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DIMETHYLTHIOPHOSPHINIC ESTERS FOR THE GAS CHROMATOGRAPHIC DETERMINATION OF MONOHYDROXY-STEROIDS WITH THE ALKALI FLAME DETECTOR

VI. STEROID PHOSPHORUS COMPOUNDS

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SUMMARY

Dimethylthiophosphinic chloride has been found to react readily at 70–90° with monohydroxy-steroids to yield dimethylthiophosphinic esters. These derivatives have good gas chromatographic properties and can be detected at the lower picogram level (10–15 pg) with the alkali flame detector. The reaction kinetics were examined, and depend on steric influences and the chemical nature of the hydroxyl group. The esters are stable to heat and hydrolysis, and since their formation is highly reproducibile and they offer a very low detection limit, they are suitable for gas–liquid chromatographic determination of various compounds of biological interest.

INTRODUCTION

We recently reported a new, sensitive and selective gas chromatographic (GC) determination of phosphorus-containing derivatives of monohydroxy-steroids with the alkali flame detector (AFD)[1,2]. This method involves formation of steroidal dimethylphosphinic esters with dimethylamino dimethylphosphine as phosphinylating reagent. A few other phosphorus-containing compounds of biological interest have been determined with the AFD[3–7]. One inconvenience of our procedure was the time-consuming synthesis of the derivatization reagent and its poor stability to moisture and air. A more readily available and more stable phosphorus-introducing reagent, with comparable reactivity, which might lead to derivatives with similarly good GC properties and detection limits, was therefore sought. Of all the compounds studied, the best results were obtained with dimethylthiophosphinic chloride.

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EXPERIMENTAL

Reagents and materials

All reagents and solvents were of analytical grade and were supplied by E. Merck (Darmstadt, G.F.R.), unless otherwise specified. Dimethylthiophosphinic chloride (Riedel de Haën, Seelze, G.F.R.) reagent grade, was used as a 1% solution in acetonitrile, which was dried over a molecular sieve (4 Å). Triethylamine was used as a 0.8% solution in dried acetonitrile.

Thin-layer chromatographic (TLC) plates, Kieselgel 60, were obtained from E. Merck. Tritium-labelled steroids (1,2-$^3H_2$, 40–80 Ci/mmole, except for estrone, which was 6,7-$^3H_2$) were purchased from New England Nuclear Chemicals (Dreieichenhain, G.F.R.).

The following trivial names and abbreviations are used:

Deoxycorticosterone (DOC): 21-hydroxy-4-pregnene-3,20-dione

Dihydrotestosterone (DHT): 17β-hydroxy-5α-androstan-3-one

Estrone (E_1): 3-hydroxy-1,3,5(10)-estratrien-17-one

3α-Etiocholanolone (3α-Et): 3α-hydroxy-5β-androstan-17-one

Testosterone (T): 17β-hydroxy-4-androsten-3-one

PS stands for the dimethylthiophosphinic ester group of the corresponding hydroxy-steroid.

Apparatus

Gas chromatograph Model 1440 (Varian, Palo Alto, Calif., U.S.A.) was used, with injector temperature 270°, a 3-ft. glass column (O.D. 1/4 in., I.D. 2 mm) containing 3.5% OV-17 or 2.5% OV-1 on Anakrom Q, coated according to ref. 8, helium carrier gas (flow-rate 30 ml/min), and isothermal column temperature 280°. The AFD (rubidium sulphate), used hydrogen (flow-rate 35–40 ml/min) and air (flow-rate 200–300 ml/min) at a temperature of 285°.

Other apparatus was an Eluchrom® (Camag, Muttenz, Switzerland), a radio-TLC scanner (Berthold, Wildbad, G.F.R.) and a liquid scintillation counter LKB 8000 (LKB-Producter, Bromma, Sweden).

