compound in their series was 1,4-androstadiene-3,11,17-trione. There are a large number of references on the analysis of steroids by electron capture gas chromatography (GC–ECD) after their convertion to more sensitive derivatives. Steroid alcohols are analyzed by GC–ECD after having been converted to their halo esters. Perfluorobutyrates are the most popular and sensitive among such derivatives. Recently, O-

pentafluorobenzyloximes were reported as very sensitive derivatives for several keto steroids[2].

The first report that has come to our attention on the practical utilization of GC–ECD for the analysis of underivatized steroids is on melengestrol acetate (6-dehydro-6-methyl-16-methylene-17α-acetoxyprogesterone, MGA®) in cattle feed supplements by Davis *et al.*[3]. Krzeminski and Cox[4] and Ryan and Dupont[5] have reported procedures for the residual level (2.5–10 ppb) determination of MGA in several bovine tissues. Because of the unusually high sensitivity of MGA to ECD, we have in this study determined its response quantitatively and compared it with several underivatized 17α-acetoxyprogesterones to elucidate structure–ECD response relationships of this class of steroids.

EXPERIMENTAL

Steroids

All the steroids were obtained from the Biological Screening Office of The Upjohn Company. They were first tested for purity by thin-layer and gas–liquid chromatography using a flame ionization detector (FID). Solutions for GC–ECD were prepared by dilution of a 1 mg/100 ml solution of each steroid in 10% benzene in cyclohexane. (The solvents were distilled in glass and of high purity). Dilutions were made with cyclohexane to obtain 40 and 100 pg/μl for the more sensitive compounds and 1 ng/μl for the less sensitive ones.

Glassware

All glassware was specially washed with chromic acid followed by cleaning in an ultrasonic cleaner. It was again rinsed with water and 3A alcohol, dried, and stored in a desiccator.

Gas–liquid chromatography and detection system

The gas chromatograph used was a Tracor Model MT 220 (Tracor, Austin, Texas, U.S.A.) equipped with a dual hydrogen FID and a ^{63}Ni (10 μCi) ECD. The operating parameters were: Column —1% OV-17 on Gas-Chrom Q 100–120 mesh, packed in a 3 mm I.D. glass U tube. The glass tube was silanized with dichlorodimethylsilane as described[6] prior to packing. The length of the column packing was 14 in. Temperatures —column, 235°; inlet, 250°; FID, 255°; ECD, 290°. Gas flow-rates —nitrogen for FID, 50 ml/min; nitrogen for ECD, 52.3 ml/min with no purge; hydrogen, 40 ml/min; air, 400 ml/min. Pulsed voltage parameters for ECD —applied voltage, 55 V; pulse rate, 270 μsec; pulse width, 9 μsec. Attenuation —10 × 64 (6.4×10^{-10} A.f.s.d.).

Initially, 1 mg/ml solutions of the steroids were subjected to GC using the FID (GC–FID) in order to establish chromatographic conditions. The diluted solutions (0.04–1.0 ng/μl) were then injected into the column connected to the ECD. The steroids under observation fell into two groups, *viz.* the highly sensitive ones, having a linear response for 40–500 pg injected onto the column, and the second group, having a lower sensitivity and falling in the linear range of 1–5 ng. Progesterone which was also included did not register any response for 5 ng. Of the steroids in the first group 40–500 pg were injected and the areas under the peak were measured using a

planimeter calibrated to represent 1 cm^2 for every ten counts. The areas were plotted against picomoles of the compound injected to find the linear dynamic range of response. The second group of steroids was treated in a similar manner except that the quantities injected into the GLC column were from 1 to 5 ng.

RESULTS

All the steroids gave good peaks on the OV-17 column with no indication of decomposition. Since the most important compound in the series was MGA, it was subjected to GLC–mass spectrometry (GLC–MS) (LKB 9000, LKB-Produkter AB, Stockholm, Bromma, Sweden) and found to chromatograph intact. The chromatographic conditions were the same for all and the retention times ranged from 2–6 min. Chromatograms of a few typical compounds and the amounts of each injected on the column are shown in Figs. 1 and 2. The structures of the steroids, their response

