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ANALYSIS OF STEROIDS BY CAPILLARY SUPERCRITICAL FLUID CHROMATOGRAPHY WITH PHOSPHORUS-SELECTIVE DETECTION

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SUMMARY

Steroids were derivatized with dimethylthiophosphinic chloride to produce steroidal thiophosphinic esters. A catalyst, 4-dimethylaminopyridine, was used to promote quantitative and reproducible thiophosphinic ester formation at low reaction temperatures. The derivatives were analyzed by capillary supercritical fluid chromatography (SFC) with phosphorus thermionic detection.

The phosphorus thermionic detector exhibited linearity over 3–4 orders of magnitude. A sensitivity of 120 fg P/s was obtained for the dithiophosphinic ester of pregnanediol at a signal-to-noise ratio of 3. The efficiency of capillary SFC and the excellent sensitivity of the phosphorus thermionic detector were demonstrated by the analysis of steroids isolated from both human urine and plasma.

INTRODUCTION

During the last several years, supercritical fluid chromatography (SFC) has been demonstrated as an effective separation method for non-volatile and thermally labile compounds. Capillary SFC has been particularly successful in the analysis of relatively non-polar mixtures of synthetic oligomers and petrochemicals which are readily soluble in the commonly used SFC mobile phases (carbon dioxide, nitrous oxide). Unfortunately, polar compounds exibit limited solubility in the preferred SFC mobile phases. For continuous growth and popularity, SFC needs more applications to polar substances, such as those encountered in biochemical and pharmaceutical analysis.

Among several ways to extend the scope of SFC toward polar solutes, sample derivatization has been attractive as a means of increasing the sample solubility in carbon dioxide or nitrous oxide. This has now been demonstrated with silylated oligosaccharides[1] and biological conjugates of steroid metabolites and bile acids[2]. An additional benefit can be realized through the use of chemical derivatization. Selection of a derivatization reagent that incorporates a heteroatom-containing moiety into the derivatized molecule allows the use of sensitive and highly selective thermionic detection. This has been recently demonstrated through the analysis of quinoxalinols, originated from α-ketoacids, by capillary SFC with nitrogen thermionic detection[3].

This communication deals with the sensitive analysis of steroids by capillary SFC with phosporus-selective detection. Steroid hydroxy groups are derivatized with

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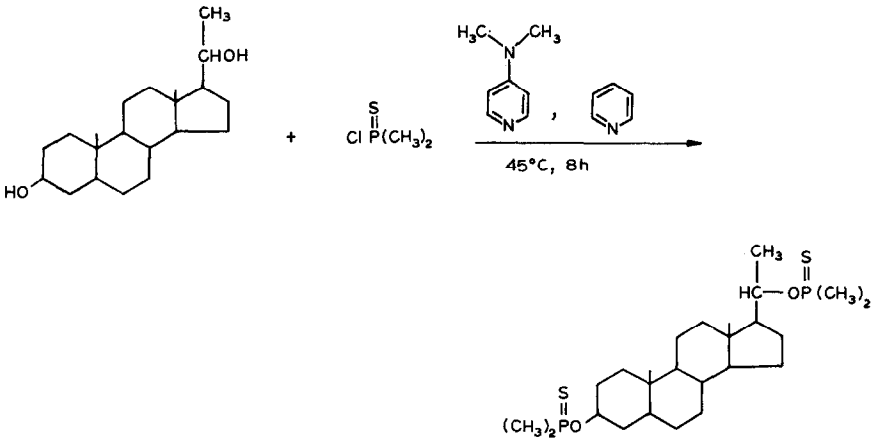

Fig. 1. Formation of a steroid thiophosphinic ester. Model steroid: pregnanediol. Catalyst: DMAP. Solvent: pyridine.

dimethylthiophosphinic chloride to form the corresponding steroid thiophosphinic esters (Fig. 1). This derivatization scheme was initially reported for the gas chromatographic (GC) analysis of monohydroxysteroids[4], but found little practical use because of the need for relatively high column temperatures. We have introduced here a nucleophilic catalyst, 4-(dimethylamino)pyridine[5], to allow this reaction to be generally applicable to all hydroxysteroids. The requirements for a derivatization reagent are somewhat less stringent in SFC than in GC. For successful SFC application the derivatization must be reproducible, but full coverage of all polar functionalities is not required. The inert capillary columns and solvating ability of the mobile phase allow for the efficient SFC analysis of solutes containing a few underivatized polar groups. In capillary GC, chromatographic efficiency is severely compromised by the presence of underivatized polar functionalities.

