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EFFECT OF STEROID HORMONES ON RESULTS FROM THE DETERMINATION OF OXYCHOLESTEROL BY TLC

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SUMMARY

Chromatographic systems comprising different stationary and mobile phases were investigated to determine their suitability for determination of oxysterols in plasma by TLC. It was found that if some steroid hormones are present in the sample, two systems and a variety of detection conditions must be used for oxysterol analysis to ensure the selectivity of the determinations.

INTRODUCTION

Oxysterols result from enzymatic and free-radical oxidation of cholesterol (in-vivo, or in-vitro) [1]. Determination of the concentrations of these compounds in the plasma and tissues of living organisms might be useful for evaluation of the oxidation stress experienced by the organism [2]. Some investigations have suggested there is a relationship between 7β-hydroxycholesterol concentration and death risk from cardiovascular causes and that it is possible to make use of the plasma concentration of this oxysterol as an independent atherosclerosis risk factor [1,3,4]. Because diagnostic laboratories are rarely equipped with expensive GC–FID or GC–MS chromatographic equipment, a TLC method has been developed for determination of oxysterols in plasma. Application of this method excludes possible errors resulting from the presence of compounds of similar structure in the material being investigated. Steroid hormones (androgens, estrogens, progesterone and derivatives of mineral and glycocorticoid function) commonly occur in biological material. This, and their similar structure and physicochemical properties to those of oxysterols, might make oxysterol determination difficult. The basic configuration of steroid hormones, cholesterol, and oxysterols is a cyclopentanoperhydrophenan-

threne oxysterols is a cyclopentanoperhydrophenanthrene nucleus substituted with hydroxyl or ketone groups at the C3 and/or C17 carbon atoms. The concentrations of these compounds in human plasma varies from several picograms to 600 ng mL^{-1}, comparable with the oxycholesterol content [5,6].

The objective of the investigations reported in this paper was to evaluate the effect of steroid hormones on the determination of oxysterols in plasma.

EXPERIMENTAL

The substances investigated were the steroid hormones whose structures are presented in Fig. 1 and the oxysterols whose structures are presented in Fig. 2.

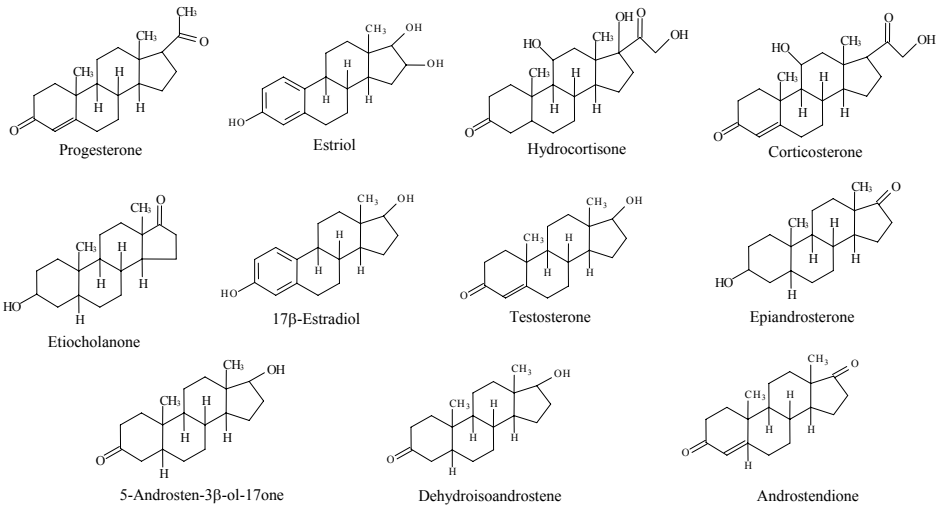

Fig. 1

The structures of the steroid hormones investigated

Blood samples (5 mL) were collected in test-tubes containing disodium versenate solution (7.5%, 100 μL) and centrifuged. The plasma was transferred to test-tubes containing 50 μg BHT (antioxidant). Plasma (5 mL) was hydrolyzed for 2 h at room temperature with ethanolic KOH solution (0.35 M, 10 mL). Lipids were extracted with *n*-hexane (2 × 10 mL) and the combined hexane extracts were washed with redistilled water

