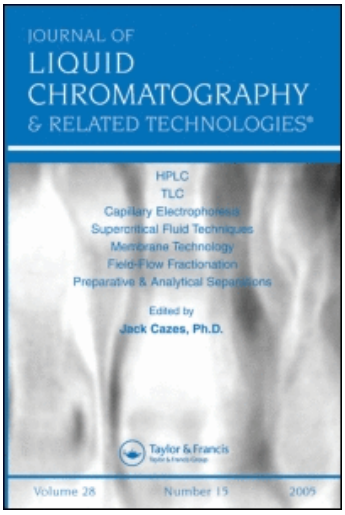

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Comparison of Spectrodensitograms of the Selected Drugs on Different Chromatographic Sorbents

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Abstract: The spectrodensitograms of α-tocopherol acetate, α-tocopherol, cholecalciferol, estradiol, testosterone, and hydrocortisone on different chromatographic sorbents (silica gel 60, silica gel $60F_{254}$, mixture of silica gel 60 and kieselguhr F_{254}, neutral aluminium oxide $60F_{254}$, neutral aluminium oxide $150F_{254}$, and RP18W) were compared. The resultant spectrodensitograms of the studied compounds indicate that applied sorbents have an influence on the wavelength of the obtained fundamental absorption band (λ_{max}) and the additional absorption bands, as well as on their intensity values [AU]. This fact indicates the need for standaridization of the spectrodensitometric investigations regarding the applied chromatographic conditions.

Keywords: TLC, Spectrodensitometry, Lipophilic vitamins, Steroid hormones, Chromatographic sorbents

INTRODUCTION

The currently most important fields of application of thin-layer chromatography are pharmacy, environmental analysis, biochemistry, clinical, and forensic chemistry.[1] Thin-layer chromatography is useful for the identification and determination of lipophilic vitamins, steroid hormones and

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their metabolites in a variety of samples, such as biological (clinical) samples, plants, and pharmaceutical formulations. Many samples can be analyzed simultaneously and quickly at relatively low cost, and multiple separation techniques and detection procedures can be applied using TLC.[1–4]

Lipohilic vitamins and steroid hormones are biologically important compounds.[5–8] Lipophilic vitamins comprise vitamins A, D, E, and K. Physiological forms of vitamins D include vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol). Vitamins D_2 and D_3 are 9,10-secosteroids, which differ structurally in the degree of saturation of an isoprenoid side chain. It is apparent, from the literature,[3] that the biological activity of vitamin D_3 is greater than that of vitamin D_2. Vitamin D_2 is of vegetable origin, whereas D_3 is formed in the skin of humans and other animals. Vitamin E has been an enigma in nutrition research for over 70 years. There are different physiological forms known as vitamins E. In nature, vitamin E occurs in eight different forms (α-, β-, γ- and δ-tocopherols and α-, β-, γ- and δ-tocotrienols) with varying biological activities. Because, tocopherols are being intensively studied, owing to their medical, biological, and physico-chemical significance, the biological properties of α-tocopherol are of particular importance. Of these eight compounds, α-tocopherol is reported to have the highest biological activity.[3]

Estradiol and testosterone, like other steroids, are derived from cholesterol. Estradiol is a sex hormone. It represents the major estrogen in humans. Testosterone is a steroid hormone from the androgen group. It is the principle male sex hormone and an anabolic steroid. Hydrocortisone is a natural corticosteroid produced by the adrenal glands which are located adjacent to the kidneys. Hydrocortisone has anti-inflammatory properties, and is used in a wide variety of inflammatory conditions such as arthritis, colitis, asthma, and bronchitis. Hydrocortisone may also be used to relieve the redness, dryness, itching, crusting, scaling, inflammation, and other discomforts of various skin conditions.

We have previously used thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), and gas chromatography (GC) for the separation of tocopherols.[9] The topological indices were used for the prediction of chromatographic separation of tocopherols.[9,10] We have also used normal phase thin-layer chromatography (NP-TLC) for the separation, and reversed phase thin-layer chromatography (RP-TLC) for the lipophilicity evaluation of the selected steroid compounds, including the steroid hormones.[11,12]

The aim of this study was to compare the spectrodensitograms of the selected drugs on the different chromatographic sorbents. The subjects of these investigations were the selected lipophilic vitamins (α-tocopherol acetate, α-tocopherol, cholecalciferol) and steroid hormones (estradiol, testosterone, hydrocortisone), which were investigated on the different chromatographic sorbents (silica gel 60, silica gel $60F_{254}$, mixture of silica gel 60

and kieselghur F_{254}, neutral aluminium oxide $60F_{254}$, neutral aluminium oxide $150F_{254}$, and RP18W).

