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STUDIES ON STEROIDS

CIII. A NEW TYPE OF DERIVATIVE FOR ELECTRON CAPTURE–GAS CHROMATOGRAPHY OF KETOSTEROIDS

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SUMMARY

Pentafluorobenzyloxyamine is presented as a new derivatizing agent for gas chromatography of ketones using electron capture detection. The typical 17-ketosteroid was readily transformed into the O-pentafluorobenzyloxime which on usual trimethylsilylation led to a 3-trimethylsilyl ether derivative exhibiting good gas chromatographic properties. The derivatization procedure was applied to the determination of dehydroepiandrosterone in human plasma by electron capture–gas chromatography employing an internal standard method and in consequence satisfactory results were obtained.

INTRODUCTION

A variety of derivatizing agents such as alkoxyamine, alkyl- and arylhydrazine have previously been proposed for gas–liquid chromatography of the carbonyl compounds[1–13]. From the structural requirement for electron capturing properties, pentafluorophenylhydrazine has also been devised as a sensitive reagent[14,15]. However, the nature of the condensation products is not always satisfactory with respect to their thermal stability. The necessity for gas chromatographic determination of the ketosteroids in the biological fluid prompted us to develop a suitable derivatizing agent. The present paper deals with the synthesis of a new reagent, pentafluorobenzyloxyamine, and its application for the quantitation of dehydroepiandrosterone in human plasma.

EXPERIMENTAL

Synthesis of derivatizing reagent

N-(2,3,4,5,6-Pentafluorobenzyloxy)phthalimide (II). To a stirred solution of

N-hydroxyphthalimide (I)[16] (2 g) and triethylamine (10 ml) in dimethylformamide (10 ml) was added dropwise 2,3,4,5,6-pentafluorobenzyl bromide (2 g) under ice-cooling, and the resulting solution was stirred at room temperature for 1 h. The reaction mixture was poured into ice-water (100 ml) and the precipitate was collected by filtration and washed with water. Recrystallization from aqueous ethanol gave II (2.2 g) as colourless needles (m.p. 145–146°; analysis: calculated for $C_{15}H_6NO_3F_5$: C, 52.59; H, 1.76; N, 4.08; found: C, 52.61; H, 2.07; N, 4.20).

2,3,4,5,6-Pentafluorobenzyloxyamine hydrochloride (III). A solution of II (2 g) dissolved in 6 *N* HCl (30 ml)–glacial acetic acid (30 ml) was heated at 100° for 2 h. The resulting solution was evaporated *in vacuo* and the residue obtained was dissolved in 10 ml water and filtered. The filtrate was evaporated to dryness and the residue was kept in a desiccator overnight. The pale brown cake thus obtained was recrystallized from ethanol to give III (0.7 g) as colourless leaflets (m.p. 115–116°; analysis: calculated for $C_7H_5NOF_5Cl$: C, 33.69; H, 2.02; N, 5.61; found: C, 34.13; H, 2.38; N, 5.76).

Materials

The steroid samples were donated by Teikoku Hormone (Tokyo, Japan). 2,3,4,5,6-Pentafluorobenzyl bromide was purchased from Aldrich (Milwaukee, Wisc., U.S.A.) and other reagents from Tokyo Kasei (Tokyo, Japan).

Gas chromatography

The apparatus used for this work was a Shimadzu Model GC-4BMPFE gas chromatograph equipped with ^{63}Ni electron capture and hydrogen flame ionization detectors and a U-shaped glass column (3 mm I.D.). The column was packed with 1% OV-1 or 2% OV-17 on a support of Gas-Chrom Q (80–100 mesh). The detector and flash heater were kept at 240°, while the column temperature was 220°. Nitrogen was used as a carrier gas at a flow-rate of 60 ml/min.

Preparation of derivatives

To a solution of the 17-ketosteroid (*ca.* 1 μg) in pyridine (2 drops) was added pentafluorobenzyloxyamine hydrochloride (III) (0.2 mg) and heated at 60° for 1 h. The reaction mixture was diluted with hexane (3 ml), washed with water (1 ml), 0.1 *N* HCl (1 ml), 0.1 *N* NaOH (1 ml), and water (1 ml), successively, and centrifuged. After evaporation of solvent, the residue was treated with hexamethyldisilazane (0.1 ml) and trimethylchlorosilane (0.1 ml) in pyridine (5 drops) according to the procedure of Sweeley *et al.*[17]. On usual work-up the residue was dissolved in hexane (1 ml) and a 2 μl aliquot was injected into the gas chromatograph.