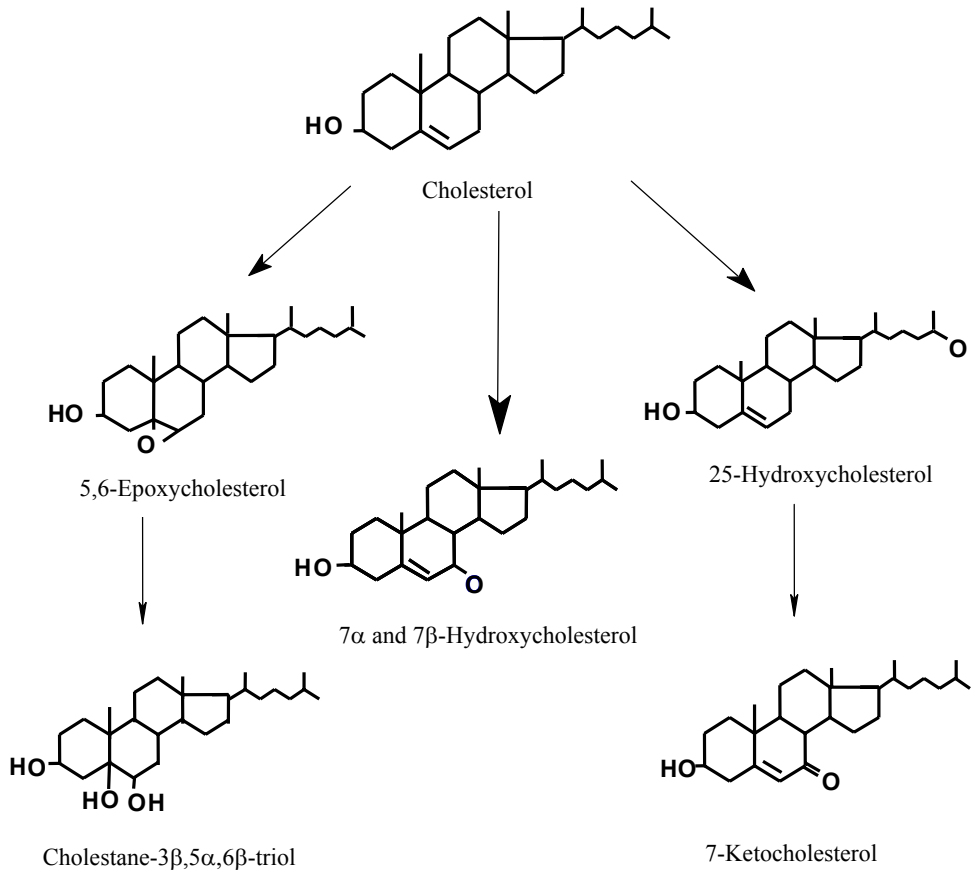

Fig. 2

The structures of the oxycholesterols investigated

(10 mL) and, after separation from the aqueous phase, evaporated to dryness. The lipid extract was dissolved in toluene (1 mL) and separated by SPE. Details of the procedure used for determination of oxycholesterols in blood plasma are available elsewhere [7].

Conditions for SPE Analysis

SPE was performed with 100 mg mL^{-1} Bakerbond SPE-SiOH columns. The columns were conditioned with *n*-hexane–2-propanol (1:1, *v*/*v*; 2 mL) and *n*-hexane (1 mL) and the sample was then applied to the column as a solution in toluene (1 mL). Compounds were eluted with (a) *n*-hexane (1 mL), (b) 2-propanol in *n*-hexane (0.5%, 10 mL), and (c) 2-

propanol in *n*-hexane (30%, 5 mL). The fraction eluted with (c) contained the oxysterols and the steroid hormones. The volume of solvent was reduced to 100 μL and the concentrated solution was analyzed by TLC with densitometry.

Conditions for TLC Analysis

Chromatography was performed on 20 cm × 20 cm plates coated with either RP-C18 (Macherey–Nagel) or silica gel F_{254} (Merck). Samples were applied to the plates by means of a Nanomat III applicator (Camag) equipped with 0.5–1 mL capillaries. RP-C18 plates were developed with 2-propanol–dichloromethane, 3:97 (*v*/*v*), as mobile phase, silica gel plates with acetone–chloroform, 1:9 (*v*/*v*), in a horizontal DS chamber (Chromdes, Lublin, Poland). After development spots were revealed by treatment with Liebermann–Burchard reagent (20 mL methanol + 2 mL conc. H_2SO_4 + 2 mL acetic anhydride) and heating at 110°C for 5 min. Plates were evaluated by use of a Shimadzu CS 93001PC densitometer.