EXPERIMENTAL

Chemicals and Sample Preparation

The components of the mobile phases: toluene, ethyl acetate (POCh, Poland; analytical grade), and methanol (Merck, Germany; for liquid chromatography) were used for TLC analysis. The commercial samples of α-tocopherol acetate (Sigma-Aldrich, USA), α-tocopherol (Grindsted, Denmark), cholecalciferol (Sigma-Aldrich, USA), estradiol (Merck, Germany), testosterone (Fluka, Switzerland), and hydrocortisone (Sigma-Aldrich, USA) were used as test solutes. Ethanol (POCh, Poland; 96%; analytical grade), acetone (Chempur, Poland; analytical grade), and chloroform (POCh, Poland; analytical grade) were used to prepare the solutions of above-mentioned compounds (about a concentration of 5 mg mL^{-1} of each standard). The standard solutions of α-tocopherol acetate, α-tocopherol, cholecalciferol, estradiol, and testosterone were prepared in ethanol. However, the standard solution of hydrocortisone was prepared in a mixture chloroform and acetone (7:3, v/v).

Thin Layer Chromatography

Adsorption Thin-Layer Chromatography

Adsorption thin-layer chromatography (NP-TLC) was performed on 20×20 cm aluminium plates precoated with 0.2 mm layer of: a silica gel 60 (E.Merck, #105553; lot: 640105678), a silica gel $60F_{254}$ (E.Merck, #105554; lot: 540034873), a mixture of silica gel 60 and kieselghur F_{254} (E.Merck, #105567; lot: 64545859), a neutral aluminium oxide $60F_{254}$ (Type E) (E.Merck, #105550; lot: 540022980), and a neutral aluminium oxide $150F_{254}$ (Type E) (E.Merck, #105551; lot: 22222572). The plates were prewashed with methanol and dried for 24 h at room temperature. The plates were then activated at 120°C for 20 min. Solutions of the standards were spotted separately onto the plates in 5 μL quantities using a microcapillary (Camag, Switzerland). The lipophilic vitamins (α-tocopherol acetate, α-tocopherol, cholecalciferol) were separated using toluene as mobile phase. However, the steroid hormones (estradiol, testosterone, hydrocortisone) were separated using toluene-ethyl acetate in volume composition 25:25 as mobile phase. The mobile phases (50 mL) were placed in classical chambers (Camag, Switzerland) and the chambers were saturated with the mobile phase vapors for 20 min. The plates were developed at room

temperature (18 ± 1°C). The plates were dried for 24 h at room temperature (18 ± 1°C) in a fume cupboard.

Reversed-Phase High Performance Thin-Layer Chromatography

Reversed-phase high performance thin-layer chromatography (RP-HPTLC) was performed on 20 × 10 cm glass HPTLC plates, coated with RP-18W (Merck, #114296; lot: OB315589). The plates were prewashed with methanol and dried for 24 h at room temperature (18 ± 1°C). Solutions of the standards were spotted separately on the plates in 5 μL quantities using a microcapillary (Camag, Switzerland). Methanol was used as mobile phase. Plates were developed to a distance of 7.5 cm at room temperature (18 ± 1°C) in a classical bottom chamber (Camag, Switzerland) previously saturated with the mobile phase for 20 min. After development, the plates were dried for 24 h at room temperature (18 ± 1°C).

Visualization of Spots by Use of a Camag Densitometer

Densitometric scanning was then performed at $\lambda = 254$ nm with a Camag Scanner TLC 3 operated in the absorbance mode and controlled by winCATS 1.4.1 software. The radiation source was a deuterium lamp emitting a continuous UV spectrum between 190 and 450 nm. The slit dimensions were 8.00 × 0.40 mm, Micro; the optimize optical system was light; the scanning speed was 20 mm s^{-1}; the data resolution was 100 μm $step^{-1}$; the measurement type was remission; and the measurement mode was absorption; the optical filter was second order. Each track was scanned three times and baseline correction (lowest slope) was used.

Retardation Factor

The chromatograms were done in five-fold manner and each track was scanned three times; the mean retardation factor R_F values were calculated.

Spectrodensitometric Analysis

Spectrum scan was also performed using a Camag Scanner TLC 3. The radiation sources were a deuterium lamp emitting a continuous UV spectrum between 190 and 450 nm and a tungsten lamp-emitting a spectrum between 370 and 800 nm. Starting wavelength was 200 nm and ending wavelength was 700 nm. The slit dimensions were 8.00 × 0.40 mm, Micro; the optimized optical system was resolution; the scanning speed was 20 nm s^{-1}; the data resolution was 1 nm $step^{-1}$; the measurement type was

emission; and the measurement mode was absorption; the optical filter was second order.