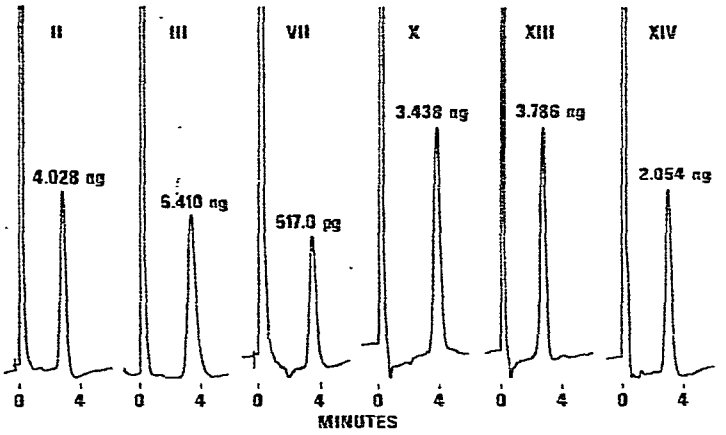


Fig. 1. Chromatograms of underivatized 17α-acetoxyprogesterones by GLC–ECD. The amounts injected onto the column are indicated for each steroid. For structural identification, see Table I.

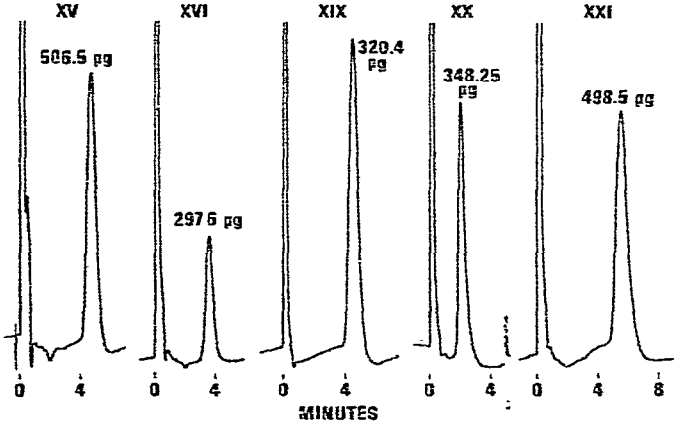
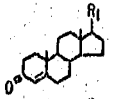
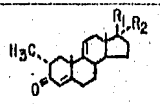
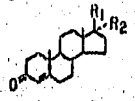
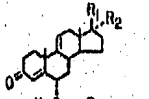
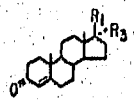
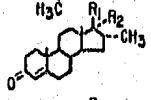
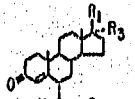
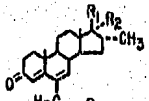
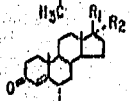
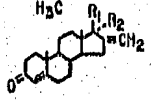
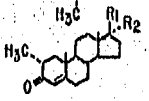
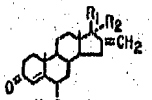
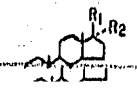
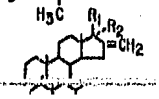


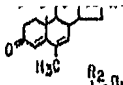
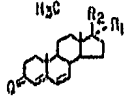
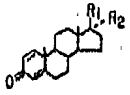
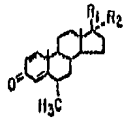
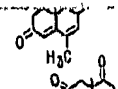
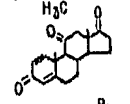
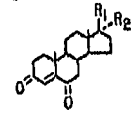
Fig. 2. Chromatograms of underivatized 17α-acetoxyprogesterones by GLC–ECD. The amounts injected onto the column are indicated for each steroid. For structural identification, see Table I.

TABLE I

STRUCTURES AND RESPONSES TO ELECTRON CAPTURE DETECTION OF SUBSTITUTED 17α-ACETOXYPROGESTERONES

$R_1 = -\overset{O}{\overset{\|}{C}}-CH_3$, $R_2 = -O-\overset{O}{\overset{\|}{C}}-CH_3$, $R_3 = -O-\overset{O}{\overset{\|}{C}}-CH_2CH_3$

Compound No.	*Structure*	*Electron capture response (C/mole)*	*Response relative to 17α-acetoxyprogesterone*	*Compound No.*	*Structure*	*Electron capture response (C/mole)*	*Response relative to 17α-acetoxyprogesterone*
I		— [a]	—	XII		6.7×10^2	1.4
II		4.8×10^2	1.0	XIII		6.7×10^2	1.4
III		5.47×10^2	1.14	XIV		1.1×10^3	2.3
IV		5.28×10^2	1.10	XV		1.1×10^4	22.9
V		6.4×10^2	1.3	XVI		6.5×10^3	13.5
VI		4.5×10^2	0.94	XVII		7.1×10^3	14.8
VII		3.5×10^3	7.3	XVIII		6.2×10^3	12.9