Method

The hydroxy-steroid (nanogram amounts) together with the radioactive tracer (*ca.* 30,000 dpm) is treated with 25 μl of dimethylthiophosphinic chloride solution and 25 μl of triethylamine solution for 1–3 h at 90° under nitrogen. The volatile components are removed *in vacuo* (*ca.* 0.4 Torr). The residue is dissolved in 100 μl of methanol and 30 mg of $NaHCO_3$ are added. The mixture is treated on a vortex mixer and incubated for 30 min at 50°. The supernatant is transferred to a TLC plate and developed with chloroform–ethyl acetate (4:1, v/v). The derivative is localized with the radio-TLC scanner and eluted with methanol on the Eluchrom. The eluate is dried under a stream of nitrogen and dissolved in acetone. An aliquot is used for determination of the recovery rate in the liquid scintillation counter and 1–2-μl samples are injected into the gas chromatograph together with an appropriate internal standard.

RESULTS AND DISCUSSION

Dimethylthiophosphinic chloride reacts with hydroxy-steroids in presence of triethylamine to the corresponding dimethylthiophosphinic esters according to eqn.1

$$ROH + ClP(CH_3)_2 \xrightarrow{N(C_2H_5)_3} ROP(CH_3)_2 + HCl \quad (1)$$

The structures of these derivatives were confirmed by ^1H, ^{31}P and ^{13}C nuclear magnetic resonance spectroscopy, infrared spectroscopy, and mass spectrometry (MS)[9].

Reaction kinetics

In our examination of the reaction kinetics we used tritium-labelled steroids (500,000 dpm ≙ 1.7 ng). The tracer was diluted with 500 ng of unlabelled steroid to obtain concentrations comparable to those used in the analytical reaction. The reaction conditions used in these experiments were the same as described above, with the exception of reaction time and temperature. Derivatives, by-products and starting materials were localized and quantified with the radio-TLC scanner.

The reaction rate clearly depends on the steric arrangement and the chemical nature of the hydroxyl group (Figs. 1 and 2), particularly at low temperatures (Fig.1). As expected we found a higher conversion rate for the relatively acidic, phenolic hydroxyl group of E_1 and the primary 21-hydroxyl group of DOC compared with the secondary hydroxyl groups of 3α-Et, DHT and T. The equatorial hydroxyl group of 3α-Et is in a sterically favoured position compared with 3β-etiocholanolone. It reacts therefore more rapidly than the axial one (not drawn in the figures). The 17β-

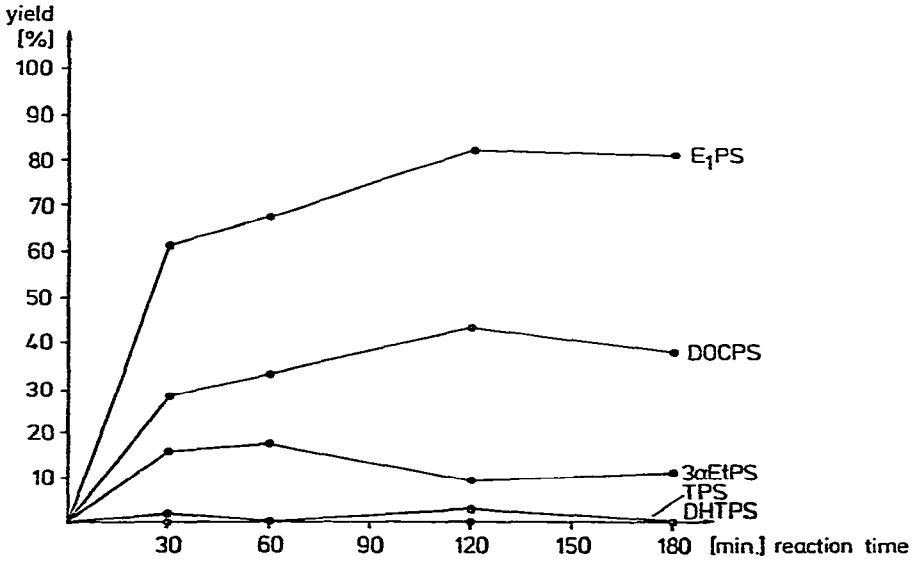

Fig. 1. Kinetics of the reaction of dimethylthiophosphinic chloride with dihydrotestosterone (DHT). deoxycorticosterone (DOC), estrone (E_1), 3α-etiocholanolone (3α-Et) and testosterone (T) at 25°.