The further purpose of this article is to demonstrate the excellent sensitivity possible with the phosphorus thermionic detector coupled to capillary SFC. To illustrate this point, steroids were isolated from physiological fluids (blood and urine) and converted to thiophosphinic esters prior to SFC analysis.

EXPERIMENTAL

Chromatographic equipment

The supercritical fluid chromatograph was a home-made instrument as described previously[6]. Mobile phase delivery and pressure control were accomplished with a Brownlee Labs. micropump with software version G (Applied Biosystems, Santa Clara, CA, U.S.A.). The mobile phase employed was SFC grade nitrous oxide (Scott Specialty Gases, Plumsteadville, PA, U.S.A.). The nitrous oxide cylinder was charged with 1500 p.s.i. of helium-head space pressure to facilitate rapid filling of the pump without externally cooling the pump heads. Injection was accomplished via an electrically actuated high-pressure valve with an internal sample loop volume of 0.06 μl (Valco Instruments, Houston, TX, U.S.A.). Split injection was used for all analyses,

with a split ratio of 5:1. The capillary column used was 10 m × 50 μm I.D. The fused-silica surface was deactivated prior to coating through treatment with polymethylhydrosiloxane (85 cSt) (Petrarch Systems, Bristol, MA, U.S.A.). The column was statically coated at 65°C with a 40 mg/ml solution of SE-33 in Freon 11 to produce a 0.50-μm film[8]. The stationary phase was crosslinked three times with azo-*tert.*-butane (Alfa Products, Danvers, MA, U.S.A.).

The pressure restriction required for the operation of a flame-based detector was accomplished by forming an integral restrictor[10] directly at the end of the coated column. The detector was a modified Perkin-Elmer Sigma 3 nitrogen–phosphorus detector. The detector modification consisted of machining a brass detector base containing a centered 1/8 in. swagelok union (Crawford Fitting, Salon, OH, U.S.A.). The detector flame jet was firmly held in the union with a graphite ferrule. The flame jet was a 2 cm × 1 mm I.D. × 3 mm O.D. quartz tube with a conical stainless-steel flame jet tip crimped at the end. The tip outlet orifice was 0.010 in. Polarization voltage and signal amplification were provided by Perkin-Elmer stand-alone nitrogen–phosphorus detection (NPD) electrometer (Perkin-Elmer, Norwalk, CT, U.S.A.). The rubidium silicate thermionic source was prepared according to the procedure of Lubkowitz *et al.*[11]. Beads containing 1.8% B_2O_3, 12.6% Na_2O, 73.3% SiO_2 and 12.2% Rb_2O exhibited optimum sensitivity and 1–2 month lifetime. Typical detector operating conditions were: 300°C heating block temperature, −260 V polarizing voltage, 25 ml/min hydrogen flow, 275 ml/min air flow.

Chemicals

Steroid standards were obtained from Sigma (St. Louis, MO, U.S.A.). Dimethylthiophosphinic chloride was received from Alpha Products. 4-(Dimethylamino)pyridine (DMAP) (Aldrich, Milwaukee, WI, U.S.A.) was employed as a catalyst, and was purified prior to use by alumina column chromatography using diethyleter as the eluent. Pyridine (MCB Manufacturing Chemists, Cincinnati, OH, U.S.A.) was distilled over potassium hydroxide prior to use and stored in glass vials over potassium hydroxide pellets.

