DRUG DEVELOPMENT AND INDUSTRIAL PHARMACY, 5(3), 263-275 (1979)

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GAS CHROMATOGRAPHIC DETERMINATION OF DEGRADATION PRODUCTS AND STABILITY OF DEHYDROEPIANDROSTERONE SULFATE IN AQUEOUS SOLUTION*

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ABSTRACT

A method for the quantification of the degradation products in aqueous solution of sodium dehydroepiandrosterone sulfate (DHA•S) using gas chromatography was described.

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*This paper constitutes Part IV of the series entitled "Pharmaceutical Studies of Dehydroepiandrosterone Sulfate." Part III : S. Maeda, M. Matsumoto and I. Sugimoto, Iyakuhin Kenkyu, 9, 98(1978). A part of this work was presented at the 3rd Conference on Pharmaceutical Technology, Shirakabako, Aug., 1978.

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One of the degradation products, 3,5-cycloandrostan-6β-ol-17-one, which was unstable in GLC column, was readily transformed into the trimethylsilylether derivative exhibiting good gas chromatographic properties. The kinetics of the degradation of sodium dehydroepiandrosterone sulfate solution to dehydroepiandrosterone and 3,5-cycloandrostan-6β-ol-17-one was studied from pH1.3 to pH11.0. There was only one degradation product, dehydroepiandrosterone, in very strongly acidic solution, but, in weakly acidic and neutral solution, dehydroepiandrosterone and 3,5-cycloandrostan-6β-ol-17-one were identified and in the more basic solution, 3,5-cycloandrostan-6β-ol-17-one was the main degradation product. It was found that, at pH5.2, DHA•S followed a series reaction, DHA•S ⟶ 3,5-cycloandrostan-6β-ol-17-one ⟶ dehydroepiandrosterone.

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INTRODUCTION

It is expected in the field of obstetrics that dehydroepiandrosterone sulfate (DHA•S) may make the soft birth canal favorable for delivery through an activation of tissue collagenase which is induced by 17β-estradiol formed from DHA•S(1).

In the previous paper(2), it was reported the stability of DHA•S in aqueous solution followed an apparent first-order reaction. The degradation products were dehydroepiandrosterone (DHA) and androsta-3,5-dien-17-one(I) in acidic solution, 3,5-

cycloandrostan-6β-ol-17-one(II) and DHA in neutral and basic solutions. In strongly acidic solution, DHA•S was very rapidly decomposed. In neutral and basic solutions, DHA•S degraded more slowly than in strongly acidic solution.

Many conjugated steroids have been separated and quantified by GLC and HPLC after the acid-catalyzed solvolysis(3-9). Those methods measured DHA, solvolysis product of DHA•S, in urine, plasma or aqueous solution. But it was not possible to quantitate DHA, I and II in aqueous solution at the same time by those methods.

This paper presents a sensitive and selective method for the quantification of DHA, I and II in aqueous solution. In the case of DHA and I, the method was based on chloroform extraction and gas chromatographic determination. In the case of DHA and II, the method was based on a derivatization of DHA and II with bis(trimethylsilyl)-acetamide (BSA) to their BSA ether derivatives and gas chromatographic determination. Further the kinetics of degradation of DHA•S in aqueous solution was reported.

EXPERIMENTAL

Materials —— DHA•S, I and II were synthesized and purified by the previous report(2). DHA(Organon oss. Holland) and pregnenolone (E.Merk, Darmstadt) were obtained commercially. Their identity and purity were checked by TLC and GLC. BSA(E.Merk,

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Darmstadt) was reagent grade for GLC. All other materials were reagent grade and deionized water was used throughout this study.

Internal Standard Solution —— Dissolve 50mg of pregnenolone accurately weighed and dilute with $CHCl_3$ to volume in a 100ml volumetric flask.

Gas chromatography —— The assay was performed on a gas chromatograph(Hitachi, model 063) equipped with a flame-ionization detector. A glass coiled column, 2.0mx3.0mm o.d., was packed with 2% OV-1 on 80-100 mesh Gas Chrom Q, conditioned at 300° overnight and injected periodically with trimethylsilylether derivatives with BSA. Nitrogen pressure as a carrier gas was maintained at $1.3kg/cm^2$, the pressures of air and hydrogen were 1.0 and $2.0kg/cm^2$, respectively. The injector and detector temperatures were both maintained at 240° throughout the procedure.