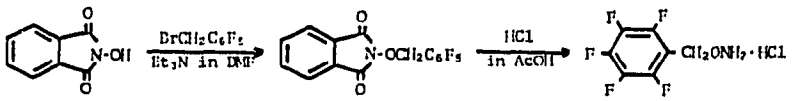
Determination of dehydroepiandrosterone in human plasma

To a 0.5 ml aliquot of plasma sample was added ethanol (3 ml), stirred for 10 min, and centrifuged. The supernatant liquid was transferred to a test tube and the precipitate was rinsed with ethanol (2 ml). The supernatant liquid and washings were combined and evaporated under a stream of nitrogen gas. To the residue dissolved in a NaCl-saturated solution (1 ml) was added 5 *N* H_2SO_4 (1 drop) and extracted with ethyl acetate (3 ml) twice. The organic layer was incubated at 37° for 48 h. To this solution was added a known amount of epiandrosterone acetate (*ca.* 1 μg) dissolved

in hexane, washed with 5% $NaHCO_3$ and water, and evaporated. The residue was treated in the manner as described above and the resulting dehydroepiandrosterone O-pentafluorobenzyloxime-trimethylsilyl derivative was applied to gas chromatography.

RESULTS AND DISCUSSION

An initial effort was directed to the synthesis of a new reagent to possess both the stability of the O-benzyloxime and the electron capturing property of the pentafluorobenzyl system. Condensation of pentafluorobenzyl bromide with N-hydroxyphthalimide (I) in the presence of triethylamine proceeded readily resulting in formation of N-(pentafluorobenzyloxy)phthalimide (II). Subsequent hydrolysis with hydrochloric acid provided the desired pentafluorobenzyloxyamine hydrochloride (III) in moderate yield.



I II III

Derivatization into the O-pentafluorobenzyloxime was easily attained in the usual manner without the formation of any undesirable by-products. The resulting oximes derived from the commonly occurring 17-ketosteroids sometimes showed tailing to a small extent due to the irreversible adsorption of the free hydroxyl group. This problem could be solved, however, by subsequent trimethylsilylation using the method of Sweeley *et al.*[17]. Thus, the typical 17-ketosteroids were transformed into the corresponding 17-O-pentafluorobenzyloxime 3-trimethylsilyl ethers, and these gave a single peak of the correct theoretical shape in the gas chromatograph. The 11-keto group failed to react with the new derivatizing agent under the conditions

TABLE I

RELATIVE RETENTION TIMES OF 17-KETOSTEROID O-PENTAFLUOROBENZYLOXIME-TRIMETHYLSILYL DERIVATIVES

Conditions: U-shaped glass column (3 mm I.D.): nitrogen flow-rate, 60 ml/min; hydrogen flame ionization detector.

Compound	*1% OV-1** *(1.0 m)*	*2% OV-17*** *(2.0 m)*
Dehydroepiandrosterone	2.71	2.87
Androsterone	2.15	2.05
Ethiocholanolone	2.12	2.07
11β-Hydroxyandrosterone	3.36	3.98
11β-Hydroxyethiocholanolone	3.23	3.88
11-Ketoandrosterone	3.00	3.38
11-Ketoethiocholanolone	2.89	3.20
Cholestane	1.00 (3.54 min)	1.00 (7.90 min)

* Column temperature 220°, injection chamber and detector temperature 240°.
** Column temperature 250°, injection chamber and detector temperature 270°.

hereby employed due to steric hindrance. The retention times relative to cholestane obtained on the OV-1 and OV-17 columns are collected in Table I.

This promising derivatization method was then applied to the determination of dehydroepiandrosterone in human plasma. As expected, the 17-O-pentafluorobenzyloxime showed a highly sensitive response on the electron capture detector. The calibration curve was constructed by plotting the ratio of the peak area of dehydroepiandrosterone to that of the internal standard (epiandrosterone acetate) against the weight ratio of the two; satisfactory linearity was observed in the range 0.6–3.0 ng of dehydroepiandrosterone (see Fig. 1).