Preparation of standard steroids

Microgram amounts of standard steroids were dissolved in 0.2 ml of dry pyridine. Under a dry nitrogen purge, 1.5 μl of neat dimethylthiophosphinic chloride and 10 μl of a 0.1 *M* DMAP solution in dry pyridine were added. The reaction vial was tightly sealed and placed in a heating block at 45°C for 8 h. After the reaction was completed, the reaction mixture was taken to dryness under a stream of nitrogen. To the residue, 50 mg of sodium bicarbonate and 0.2 m of methanol were added. The mixture was heated at 50°C for 1 h to react any remaining dimethylthiophosphinic chloride. The mixture was again taken to dryness under a stream of dry nitrogen. The residue was dissolved in 0.3 ml of methanol and transferred to a syringe attached to a Sep-Pak C_{18} cartridge (Waters Assoc., Milford, MA, U.S.A.). The methanol solution was mixed with 0.7 ml of water and passed through the Sep-Pak cartridge. Excess reagent and reaction by-products (mainly dimethylthiophosphinic anhydride) were eluted with 20 ml methanol–water (30:70, v/v). The derivatized steroids were recovered with 5 ml of methanol, take to dryness, and reconstituted in 0.1 ml of methanol.

Preparation of urine sample

A 10-ml aliquot of a 24-h collection of human pregnancy urine was processed according to the procedure described by Axelson *et al.*[12]. Briefly, the urine was filtered through a Sep-Pak C_{18} cartridge and the steroids were eluted with methanol. After a cation-exchange step (SP- Sephadex, H^+ form), the steroid conjugates were fractionated on a TEAP-LH-20, strong anion-exchange column in the hydroxide form[12]. Three fractions were collected: (1) unconjugated neutral and phenolic steroids, (2) glucuronide conjugated steroids, and (3) mono- and disulphate conjugated steroids The glucuronide conjugates were enzymatically hydrolyzed by β-glucuronidase (Sigma) at 37°C for 24 h. After hydrolysis, Sep-Pak C_{18} and TEAP-LH-20 steps were employed to purify the liberated steroids. Steroid sulfates were cleaved by solvolysis with acidified tetrahydrofuran for 1 h at 50°C. The neutral and phenolic steroids were purified through anion exchange with the TEAP-LH-20 material.

All three fractions were taken to dryness under a stream of nitrogen and reconstituted in 0.2 ml of dry pyridine. Under a nitrogen purge, 3 μl of neat dimethylthiophosphinic chloride and 10 μl of 0.1 *M* DMAP in pyridine were added. The reaction proceeded at 45°C for 12 h. After completion, each fraction was processed in an identical fashion as described in the preparation of standard derivatives. Finally, both fractions 1 and 3 were brought to a final volume of 25 μl with methanol. Fraction 2 was reconstituted to 50 μl with methanol.

Preparation of plasma sample

A 5-ml sample of heparinized human male plasma was processed according to the procedure of Axelson and Sahlberg[13]. The plasma was diluted to 15 ml total volume with distilled water. This solution was heated to 60–64°C for 10 min and passed through a Sep-Pak C_{18} cartridge also held at 64°C. The cartridge was washed with 5 ml of water at 64°C, and steroids were eluted with 8 ml of methanol at room temperature. The Sep-Pak extraction was performed at elevated temperatures to minimize steroidal-protein interactions. Silanized glassware was used throughout the plasma steroid preparation to prevent losses due to irreversible adsorption of conjugated steroids to glass surfaces.

The steroids were fractionated via ion-exchange procedures identical to the urinary steroid preparation. However, the unconjugated plasma steroid fraction was not analyzed, since this fraction contained lipids with unconjugated steroids as minor components. The majority of the plasma steroids are known to exist as either glucuronides or sulfate conjugates. The conjugated fractions were derivatized with dimethylthiophosphinic chloride as described for the urinary steroids. Both fractions were reconstituted to a final volume of 15 μl with methanol.