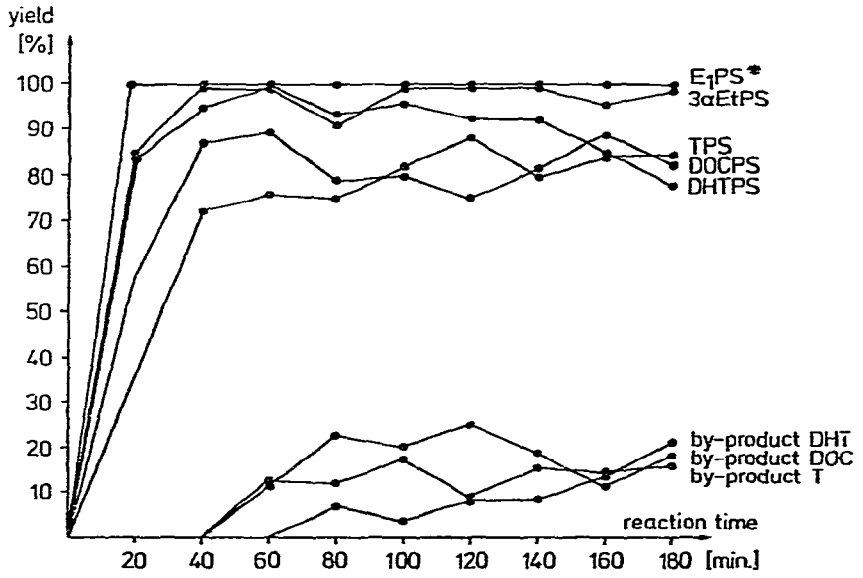

Fig. 2. Kinetics of the reaction of dimethylthiophosphinic chloride with DHT, DOC, E_1, 3α-Et and T at 90°. For abbreviations see Fig. 1. *In this experiment a ten-fold higher concentration of the derivatization reagent was used.

hydroxyl groups of DHT and T are moderately hindered by the angular methyl group (C-18). These results are in accordance with those obtained by silylation of different reactive hydroxyl groups using silylating agents with varying silylating potentials[10].

With respect to the yield of the individual thiophosphinic steroidal esters the optimum reaction conditions depend on time and temperature. Side reactions are observed in the case of T, DHT and DOC, especially at higher temperatures and longer reaction times. This reduces slightly the yield of the respective dimethylthiophosphinic esters. The by-products of T and DHT were examined by combined GC–MS and identified as the corresponding dehydrated steroids.

Optimum reaction conditions for the individual steroids are as follows: DHT, 70° for 180 min; DOC, 70° for 180 min (or 90° for 60 min); E_1, 90° for 60 min; 3α-Et, 90° for 60 min; and T, 90° for 120 min.

Removal of interfering by-products

Separation of the derivatives from the free steroids was only necessary in the kinetic studies and could be achieved very simply by TLC with the solvent system described.

Treatment of the reaction products with methanol–sodium bicarbonate was necessary, because the by-product dimethylthiophosphinic anhydride formed could not be removed satisfactorily from the reaction mixture. Methanolysis of the anhydride is complete within 30 min at 50°. Traces of non-steroidal phosphorus-containing reaction products could not be removed totally by the TLC step. These compounds are eluted from the GC column with the solvent front, but they cause a weak tailing, which may affect the detection limit.

Thermal and hydrolysis stability

The thermal stability of the esters is good and the stability against hydrolysis is extremely so, as they did not react during a 3-h treatment with aqueous dioxane at 95°. The crystallized esters can be stored in screw-cap vials without decomposition for more than a year.