RESULTS AND DISCUSSION

The retardation factor values calculated from the densitometric measurements for the examined drugs by NP-TLC and RP-TLC techniques on the different chromatographic sorbents are presented in Table 1. It was affirmed that in NP-TLC the R_F values of the investigated vitamins decrease in the following order: α- tocopherol acetate, α-tocopherol, cholecalciferol. In the case of the steroid hormones investigated on silica gel 60, silica gel $60F_{254}$, as well as mixture of silica gel 60 and kieselghur F_{254}, their neutral aluminium oxide $60F_{254}$, their R_F values decrease in the following order: estradiol, testosterone, and hydrocortisone. However, testosterone has higher R_F values in relation to estradiol, both on neutral aluminium oxide $60F_{254}$, and neutral aluminium oxide $150F_{254}$. The lipophilic vitamins have similar R_F values in the range from 0.45 to 0.49 in reversed phase thin layer chromatography (RP-TLC). However, the R_F values of the studied steroid hormones by RP-TLC are in the range from 0.66 to 0.74.

The spectrodensitogram characteristics of the investigated lipohilic vitamins and steroid hormones on the different chromatographic sorbents are presented in Table 2.

The α-tocopherol acetate spectrum on silica gel (silica gel 60 and silica gel $60F_{254}$) characterizes a fundamental absorption band (λ_{max}) at 203 nm. The remaining absorption bands have the values equal to 222 and 284 nm. The similar absorption bands for the α-tocopherol acetate are observed on a mixture of silica gel 60 and kieselghur F_{254} as well as on RP18W. Insignificant differences are observed in the intensities of the additional bands. The fundamental absorption band (λ_{max}) of α-tocopherol acetate is somewhat shifted and it is equal to 208 nm on neutral aluminium oxide $60F_{254}$, and neutral aluminium oxide $150F_{254}$. The remaining absorption bands are at 225 and 285 nm. Insignificant difference occurs for the fundamental absorption band (λ_{max}) of α-tocopherol acetate on neutral aluminium oxide $60F_{254}$, and neutral aluminium oxide $150F_{254}$ in relation to the remaining investigated sorbents (namely, silica gel 60, silica gel $60F_{254}$, mixture of silica gel 60 and kieselghur F_{254}, and RP18W). General differences are observed in the band intensity [AU] of the studied compound on above-mentioned sorbents. The highest values of the band intensity [AU] of α-tocopherol acetate are observed on aluminium oxide. For example, the α-tocopherol acetate spectra on silica gel 60, neutral aluminium oxide $60F_{254}$, and RP18W are presented in Fig. 1.

The α-tocopherol spectrum on silica gel (silica gel 60 and silica gel $60F_{254}$) characterizes a fundamental absorption band (λ_{max}) at a wavelength of 203 nm. The remaining absorption bands have numerical values equal to about 222 and 290 nm. The similar absorption bands for the α-tocopherol

Table 1. R_F values[a] of selected lipophilic vitamins and steroid hormones investigated by normal and reversed phases TLC

		NP-TLC					
		Stationary phase					
Compounds	Mobile phase[b]	Silica gel 60 (#105553)	Silica gel $60F_{254}$ (#105554)	Mixture of silica gel 60 and kieselguhr F_{254} (#105567)	Neutral aluminium oxide $60F_{254}$ (#105550)	Neutral aluminium oxide $150F_{254}$ (#105551)	RP-TLC on RP18W (#114296) plates; mobile phase:methanol
α-Tocopherol acetate	A	0.48	0.38	0.63	0.89	0.91	0.45
α-Tocopherol	A	0.44	0.36	0.60	0.44	0.48	0.49
Cholecalciferol	A	0.11	0.07	0.17	0.13	0.14	0.48
Estradiol	B	0.66	0.55	0.78	0.25	0.52	0.68
Testosterone	B	0.48	0.39	0.62	0.41	0.60	0.66
Hydrocortisone	B	0.15	0.12	0.24	0.01	0.03	0.74

[a]Mean of n = 5 individual experiments.

[b]Mobile phases: A) toluene; B) toluene-ethyl acetate, 25:25 (v/v).