VIII		3.5×10^3	7.3
IX		2.7×10^3	5.6
X		9.3×10^2	1.9
XI		9.0×10^2	1.9
XIX		2.2×10^4	45.8
XX		8.1×10^3	16.9
XXI		1.2×10^4	25.0

* No response for 5 ng injected on the column.

to ECD in C/mole and the relative responses compared to 17α-acetoxyprogesterone (AP, II) are shown in Table I. The responses are the average of at least four points from the linear range of plots of concentrations *versus* response (peak areas). For MGA, the plot was linear from 40–300 pg injected onto the column and the *Y* intercept was zero. For all the other compounds, the *Y* intercept was negative. This deviation was minimal for those steroids which showed fair to good response in the picogram quantities and greater for those that had response only in the nanogram quantities.

DISCUSSION

The theories on the fundamental processes taking place in an ECD operated in the pulsed mode are well documented. Wentworth *et al.*[7,8] proposed a series of reactions taking place in the detector on the basis of kinetic derivations using steady-state approximations. They derived the following expression relating the concentration of capturing species and changes in plasma current

$$\frac{I_b - I_e}{I_e} = Ka \tag{1}$$

where

I_b = maximum initial current before addition of capturing species
I_e = current remaining after the introduction of a capturing species
K = capture coefficient
a = instantaneous concentration of capturing species

The capture coefficient K can be obtained by integration of eqn. 1 with respect to the volume of gas passing through the detector cell during the residence time of the peak and the following equation was derived[8]

$$K = \frac{I_b - I_e}{I_e} \cdot (W_{1/2}) \cdot \frac{Fr}{SMCs} \tag{2}$$

where

$W_{1/2}$ = peak width at half-height (in.)
Fr = flow-rate in the detector in l/min
S = sample size (μl)
M = molar concentration
Cs = chart speed (in./min).

Even though the capture coefficient is a useful figure to determine the temperature dependence of electron capture, activation energies, and other thermodynamic properties, the sensitivities of steroid derivatives to ECD are more commonly expressed in C/mole. In order to compare the response of MGA and the other steroids in this study with the reported values of steroid derivatives, the responses in C/mole were calculated knowing the electrometer specifications for the amount of current required for full scale deflection, the chart speed, the area of the peak, and the concentration of the sample.

Effect of 17α-esters

Progesterone showed no response when 5 ng were injected onto the column.

17α-Acetoxyprogesterone (AP, II) on the other hand, showed fair response and had a sensitivity of 4.8×10^2 C/mole (80 mm peak for 4 ng). C-17 α-propionates (III and IV) showed the same response as the acetate. Introduction of a methyl group at C-2 or C-6 of AP did not have any effect (V and VI).

Effect of additional double bonds

Lovelock *et al.*[1] in their study on the affinity of steroids for electrons with thermal energies had observed that only those steroids possessing the structure $-CO-CH=CR_1R_2$ had any marked electron absorption and that this structure conferred electron absorption provided that there was in the molecule an opportunity for further electronic interaction. In view of the above observations, one would expect a greater response for 6-dehydro AP (VII) than for AP. 6-Dehydro AP and 6-dehydro-6-methyl AP (VIII) indeed were seven times more sensitive (3.5×10^3 C/mole) than AP. 17β-Acetoxy-17-isoprogesterone (IX) had a response of 2.7×10^3 C/mole, not significantly lower than its 17 epimer (VII). A comparison of Δ^1 AP (X, 9.3×10^2 C/mole) and Δ^1-6α-methyl AP (XI, 9.0×10^2 C/mole) with the 3-keto $\Delta^{4,6}$-ones (VII–IX) having otherwise identical substituents on the D ring showed the $\Delta^{4,6}$ compounds had a more than threefold greater affinity for electrons than the $\Delta^{1,4}$ compounds. The 1,4-dien-3-ones were only about twice as sensitive as the 3-keto Δ^4 compounds (II–VI) in their affinity for electrons. This result is in agreement with the observations of Lovelock *et al.*[1]. The UV spectra of these two classes of steroids show greater conjugation in the 3-keto $\Delta^{4,6}$ systems than in the 3-keto $\Delta^{1,4}$ ones. The former have absorption maxima at ≈285 nm with an extinction coefficient of 27,000–29,000; the latter have absorption maxima at ≈245 nm and an extinction coefficient of ≈15,000. Therefore, the greater electron affinity by the 3-keto $\Delta^{4,6}$ steroids may be explained on the basis that the absorbed electron may be delocalized more readily in this system than in the 3-keto $\Delta^{1,4}$ system.