Kinetic Studies —— A 200ml volumetric flask containing a approximately 190ml of appropriate buffer was placed in a constant temperature bath kept at 39.7±0.2°. After thermal equilibrium had been attained, 200mg of DHA•S was added and dissolved. The solution was made to volume with buffer and again rendered homogeneous. At appropriate intervals, 5ml of sample solution was withdrawn and the concentrations of undegraded DHA•S and degraded products were determined.

Buffer Composition —— Buffers were prepared with reagent grade chemicals and they contained the following fraction per liter. pH1.3 : 1N HCl 77.4ml, NaCl 0.88g and KCl 4.47g pH2.5 : 0.2M HCl 5ml and KCl 11.36g pH4.0 : 1N CH_3COOH 120ml, CH_3COONa•

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$3H_2O$ 2.72g and KCl 0.75g, pH4.7 : 1N CH_3COOH 50ml, $CH_3COONa \cdot 3H_2O$ 6.8g and KCl 3.7g, pH5.2 : 1N CH_3COOH 30ml, $CH_3COONa \cdot 3H_2O$ 13.88g and KCl 1.34g, pH6.0 : KH_2PO_4 10.89g, $Na_2HPO_4 \cdot 12H_2O$ 4.77g and KCl 2.25g, pH7.2 : KH_2PO_4 3.13g, 1N NaOH 13.6ml and NaCl 0.05g, pH9.5 : 1N NaOH 13.0ml, H_3BO_4 1.24g and NaCl 1.14g, pH11.0 : $Na_2HPO_4 \cdot 12H_2O$ 17.82g and 1N NaOH 5ml. A constant ionic strength of 0.15 was maintained by adding KCl and/or NaCl.

Assay Procedure of DHA•S —— DHA•S was assayed by the previous paper(2).

Chromatographic Methods

Method 1 —— Five milliliters of sample solution was pipetted into a 50ml separatory funnel. Exactly 20ml of $CHCl_3$ was added and the funnel was shaken, the layers were allowed to separate. After triple extraction with $CHCl_3$, the $CHCl_3$ layer was dried with anhydrous sodium sulfate, and evaporated under vacuum. The residue was dissolved in 1ml of internal standard solution. Then 1.0μl of the solution was injected into the gas chromatograph.

Method 2 —— Five milliliters of sample solution was pipetted into a 50ml separatory funnel. Exactly 20ml of $CHCl_3$ was added and the funnel was shaken, the layers were allowed to separate. After triple extraction with $CHCl_3$, the $CHCl_3$ layer was dried with anhydrous sodium sulfate and evaporated under vacuum. One milliliter of internal standard solution was added to the residue and again evaporated to dryness. The residue was

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redissolved in 100μl of BSA. After this mixture was allowed in a boiling water bath for 30min, then 1μl of the solution was injected into the gas chromatograph.

In the acetate buffer, the extracted $CHCl_3$ layer was neutrified with Na_2CO_3, washed with water and evaporated under vacuum. The residue was treated with the manner as described above and the resulting solution was applied to the gas chromatograph.

Results and Discussion

Determination Method

Separation —— A typical gas chromatogram of DHA(t_R=9.0min), I(t_R=5.4min) and the internal standard (t_R=14.4min) by method 1 is shown in Figure 1. Figure 2 shows a typical gas chromatogram of DHA-BSA(t_R=6.0min), II-BSA(t_R=11.1min) and internal standard-BSA (t_R=18.0min) ethers by method 2.