A standard mixture of oxysterols and steroid hormones was analyzed under the same conditions.

RESULTS

Oxysterol and steroid hormone R_F values, and the spot colors obtained after use of Liebermann–Burchard reagent, are presented in Table I.

The optimum conditions for TLC separation and densitometric determination of the oxysterols investigated are listed in Table II. Two sets of conditions were discovered which enabled TLC determination of oxysterol concentrations without interference from steroid hormones. The first was separation of the oxysterol fraction on silica gel with acetone–chloroform, 9:1 (*v*/*v*), as mobile phase (system I); 7-ketocholesterol (7-keto) was determined by densitometry immediately after development of the chromatogram and 7-hydroxycholesterol (sum of the *α* and *β* isomers; 7*β*-OH) by densitometry after use of Liebermann–Burchard reagent (the densitogram was obtained 10 min after development of the chromatogram). The oxysterol fraction could also be separated on RP-18 with 2-propanol–dichloromethane, 3 + 97 (*v*/*v*), as mobile phase (system II); these conditions enabled determination of 5,6-epoxycholesterol (sum of the *α* and *β* isomers, 5,6*α*-EP and 5,6*β*-EP) and 25-hydroxycholesterol (25-OH) and, after use of Liebermann–Burchard reagent, 3*β*,5*α*,6*β*-cholestantriol (Triol) (the densitogram was obtained 10 min after development of the chromatogram).

Table I

Comparison of results from separation and determination of selected oxycholesterol and steroid standards analyzed under the same TLC conditions

Compound	R_F for TLC system:		Spot color after Liebermann reaction[a]	
	I[b]	II[c]	UV at 366 nm	Visible
Oxycholesterols				
Triol	0.02	0.04	Violet–pink	–
7*β*-OH	0.21	0.29	Brown	Blue
7-Keto	0.39	0.39	Blue	–
5,6*α*-EP and 5,6*β*-EP	0.39	0.41	Pink	–
25-OH	0.42	0.46	Salmon	–
Cholesterol	0.71	0.64	Violet	–
Steroid hormones				
Progesterone	0.88	0.59	Yellow–brown	Green–gray
Estriol	0.11	0.29	Yellow	Violet
Hydrocortisone	0.08	0.03	Yellow	Gray
Corticosterone	0.19	0.04	Yellow–green	Gray
Etiocholanone	0.46	0.60	Green	Violet–gray
17*β*-Estradiol	0.48	0.59	Orange–brown	Carmine
Testosterone	0.49	0.59	Yellow	Green–gray
Epiandrosterone	0.52	0.60	Orange–red	Violet–gray
5-Androsten-3*β*-ol-17-one	0.56	0.60	Orange	Yellow
Androstendione	0.79	0.66	Violet	Violet–gray

[a] All steroid hormones except estriol, etiocholanone, and epiandrosterone were determined in UV light at λ = 254 nm

[b] I – silica gel with chloroform–acetone, 9:1 (*v*/*v*), as mobile phase; development distance 13.5 cm

[c] II – RP-18 with 3% 2-propanol in dichloromethane as mobile phase; development distance 14 cm

CONCLUSIONS

Steroid hormones are compounds with molecular structures similar to those of cholesterol oxidation products – they have two or three hydroxyl and/or carbonyl groups and polarity similar to that of oxysterols. During their separation from biological material they are concentrated in the oxysterol fraction.

Table II

Data from TLC determination of oxycholesterol standards

TLC system[a]	Compounds determined	$R_F \pm SD$	Scanning[b] at λ (nm)	Detection limit (ng spot^{-1})
System I	7-Keto	0.39 ± 0.02	239	25
	Sum of 7α- and 7β-OH	0.21 ± 0.01	650	6
System II	Triol	0.04 ± 0.01	432	6
	Sum of 5α,6α-EP and 5β,6β-EP	0.04 ± 0.03	432	6
	25-OH	0.46 ± 0.03	432	6

[a] System I – silica gel with acetone-chloroform, 1:9 (*v*/*v*), as mobile phase; development distance 13.5 cm. System II – RP-18 with 2-propanol–CH_2Cl_2, 3:97 (*v*/*v*), as mobile phase; development distance 14 cm

[b] After use of System I zigzag scanning was performed in reflectance mode. After use of System II linear scanning was performed in fluorescence mode

Analysis of oxysterols, by TLC with densitometric detection, in the presence of steroid hormones thus requires application of two chromatographic systems and different detection conditions.

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