There were two 3-keto $\Delta^{4,9-11}$ compounds in the series, one with a methyl group at C-2 (XII) and the other with methyl at C-6 (XIII). These were included to determine the effect of an isolated double bond. The response values for these two compounds were intermediate between the AP type (II–VI) and the Δ^1 AP type of compounds (X and XI). Obviously, the electronic interaction in the 3-keto $\Delta^{4,9-11}$ compounds is more than in the 4-en-3-one systems, but less than the 1,4-dien-3-one systems.

Effect of substitution at C-16

It was pointed out earlier that a methyl group in the A or B ring did not affect the sensitivity, but when an α methyl was substituted at C-16 in AP (XIV), a twofold difference in sensitivity was observed (1.1×10^3 C/mole). In the 4,6-dien-3-one series, the effect of C-16 methyl substitution was even more pronounced. Thus, 6-dehydro-16α-methyl AP (XV) was thrice as sensitive (1.1×10^4 C/mole) as 6-dehydro AP (VII) and other similar compounds (VIII and IX). It is difficult to explain that while a methyl group at C-2 was immaterial, a methyl group at C-16 was so influential in the absorption of electrons.

The effect of C-16 methylene substitution was much greater than that of methyl substitution. The three 16-methylene AP tested (XVI–XVIII) were fourteen times as sensitive as the 16-nonsubstituted ones (II–VI) and about seven times as

sensitive as 16α-methyl AP (XIV). Among the 4,6-dien-3-one steroids, the C-16 methylene steroid (XIX, MGA) was twice as sensitive as the corresponding C-16 methyl one (XV) and about seven times more sensitive as the 16-nonsubstituted ones (VII–IX). Its response in C/mole was 2.2×10^4, a very high figure for an underivatized steroid.

Effect of additional keto groups

Lovelock *et al.*[1] had reported that the presence of even three isolated keto groups in a saturated steroid conferred little more electron absorption than the slight amount expected of a simple ketone. He had also pointed out that in a 4-en-3-one system, a keto group in the 11 position was important and the most active compound in his series was 4-androstene-3,11,17-trione (XX). Our results showed this compound to be fairly active, giving a response of 8.1×10^3 C/mole. This was only slightly better than the 16-methylene 17α-acetoxyprogesterones (XVI–XVIII). 6-Keto AP (XXI), however, was more sensitive than 4-androstene-3,11,17-trione. Its response of 1.2×10^4 C/mole can be attributed to the good electronic interaction of the C-6 keto group with the orbitals of the 4-en-3-one system.

The three steroids that had the highest responses were 6-dehydro-6β,16α-dimethyl AP (XV, 1.1×10^4 C/mole), 6-keto AP (XXI, 1.2×10^4 C/mole), and 6-dehydro-6-methyl-16-methylene AP (XIX, MGA, 2.2×10^4 C/mole). The responses for these underivatized steroids were very high: 10–20 pg were easily detectable. For comparison, the responses for these three steroids are shown in Table II alongside

TABLE II

COMPARISON OF ELECTRON CAPTURE RESPONSES OF UNDERIVATIZED MELENGESTROL ACETATE AND RELATED STEROIDS AND LITERATURE VALUES FOR STEROID HEPTAFLUOROBUTYRATES AND O-PENTAFLUOROBENZYLOXIMES