By preliminary experiment, there were four peaks in the gas chromatogram of II by method 1. It might be unstable in the column at 240°. So it was attemped to derive II to trimethylsilylether derivative with BSA. At first, II did not sufficiently react with BSA in the presence of organic solvent, that is, $CHCl_3$, ethyl acetate or pyridine, even on heating the reaction mixture in a boiling water bath for 30min, while DHA and pregnenolone(internal standard) reacted easily. Accordingly, vigorous investigations were carried out to develop a suitable condition. When directly dissloved II in BSA, heating in a boil-

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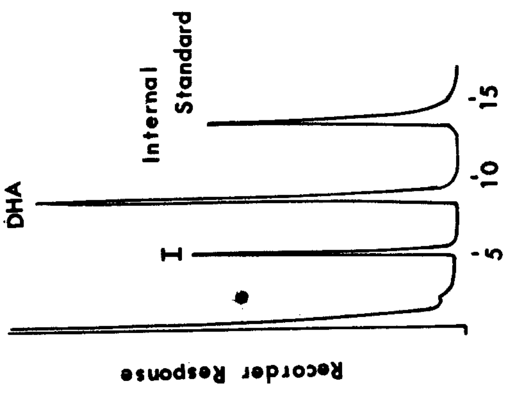

FIGURE 1

Gas chromatogram of DHA and I

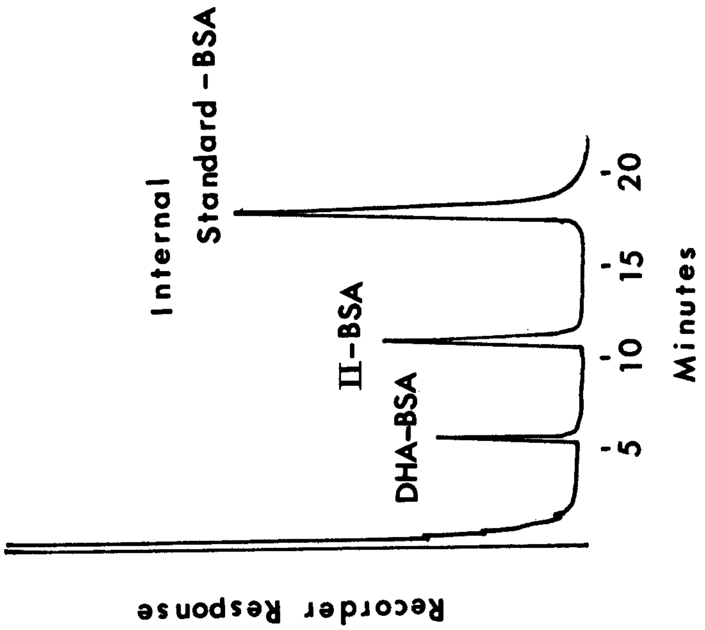

FIGURE 2

Gas chromatogram of DHA-BSA, II-BSA and Internal Standard-BSA Ethers ing water bath for approximately 15min, one sharp peak exhibiting good gas chromatographic properties was obtained.

Calibration Graphs —— The calibration graphs obtained with 0.05-2.0mg of DHA, I or II in 1ml of $CHCl_3$ are shown in Figure 3. All of the graphs were straight lines and passed through the origin. A correlation coefficient was 0.991 or better.

Recoveries of DHA and I in methanol mixture solution were 93-101% and 93-100%, respectively by method 1 and those of DHA and II in methanol mixture solution were 96-103% and 89-104%, respectively by method 2.

Stability in Aqueous Solution —— In the previous paper, it was found that the degradation of DHA•S followed the apparent first-order reaction(2). As it became possible to assay the degradation products as metioned before, those products and residual DHA•S were determined at appropriate time intervals in the present study. Figure 4 shows the plots of DHA•S and its degredation products at 40° over the 3.1-11.0 pH range. At pH3.1, DHA was only one degradation product, but at pH3.9 or weakly acidic solution, the mechanism of degradation was more complex. At those solutions DHA and II were identified. At neutral or weakly basic solution, II was the main product. Inspection of these plots(Figure 4) indicated that the reaction might be fitted to a series first-order kinetics.

Accordingly, the following reaction is taken into account:

$$\mathrm{DHA \cdot S} \xrightarrow{k_1} \mathrm{II} \xrightarrow{k_2} \mathrm{DHA}$$

where k_1 and k_2 are apparent first-order rate constants.