Table 2. Spectrodensitogram characteristics of selected lipophilic vitamins and steroid hormones on different chromatographic sorbents

	Spectrodensitogram characteristic		
		Remaining absorption bands	
Sorbent[a]	λ_{max} (nm)[b]	λ (nm)	Intensity (AU)
α-Tocopherol acetate			
Silica gel 60	203	222; 284	68.1; 43.6
Silica gel 60F_{254}	203	222; 284	69.0; 44.5
Mixture of silica gel 60 and kieselguhr F_{254}	204	221; 284	74.7; 29.1
Neutral aluminium oxide 60F_{254}	208	225; 285	90.8; 68.6
Neutral aluminium oxide 150F_{254}	208	225; 285	86.6; 56.8
RP18W	203	222; 285	57.8; 47.7
α-Tocopherol			
Silica gel 60	202	223; 291	64.0; 66.2
Silica gel 60F_{254}	202	222; 290	64.3; 65.9
Mixture of silica gel 60 and kieselguhr F_{254}	204	222; 292	65.7; 48.0
Neutral aluminium oxide 60F_{254}	294	208; 226	89.2; 76.1
Neutral aluminium oxide 150F_{254}	208	225; 295	81.9; 80.1
RP18W	202	225; 291	50.8; 47.7
Cholecalciferol			
Silica gel 60	266	220	49.4
Silica gel 60F_{254}	270	220	50.5
Mixture of silica gel 60 and kieselguhr F_{254}	270	219	53.5
Neutral aluminium oxide 60F_{254}	271	221	36.0
Neutral aluminium oxide 150F_{254}	271	222	42.1
RP18W	264	200	75.2
Estradiol			
Silica gel 60	200	221; 281	57.2; 52.8
Silica gel 60F_{254}	200	221; 282	55.1; 46.3
Mixture of silica gel 60 and kieselguhr F_{254}	200	221; 282	61.0; 30.8
Neutral aluminium oxide 60F_{254}	204	225; 284	92.9; 77.4
Neutral aluminium oxide 150F_{254}	206	224; 284	77.5; 52.1
RP18W	200	221; 282	62.5; 53.8
Testosterone			
Silica gel 60	251	303	8.2
Silica gel 60F_{254}	251	300	6.8
Mixture of silica gel 60 and kieselguhr F_{254}	249	304	2.7
Neutral aluminium oxide 60F_{254}	247	—	—
Neutral aluminium oxide 150F_{254}	247	310	3.6
RP18W	251	303	8.6

(*continued*)

Table 2. Continued

Sorbent[a]	Spectrodensitogram characteristic		
		Remaining absorption bands	
	λ_{max} (nm)[b]	λ (nm)	Intensity (AU)
Hydrocortisone			
Silica gel 60	249	297	18.8
Silica gel 60F_{254}	249	300	14.8
Mixture of silica gel 60 and kieselguhr F_{254}	247	299	5.6
Neutral aluminium oxide 60F_{254}	247	316	10.6
Neutral aluminium oxide 150F_{254}	247	313	8.1
RP18W	251	296	25.7

[a]Sorbents: Silica gel 60 (#105553); Silica gel 60F_{254} (#105554); Mixture of silica gel 60 and kieselguhr F_{254} (#105567); Neutral aluminium oxide 60F_{254} (#105550); Neutral aluminium oxide 150F_{254} (#105551); and RP18W (#114296).

[b]Intensity of all absorption maximum is equal to 95 AU.

are observed on a mixture of silica gel 60 and kieselghur F_{254} as well as on RP18W. Insignificant differences are observed in the intensities of the additional absorption bands in the case of a mixture of silica gel 60 and kieselguhr F_{254}. The fundamental absorption band (λ_{max}) of α-tocopherol occurs at the wavelength equal to 294 nm on neutral aluminium oxide 60F_{254}; and the remaining absorption bands are at 208 and 226 nm. On neutral aluminium oxide 150F_{254}, the fundamental absorption band (λ_{max}) of α-tocopherol occurs at the wavelength equal to 208 nm, and the remaining absorption bands are at 225 and 295 nm. Considerably difference occurs for the fundamental absorption band (λ_{max}) of α-tocopherol on neutral aluminium oxide 60F_{254} in relation to all remaining investigated sorbents. General differences are observed in the band intensities [AU] of the studied compound on above-mentioned sorbents. The highest values of the band intensity [AU] of α-tocopherol are observed on aluminium oxide, and the lowest on RP18W. For example, the α-tocopherol spectra on silica gel 60, neutral aluminium oxide 60F_{254}, and RP18W are presented in Fig. 2.