RESULTS AND DISCUSSION

A reaction temperature of 45°C was chosen for the formation of steroid thiophosphinic esters to allow this derivatization scheme to be generally applicable to all classes of steroids. It was observed that reaction temperatures of 70°C or greater caused decomposition of the labile corticosteroids. The reaction time for the thiophosphinic ester formation was optimized using 5α-pregnane-3β,20β-diol as the model steroid (Fig. 2). At reaction times of less than 8 h, a mixture of products was

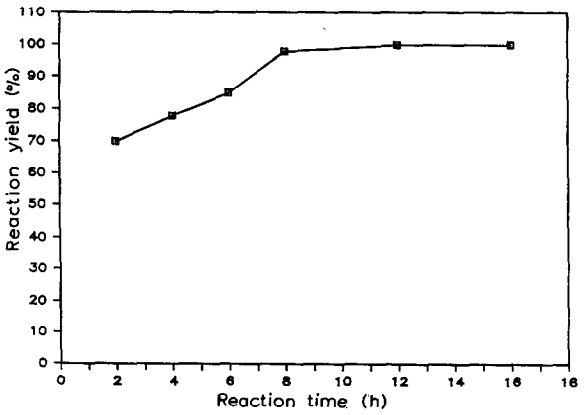

Fig. 2. Reaction time optimization. Model steroid: pregnanediol. Reaction temperature: 45°C.

obtained. This was determined by the appearance of three peaks in the SFC run for pregnanediol. Two early-eluting peaks corresponded to a single-ester formation at either the 3 and 20 positions, respectively, and a later-eluting peak corresponded to the fully derivatized diester of pregnanediol. At reaction times of 8 h or greater, the test chromatogram for pregnanediol contained only one peak, with a retention time matching that for the fully derivatized steroid. To ensure reproducible reaction of more complex steroids, reaction times of 12 h were used for all steroid isolated from physiological fluids.

The linearity and sensitivity of the phosphorus thermionic detector were examined using two representative steroid probes, androstrone and pregnanediol (Fig. 3A and B). All points on the curves were the average of five peak area measurements at each concentration. All peak area measurements had a relative standard deviation (R.S.D.) of 5% or less. Fig. 3A is the calibration curve for the thiophosphinic ester of androsterone (And-PS). The response was linear over 3–4 orders of magnitude with a slope of 0.97 and a minimum detectable quantity (MDQ) of 60 pg And-PS. This corresponds to a sensitivity of 250 fg P/s. with a peak to peak signal-to-noise ratio of 3. Fig. 3B is the calibration curve for the dithiophosphinic ester of pregnanediol (Preg-PS). The detector response was linear over 3–4 orders of magnitude, with a slope of 1.03 and a MDQ of 20 pg Preg-PS. Sensitivity for Preg-PS was 120 fg P/s at a signal-to-noise ratio of 3. As expected, the phosphorus thermionic detector exhibits twice the response for Preg-PS which contains twice the amount of phosphorus as compared to And-PS. Therefore, multiply derivatized steroids should afford even greater sensitivity.

To determine the reproducibility of the thiophosphinic ester reaction, several standard pregnanes (C_{21} steroids) and estrogens were analyzed. Fig. 4 is the standard chromatogram of various pregnane derivatives. The three peaks after 30 min all correspond to doubly derivatized steroids. Compound 3, a pregnanetetrol, reacts at the 3 and 20 positions, while compound 4, β-cortolone, reacts at the 3 and 21 positions. The sterically hindered 11β position and the tertiary hydroxyl group at the 17 position do not react under these circumstances. From these results, ester formation at sterically favorable primary and secondary hydroxyl groups can be predicted with a fair

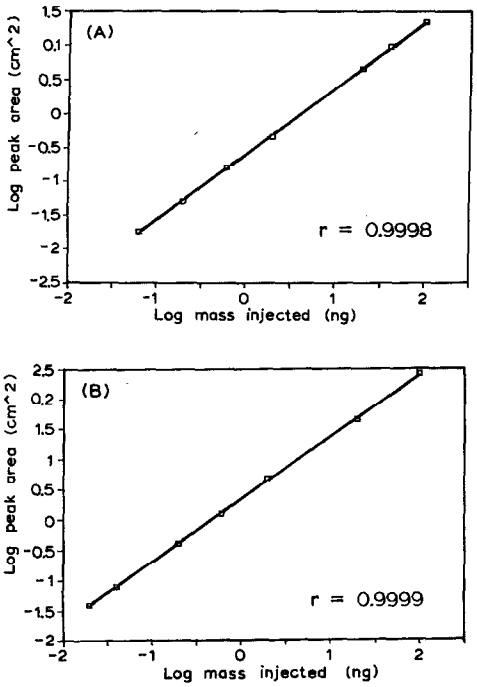

Fig. 3 Linearity of thermionic detection response. (A) androsterone; (B) pregnanediol.