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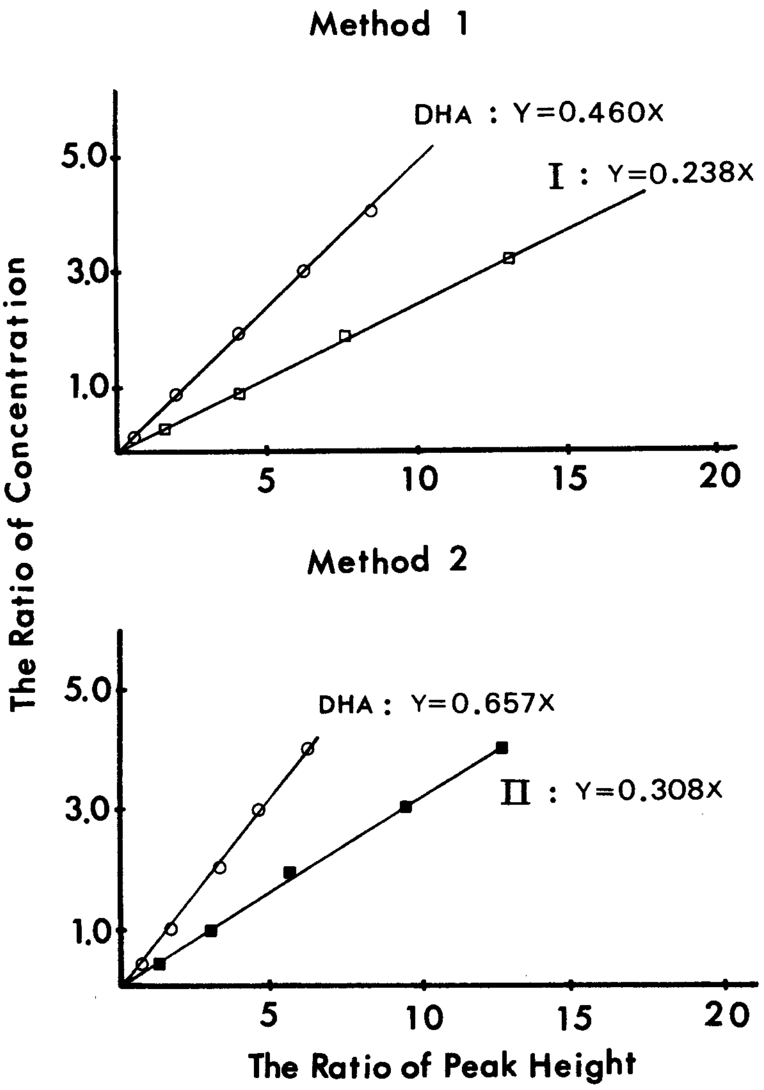

FIGURE 3

Calibration graphs for DHA, I and II

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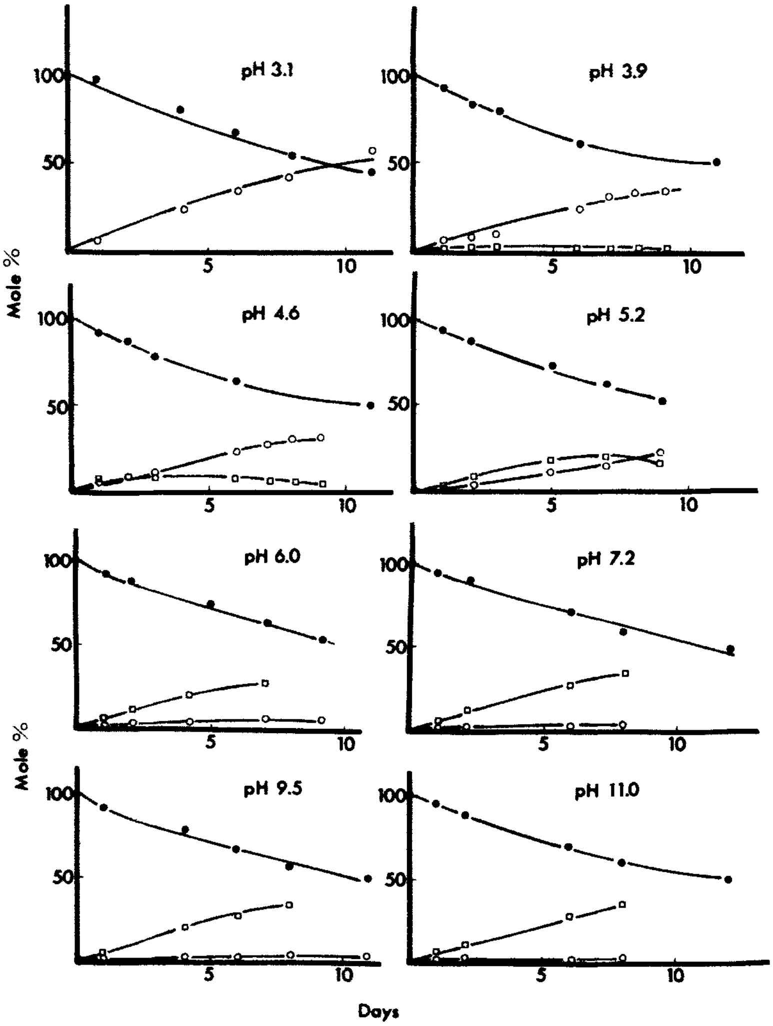