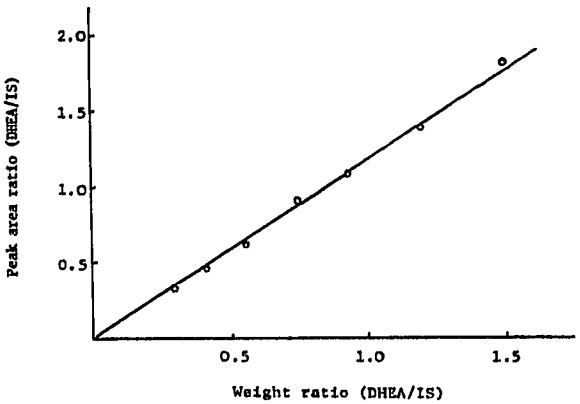


Fig. 1. Calibration curve for dehydroepiandrosterone (DHEA). Internal standard (IS) = epiandrosterone acetate.

Examinations were then made of the recovery rate of dehydroepiandrosterone sulfate added to human plasma. A plasma sample was treated with ethanol for deproteinization and extraction, and then solvolyzed with sulfuric acid in ethyl acetate[18]. The deconjugated dehydroepiandrosterone was separated, transformed into the oxime-trimethylsilyl derivative, and determined by the internal standard method in the manner previously established. A typical gas chromatogram of dehydroepiandrosterone in human plasma, together with the internal standard, is illustrated in Fig. 2. One microgram of dehydroepiandrosterone sulfate added to 0.5 ml of plasma was satisfactorily recovered as listed in Table II. With a human plasma sample, the reproducibility of the present method for quantitation of dehydroepiandrosterone was examined also. As can be seen in Table III the amount of dehydroepiandrosterone was determined with a satisfactory reproducibility.

It has been demonstrated that the new reagent, pentafluorobenzyloxyamine hydrochloride, reacts readily with the ketosteroid to form the oxime, whose response on the electron capture detector is remarkably sensitive. Introduction of the pentafluorobenzyl moiety that confers the electron capturing property on the molecule also serves to clean up the ketones in the biological material. An additional advantage of this reagent lies in the higher thermal stability of the resulting oxime. Furthermore, an excess of the reagent can be easily eliminated by washing with acid prior to injection in the gas chromatograph. The relative ease with which the unreacted re-

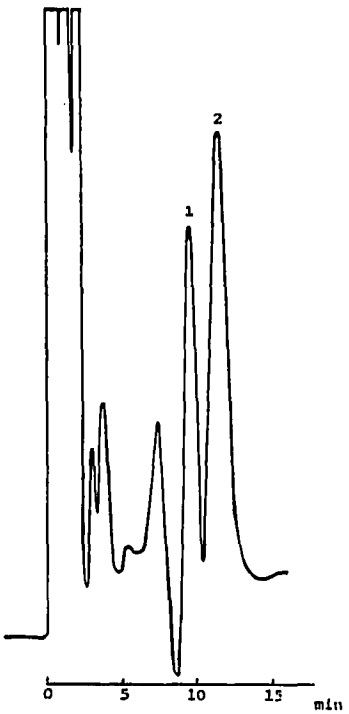


Fig. 2. Gas chromatogram of dehydroepiandrosterone extracted from human plasma. 1 = O-Pentafluorobenzyloxime-trimethylsilyl ether derivative of dehydroepiandrosterone, 2 = O-pentafluorobenzyloxime derivative of epiandrosterone acetate (internal standard). Conditions: U-shaped glass column (1.5 m × 3 mm I.D.) packed with 1% OV-1 on Gas-Chrom Q (80–100 mesh); column temperature, 220°; injection chamber temperature, 240°; detector temperature, 240°; nitrogen flow-rate, 60 ml/min.

TABLE II

RECOVERY TEST FOR DEHYDROEPIANDROSTERONE SULFATE ADDED TO HUMAN PLASMA

Amount added	*Experiment*					*Mean*
(μg)	*1*	*2*	*3*	*4*	*5*	
1.028	0.883 (85.9)*	0.877 (85.3)	0.847 (82.4)	0.866 (84.2)	0.858 (83.5)	0.869 (84.5)

* The figures in parentheses represent the recovery rate (%).

TABLE III

REPRODUCIBILITY OF THE METHOD FOR DETERMINATION OF DEHYDROEPIANDROSTERONE IN HUMAN PLASMA

Amount found	*Experiment*							*Mean (± standard deviation)*
(μg)	*1*	*2*	*3*	*4*	*5*	*6*	*7*	
	1.36	1.30	1.45	1.24	1.46	1.39	1.32	1.36 ± 0.08

agent can be removed is favorable for electron capture–gas chromatography. The potential utility as a derivatizing reagent will be helpful for identification and determination of the ketones, and in particular the ketosteroids from biological sources.

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