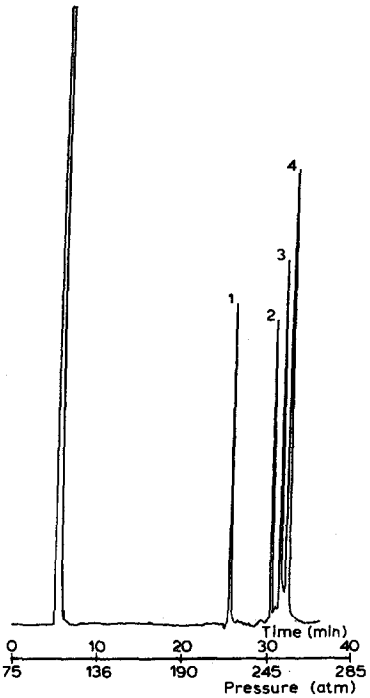

Fig. 4. Standard chromatogram of pregnanes. Peak identifications: 1 = androsterone; 2 = pregnanediol; 3 = 5β-pregnane 3α,11β,17α,20β-tetrol; 4 = 5β-pregnane 3α,17α,20β,21-tetrol-11-one (β-cortolone). Mobile phase: nitrous oxide. Column temperature: 100°C. Pressure ramp: 8 atm/min.

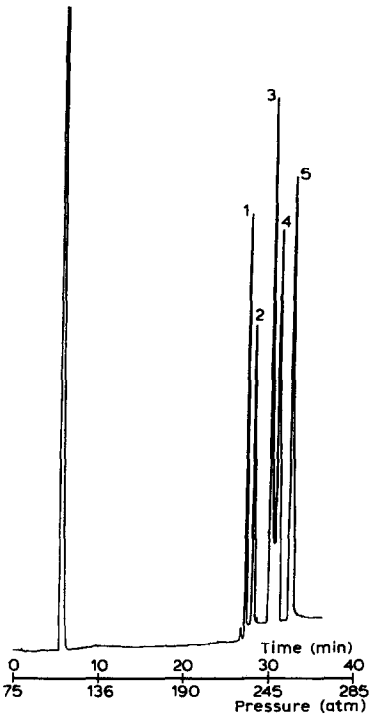

Fig. 5. Standard chromatogram of estrogens. Peak identificatons: 1 = 6-dehydroestrone, 2 = estrone, 3 = estradiol, 4 = 6-ketoestradiol, 5 = estriol. Mobile phase: nitrous oxide. Column temperature: 100°C. Pressure ramp: 8 atm/min.

degree of confidence. All derivatized steroids elute with fairly symmetric peak profiles, indicating that the underivatized hydroxyl groups do not degrade chromatographic efficiency. Fig. 5 is a chromatogram of several standard estrogens. Dimethylthiophosphinic chloride is an excellent reagent for estrogens, in that all the standards steroids were reproducibly and fully derivatized and separated efficiently.

Fig. 6A–C demonstrate chromatograms of the unconjugated, glucuronide, and sulfate steroid fractions, respectively, isolated from human pregnancy urine. All the fractions were spiked at the 10-ng level with androsterone or the respective conjugated androsterone compound as an internal standard. A few steroids have been tentatively identified by retention time comparisons with standard steroids (see figure captions). All the chromatograms were obtained using split injection with a 5:1 split ratio. The steroids in the glucuronide fraction were roughly twice as concentrated as those in the remaining fractions. Consequently, the glucuronide fraction was diluted to twice the final volume of the other fractions to maintain similar chromatographic resolution.