Reproducibility of the reaction

The applied amount of steroid correlates strongly with the observed peak height of the derivative. Calculation of the correlation coefficient of the reference curve gave a value of 0.99 using 3α-Et.

The precision was determined with 500 ng of steroid (3α-Et) in the reaction mixture; the reproducibility was 4.4% ($n = 9$). The mean recovery of the overall procedure was greater than 70%.

Gas chromatographic properties and detection limit

The dimethylthiophosphinic esters of the monohydroxy-steroids are eluted after the non-esterified steroids on OV-1 and OV-17. The retention times are prolonged with a factor of 3.1–3.5 on OV-1 and 3.6–4.2 on OV-17, relative to the hydroxy-steroids at isothermal conditions. The gas–liquid chromatographic (GLC) peaks exhibited very little tailing (Fig. 3).

For the dimethylthiophosphinic esters of E_1 and 3α-Et the detection limit was 10–15 pg with a four- to five-fold signal-to-noise ratio. The lowest detection limit (600 fg) was obtained with a non-steroidal derivative, the dimethylthiophosphinic ester of the homovanillic acid methyl ester[11].

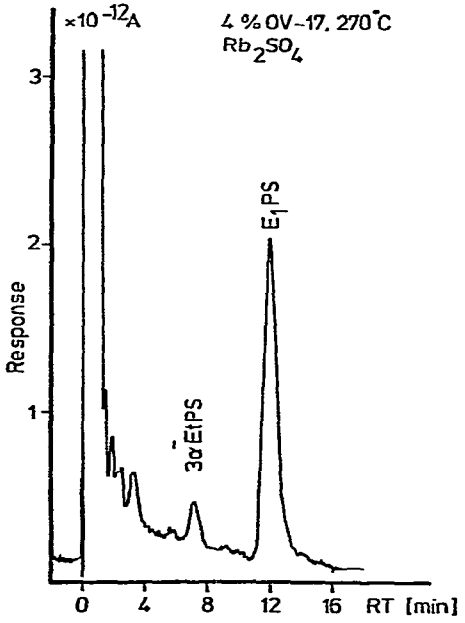

Fig. 3. Gas chromatogram of 3α-etiocholanolone (3α-Et) as the dimethylthiophosphinic ester (3α-EtPS) from human plasma. Estrone dimethylthiophosphinic ester (E_1PS) was used as internal standard in the GLC step. The injected amount of the 3α-Et derivative was 60 pg.

Application of the method to biological materials

The clinical application of this method was demonstrated by the quantitative determination of 3α-Et from human plasma, in the diagnosis of etiocholanolone fever[12,13].

Heparinized plasma is adsorbed on diatomaceous earth and extracted with diethyl ether–chloroform (3:1, v/v). The extract is evaporated, transferred to a TLC-plate, developed with benzene–ethylacetate (6:4, v/v), derivatized and quantitated with the AFD (Fig. 3), as described in the experimental section. Estrone dimethylthiophosphinate is used as internal standard for the GLC-step. The recovery rate is determined by tritiated 3α-Et.

The adsorption on kieselguhr simplifies the extraction of the unconjugated 3α-Et, affording a recovery rate greater than 90%. Interfering steroids like androsterone, dehydroepiandrosterone, DHT and T, are well separated on the first TLC plate. The within-assay precision of the method was 4.8% ($n = 5$) and the overall recovery 40–50%.

Plasma levels of free 3α-Et determined with this method from healthy volunteers were in the ranges 220–370 pg/ml for males and 260–360 pg/ml for females. These values agree well with those found by radioimmunoassay[14] and the double-isotope technique[15].

Comparison of this new method with other GLC assays[16,17] indicates its superior sensitivity and specificity. The general applicability of the derivatization procedure was shown by its use with a number of phenolic carboxylic acids and catecholamine metabolites[11].

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