The cholecalciferol spectrum on silica gel 60 characterizes the fundamental absorption band (λ_{max}) at a wavelength equal to 266 nm. The remaining absorption band occurs at the wavelength equal to 220 nm. The fundamental absorption band (λ_{max}) of cholecalciferol on silica gel 60F_{254}, mixture of silica gel 60 and kieselghur F_{254}, neutral aluminium oxide 60F_{254}, and neutral aluminium oxide 150F_{254} is at 270 or 271 nm; however, the additional band is at the wavelength in the range from 219 nm to 221 nm. Cholecalciferol spectra obtained using NP-TLC are very similar, and they differ in the

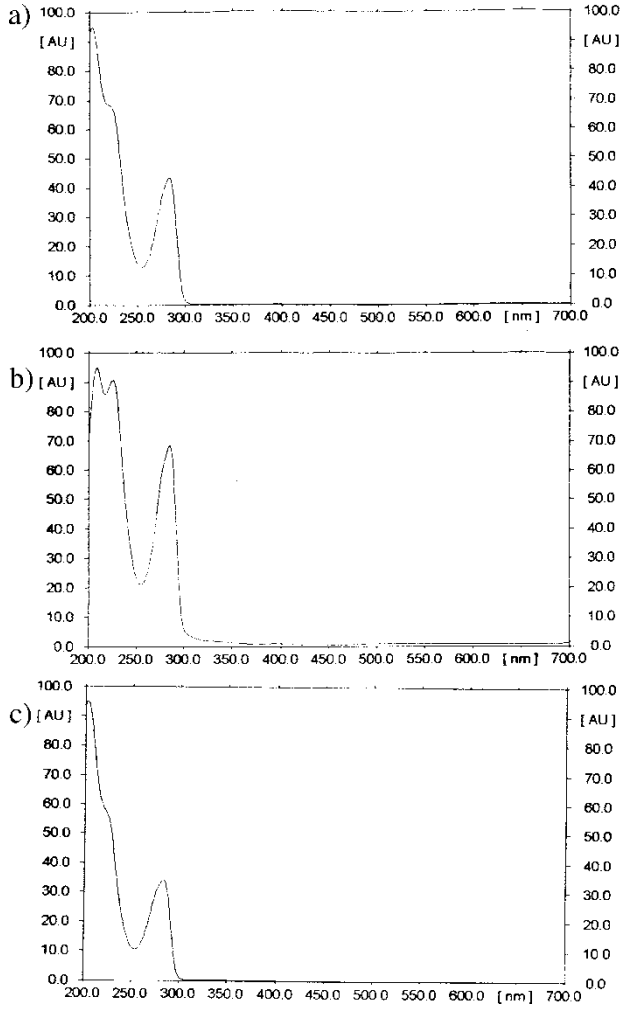

Figure 1. The α-tocopherol acetate spectra on: a) silica gel 60, b) neutral aluminium oxide $60F_{254}$, and c) RP18W.

intensity of the additional of absorption band, which is the lowest on neutral aluminium oxide $60F_{254}$. The cholecalciferol spectrum obtained by RP-TLC on RP-18W plates decidedly differs from spectra obtained on sorbents by NP-TLC. The fundamental band (λ_{max}) of cholecalciferol on RP18W occurs at the wavelength equal to 264 nm, and the remaining absorption band occurs at 200 nm. The intensity of the additional absorption band of cholecalciferol is the highest on RP18W and is equal about 75 AU. For example, the cholecalciferol spectra on silica gel 60, neutral aluminium oxide $60F_{254}$, and RP18W are presented in Fig. 3.