The excellent sensitivity of the phosphorus thermionic detector was demonstrated by the analysis of plasma steroids (Fig. 7A and B). Each fraction was spiked at the 5-ng level with the respective conjugated androsterone compound. Again, the sample introduction method was a split injection with a 5:1 split ratio. Steroids are generally present at ng per ml levels in plasma; therefore, the smaller peaks in the chromatograms correspond to low- or sub-nanogram amounts of steroid thio-

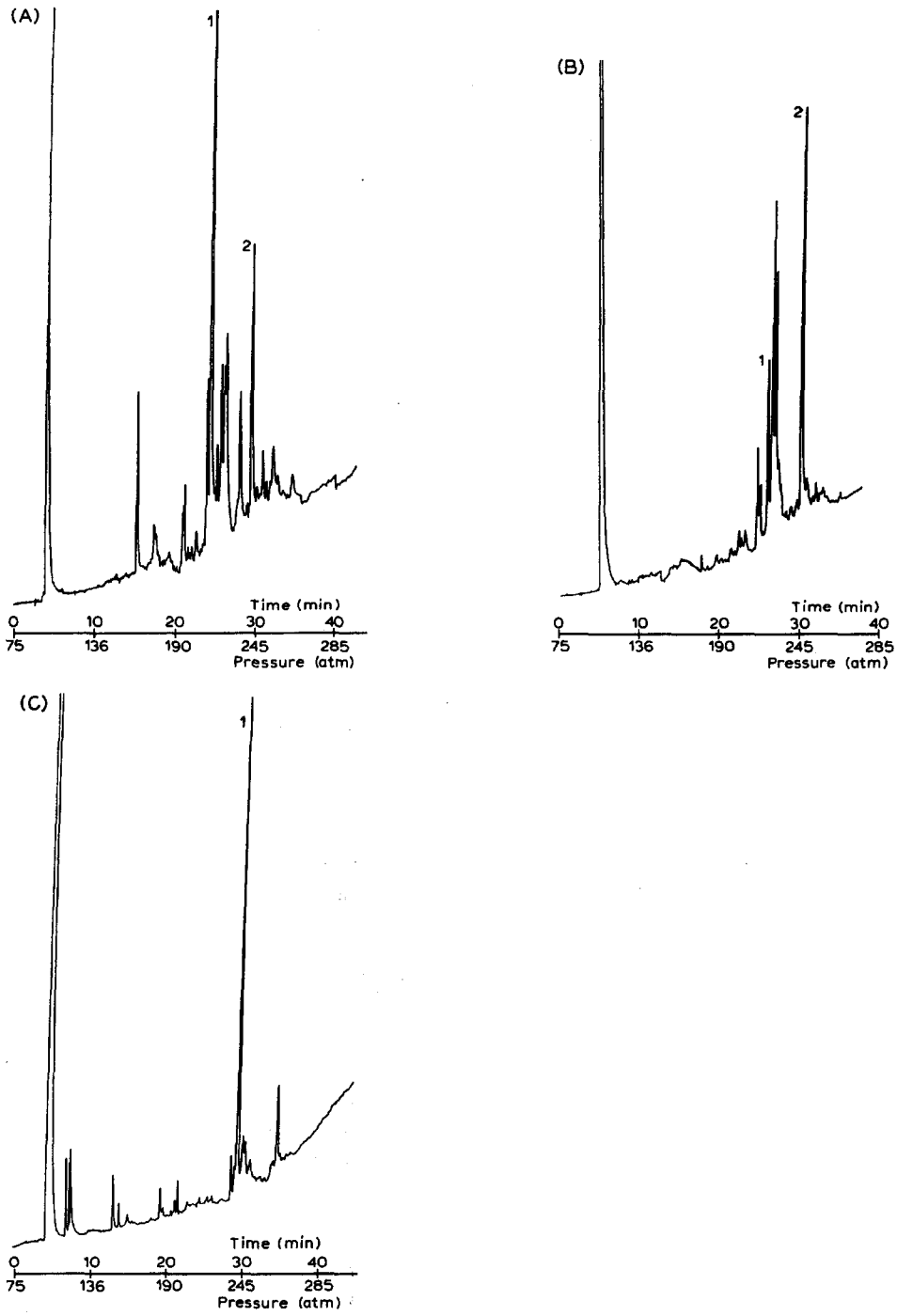

Fig. 6. Chromatograms of urinary steroids. (A) Unconjugated fraction: 1 = androsterone (spike); 2 = estradiol. (B) Glucuronide fraction: 1 = androsterone (spike); 2 = estriol. (C) Sulfate fraction: 1 = 5-androsten-3β-ol-17-one (spike). Chromatographic conditions as in Figs. 4 and 5.