FIGURE 4

Stability of DHA•S at 40° at various pH values
—— ● —— DHA•S, —— o —— DHA, ——□—— II

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When the rate constants for the reaction at pH5.2 were calculated assuming a series first-order kinetics, k_1 and k_2 were calculated to be 0.068 day^{-1} and 0.20 day^{-1}, respectively (*Figure 5*).

Further to confirm the series reaction, the stability of II in pH5.2 buffer solution was examined. The reaction product of II was only DHA, the reaction followed apparent first-order kinetics and the apparent rate constant was 0.16 day^{-1}. This rate constant for II $\longrightarrow$ DHA fairly coincided with k_2 (0.20 day^{-1})

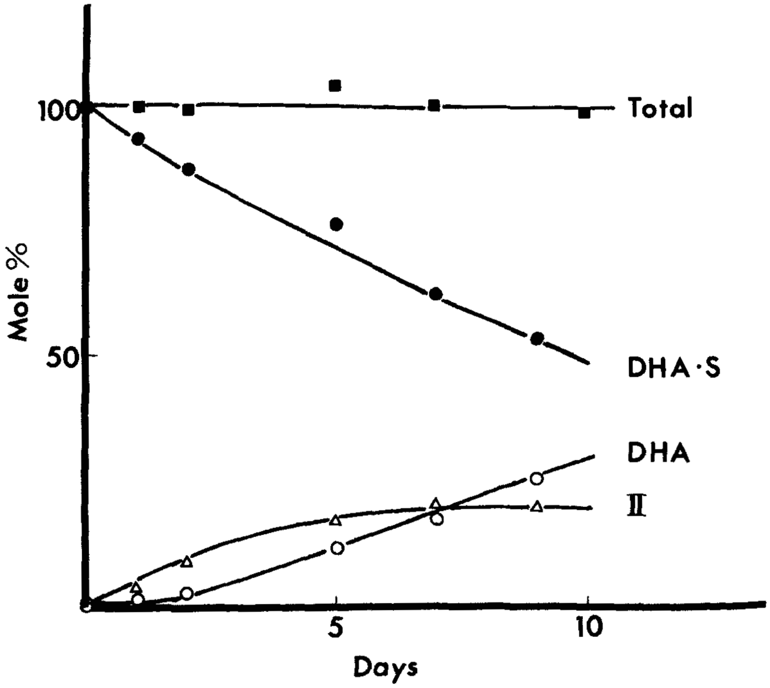

FIGURE 5

Series First-Order Reaction of DHA·S at pH 5.2 and 40°

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calculated assuming a series first-order reaction as mentioned before. Total stoichiometric concentration of DHA•S and its degradation products, II and DHA, was constant during experiment as shown in Figure 5, so it was confirmed that there was no other degradation product in this reaction. Figure 5 was the theoretical curves of the concentration of DHA•S, II and DHA as a function of the time at pH5.2 where k_1=0.068 day^{-1} and k_2=0.20 day^{-1}. These theoretical curves were compared with experimental data points in Figure 5.

The goodness of fit suggested that the reaction followed a series first-order reaction, DHA•S $\longrightarrow$ II $\longrightarrow$ DHA, at pH5.2.

ACKNOWLEDGMENTS

The authors express their deep gratitude to Dr. I. Utsumi, director of this center and are grateful to Miss Y. Nomada for assistance in this study.

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