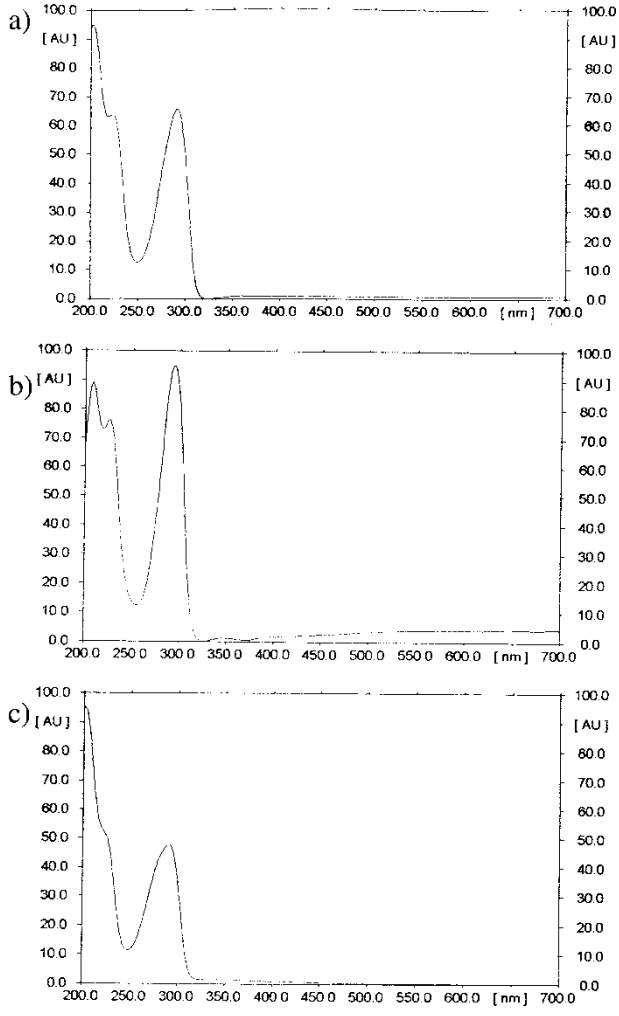

Figure 2. The α-tocopherol spectra on: a) silica gel 60, b) neutral aluminium oxide $60F_{254}$, and c) RP18W.

Estradiol spectrum on silica gel (silica gel 60 and silica gel $60F_{254}$), mixture of silica gel 60 and kieselguhr F_{254}, as well as on RP18W characterizes of the absorption maximum (λ_{max}) at the wavelength equal to 200 nm. The remaining absorption bands have the numerical values equal to 221 and 281 or 282 nm. The intensity of the additional bands is differentiated. In the case of neutral aluminium oxide $60F_{254}$ and neutral aluminium oxide $150F_{254}$ insignificant shift of absorption bands is observed. The fundamental absorption band (λ_{max}) of estradiol occurs at the wavelength equal to 204 and 206 nm, respectively on neutral aluminium oxide $60F_{254}$ and neutral

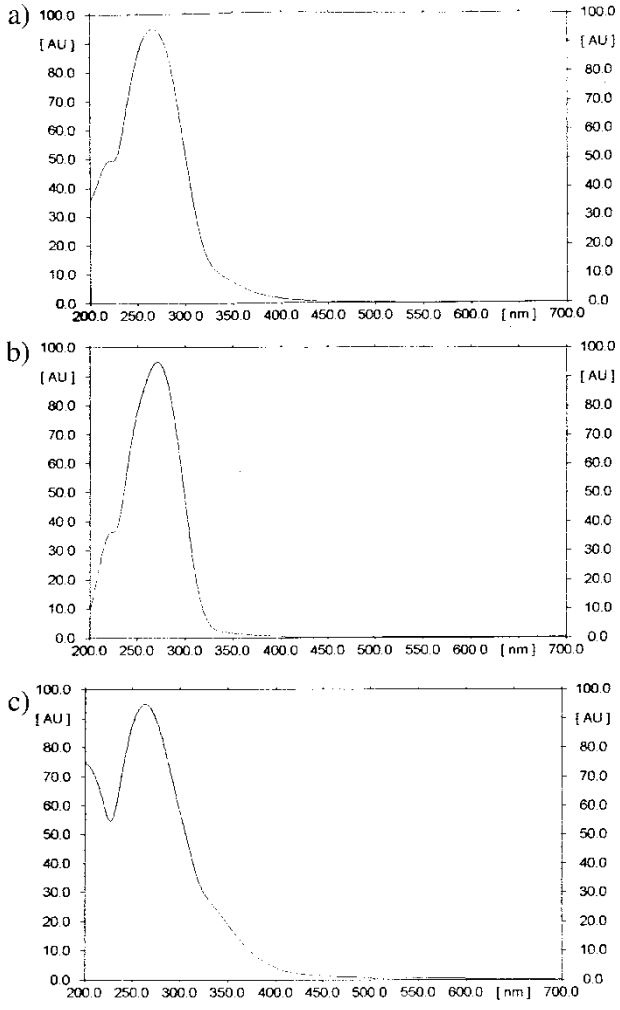

Figure 3. The cholecalciferol spectra on: a) silica gel 60, b) neutral aluminium oxide $60F_{254}$, and c) RP18W.

aluminium oxide $150F_{254}$. The additional absorption bands occur at the wavelength equal to 224 or 225 nm and at 284 nm. It was stated, that the highest intensity of the additional absorption bands has taken place on neutral aluminium oxide $60F_{254}$. For example, the estradiol spectra on silica gel 60, neutral aluminium oxide $60F_{254}$, and RP18W are presented in Fig. 4.