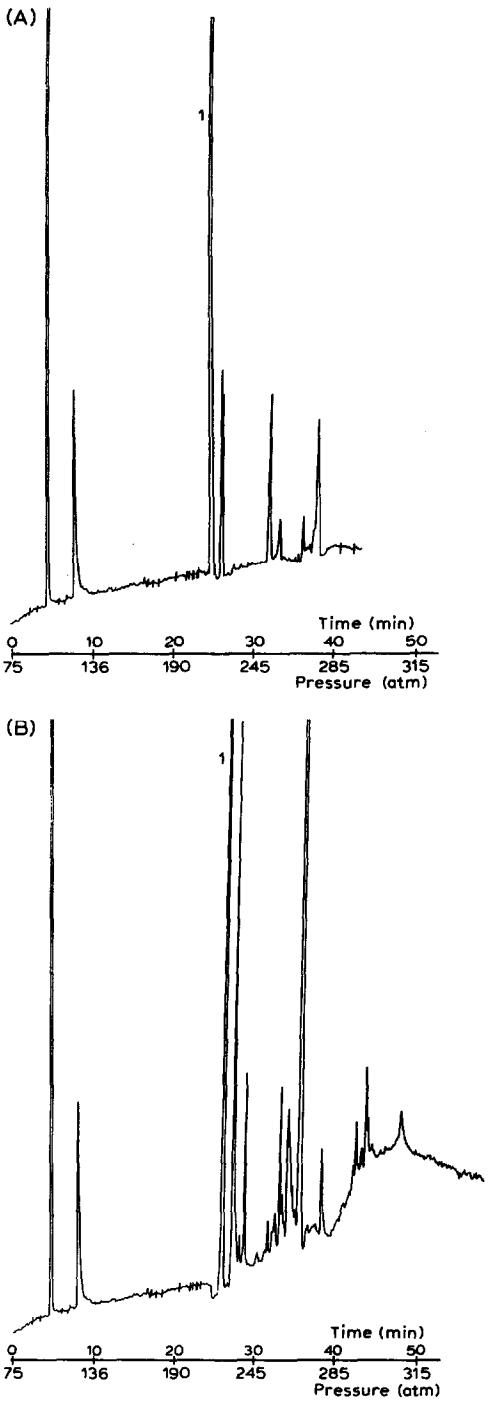

Fig. 7. Chromatograms of plasma steroids. (A) Glucuronide fraction: 1 = androsterone (spike). (B) Sulfate fraction: 1 = 5-androsterone-3β-ol-17-one (spike). Electrometer setting: $2 \cdot 10^{-12}$ A. Other chromatographic conditions as in Fig. 6.

phosphinic esters. These chromatograms serve to illustrate that, under commonly used SFC pressure programming conditions, high sensitivity thermionic detection can be obtained with minimal baseline noise and drift.

Chemical derivatization has been demonstrated as a viable solution to improving the solubility of polar compounds in the common SFC mobile phases carbon dioxide, nitrous oxide. When the derivatization also incorporates a heteroatom-containing moiety into the molecules, highly sensitive thermionic detection can be utilized. The phosphorus mode of the thermionic detector exhibits sufficient sensitivity to permit the analysis of steroids at ppb levels. Capillary SFC provides excellent efficiency for the analysis of these compounds isolated from complex physiological matrices. The capillary SFC analysis of other hydroxy- or amine-containing[14] biochemically important compounds is considerably more attractive when the dimethylthiophosphinic chloride derivatization scheme is utilized in conjunction with phosphorus thermionic detection.

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