Steroid	*Electron capture response (C/mole)*			
	Underivatized steroids	*Heptafluorobutyrates*		*O-Pentafluorobenzyloxime (Ref. 2)*
		Ref. 9	*Ref. 10*	
MGA (XIX)	2.2×10^4			
6-Dehydro-6β,16α-dimethyl-17α-acetoxyprogesterone (XV)	1.1×10^4			
6-Keto-17α-acetoxyprogesterone (XXI)	1.2×10^4			
Testosterone		3-enol, mono 0.96×10^3		
Testosterone		3-enol, 17-di 4.5×10^3	3-enol, 17-di 3.9×10^4	4.7×10^4
Androstenedione		3-enol 4.0×10^3	3-enol 3.1×10^4	9.0×10^4
Progesterone		3-enol 2.9×10^3	3-enol 2.6×10^4	9.6×10^4
Androsterone		3-hydroxy, mono 1.7×10^2	—	1.5×10^4
Pregnenolone		3-hydroxy, mono 0.87×10^2	—	4.2×10^4

reported values for steroidal heptafluorobutyrates and O-pentafluorobenzyloximes. These three underivatized steroids have higher sensitivity than the values reported by Exley and Chamberlain[9] for heptafluorobutyrates and close to the values reported by Dehennin and Scholler[10]. The extremely high sensitivity of MGA to ECD was, as reported earlier, made use of in the analysis of cattle feed supplements and residual level determination in bovine tissues.

Mechanism of electron capture by MGA

It was of interest to calculate the value of the electron capture coefficient, K, for MGA and to determine the mechanism of electron capture, *i.e.* whether it was of the dissociative or nondissociative type. The values of K were calculated from eqn. 2. A plot of $(I_b - I_e)/(I_e)$ *versus* S was not linear even though other plots of response factors (peak heights, peak areas or C/mole) *versus* concentrations were linear up to 1.6 pmoles injected onto the column. Because of this deviation, the values for the electron capture coefficient were not constant; they varied from 3–7.6 $\times$ 10^{10} l/mole. In a recent paper on the theory and practice of the ECD, Lovelock[11] has stated that the well-known Wentworth equation, which served so well in the early days when less efficient ionization sources were used, does not apply when nearly all of the sample input is ionized. He has also shown that small changes in either the flow-rate of the carrier gas or the ionization current can significantly affect the proportion of molecules ionized and, hence, the detector response factor. These factors may explain the reason for the variation in the value of K.

The electron capture coefficient for MGA was independent of the detector temperature from 260–360°. The mechanism of electron capture is usually determined from a plot of ln $KT^{3/2}$ *versus* $1/T$. For MGA, such a plot had a zero slope and therefore the mechanism of electron capture did not clearly fall in the category of the nons dissociative or the dissociative type. This temperature independency also suggest-low activation energy for MGA-electron reaction in the detector cell.

CONCLUSIONS

A number of biologically active substituted 17α-acetoxyprogesterones were found to respond to a ^{63}Ni GC–ECD in their underivatized forms. The response was fair for 17α-acetoxyprogesterone (II) and excellent for 6-dehydro-6-methyl-16-methylene-17α-acetoxyprogesterone (XIX). The following structure–electron capture response relationships were established:

(1) 17α-acetate was an important functionality as progesterone showed practically no response to ECD (5 ng on-column).

(2) α- or β-methyl substituents in the A and B rings (2, 6, and 7 positions) had no effect in 4-en-3-one and 4,6-dien-3-one systems.

(3) 1,4-dien-3-one and 4,9–11-dien-3-one systems were better electron absorbers than 4-en-3-one systems, but 4,6-dien-3-one compounds were definitely more sensitive to ECD.

(4) C-16 α-methyl substitution doubled and tripled the sensitivity of the 4-en-3-one and 4,6-dien-3-one acetoxyprogesterones, respectively.

(5) C-16 methylene substitution gave the greatest increment in sensitivity of all groups tested. It increased the sensitivity of 17α-acetoxyprogesterone fourteen

times and that of Δ^6-17α-acetoxyprogesterone seven times. C-16 methylene-17α-acetoxyprogesterone was twice as sensitive as C-16 methyl-17α-acetoxyprogesterone. Similarly, C-16 methylene-Δ^6-17α-acetoxyprogesterone was twice as sensitive as C-16 methyl-Δ^6-17α-acetoxyprogesterone. The most sensitive compound in the series was 6-dehydro-6-methyl-16-methylene-17α-acetoxyprogesterone (melengestrol acetate). Its sensitivity was about the same as the steroid heptafluorobutyrates reported in the literature.

(6) A keto group at C-6 was an effective contributor to a 4-en-3-one system. Its sensitivity was 25 times greater than 17α-acetoxyprogesterone.

ACKNOWLEDGEMENTS

The author wishes to thank J. A. Campbell of The Upjohn Company for helpful suggestions in the selection of the steroids and in the preparation of the manuscript.

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