Testosterone spectrum on silica gel (silica gel 60 and silica gel $60F_{254}$), and on RP18W characterizes of the fundamental absorption band (λ_{max}) at the wavelength equal to 251 nm. The additional absorption band occurs at

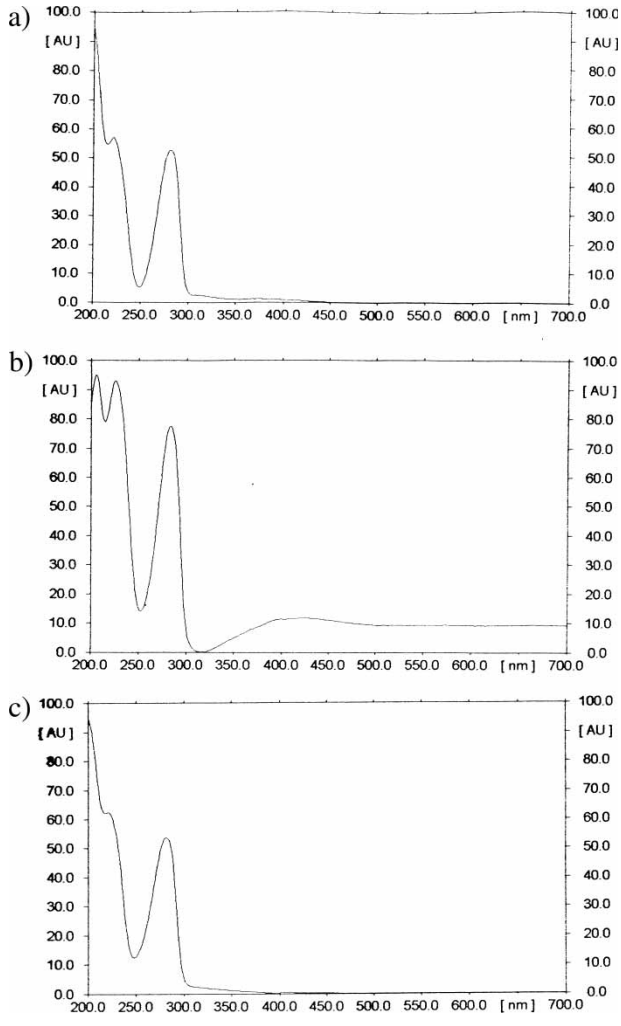

Figure 4. The estadiol spectra on: a) silica gel 60, b) neutral aluminium oxide $60F_{254}$, and c) RP18W.

the wavelength equal to 300 or 303 nm. On mixture of silica gel 60 and kieselguhr F_{254} the fundamental absorption band (λ_{max}) occurs at 249 nm. On neutral aluminium oxide $60F_{254}$ and neutral aluminium oxide $150F_{254}$ the fundamental absorption band (λ_{max}) occurs at 247 nm; however, the additional band at the wavelength equal to 310 nm occurs only on neutral aluminium oxide $150F_{254}$. From presented comparisons it results that testosterone on aluminium oxide $60F_{254}$ has only one absorption band. For example, the testosterone spectra on silica gel 60, neutral aluminium oxide $60F_{254}$, and RP18W are presented in Fig. 5.

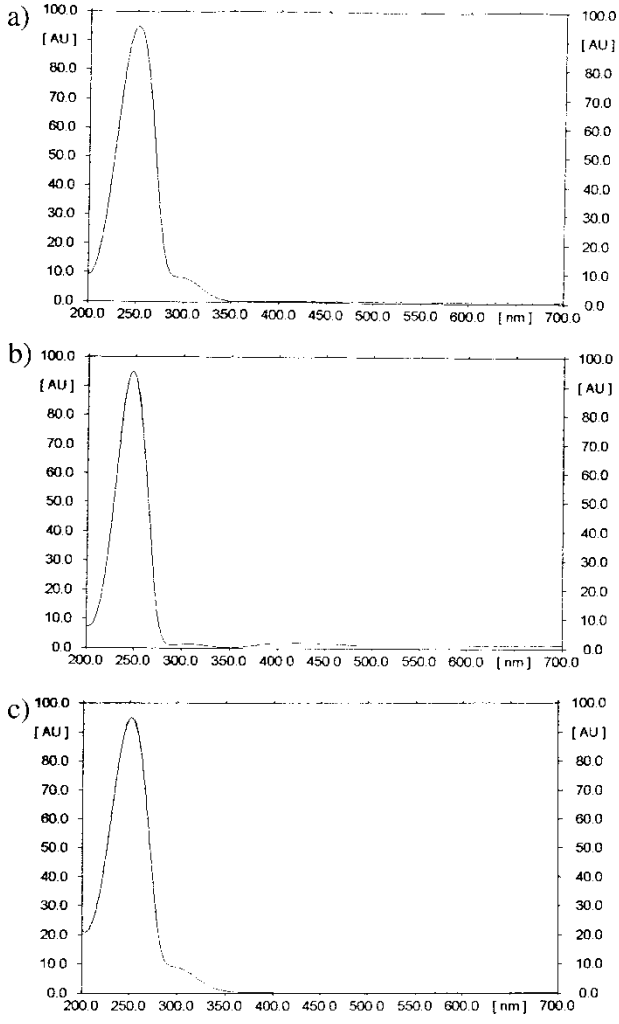

Figure 5. The testosterone spectra on: a) silica gel 60, b) neutral aluminium oxide $60F_{254}$, and c) RP18W.

Hydrocortisone spectrum on silica gel (silica gel 60 and silica gel $60F_{254}$) characterizes of the fundamental absorption band (λ_{max}) at the wavelength equal to 249 nm. The additional absorption band occurs at the wavelength equal to 297 or 300 nm. On mixture of silica gel 60 and kieselguhr F_{254}, neutral aluminium oxide $60F_{254}$ and neutral aluminium oxide $150F_{254}$ the fundamental absorption band (λ_{max}) occurs at 247 nm; however, the additional band at wavelength equal to 299, 316, and 313 nm, respectively. The obtained hydrocortisone spectra by NP-TLC are very similar, and they differ in the intensity of the additional of absorption band, which is the

lowest on mixture of silica gel 60 and kieselguhr F_{254}. Hydrocortisone spectrum obtained by RP-TLC on RPW plates insensibly differs from spectra obtained on sorbents in NP-TLC. The fundamental absorption band (λ_{max}) of hydrocortisone on RP18W occurs at the wavelength equal to 251 nm; and the additional band occurs at the wavelength equal to 296 nm. The intensity of the additional band is equal to 25.7 AU. For example, the hydrocortisone spectra on silica gel 60, neutral aluminium oxide $60F_{254}$, and RP18W are presented in Fig. 6.

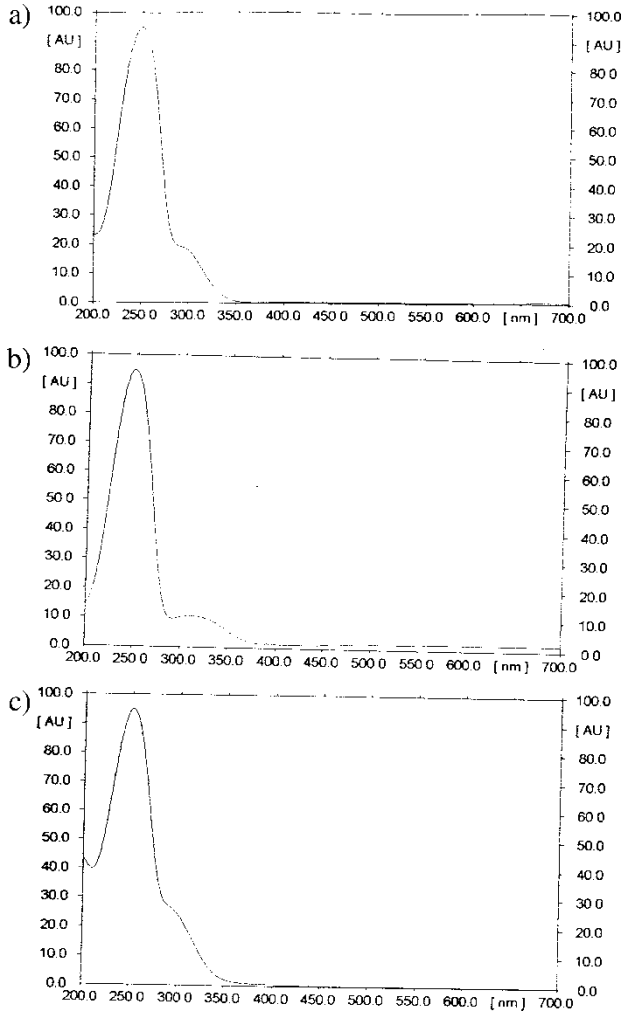

Figure 6. The hydrocortisone spectra on: a) silica gel 60, b) neutral aluminium oxide $60F_{254}$, and c) RP18W.

The obtained spectrodensitograms of the studied compounds indicate that applied sorbents influence on the wavelength of the obtained fundamental absorption band (λ_{max}) and the additional absorption bands, as well as on their intensity values [AU]. This fact indicates on the necessary standaridization of the spectrodensitometric investigations regarding the applied chromatographic conditions. Therefore the spectrodensitograms of the investigated compounds can be correctly compared only on the same chromatographic sorbent. This fact has the fundamental significance in the identification analysis.

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