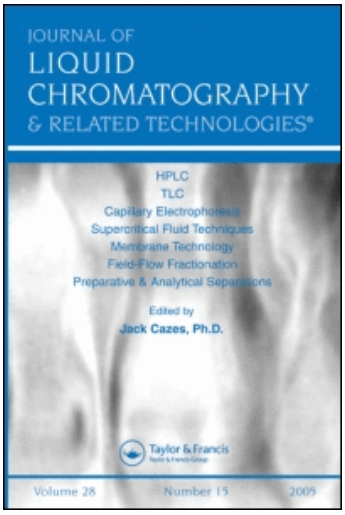

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Spectrodensitometry Application to Analytical Identification of Estradiol, Hydrocortisone, Testosterone and Cholesterol on Diol Plates

A. Pyka[a]
[a] Department of Analytical Chemistry, Faculty of Pharmacy, Medical University of Silesia, Sosnowiec, Poland

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Spectrodensitometry Application to Analytical Identification of Estradiol, Hydrocortisone, Testosterone and Cholesterol on Diol Plates

A. Pyka

Department of Analytical Chemistry, Faculty of Pharmacy,
Medical University of Silesia, Sosnowiec, Poland

Abstract: The selected steroid compounds such as: estradiol (E), hydrocortisone (H), testosterone (T), and cholesterol (CH) were separated by using thin-layer chromatography on glass plates precoated with Diol F_{254s} (E. Merck, #1.05636) and using a chloroform as mobile phase. The densitometric detection of these compounds with and without the use of sulphuric acid solutions as visualizing reagents was compared. A robust and sensitive detection procedure for selected steroid compounds using the sulphuric acid as a visualizing reagent was described. Spot intensities on the plates were quantified after dipping in sulphuric acid solutions and heating at temperatures from 80°C to 140°C for times ranging from 5 to 30 min. The best detection conditions for high signal intensity [AU] were determined. Particularly robust and sensitive detection of investigated compounds separated was observed by dipping the plate for 15 s in the solution of sulphuric acid in methanol in the volume composition 1:19, and for temperature equal to 120°C and for heating for 10 min. The spectrodensitograms of hydrocortisone, estradiol, testosterone, and cholesterol on DiolF_{254} plates and by the use of sulphuric acid as visualizing reagents are different than the spectrodensitograms obtained on the plate without the use of a visualizing reagent. The obtained spectrodensitograms of investigated compounds after the detection with the use of sulphuric acid as visualizing reagents differ in the number and intensity of additional absorption bands. This fact has analytical and pharmaceutical significance

Correspondence: A. Pyka, Department of Analytical Chemistry, Faculty of Pharmacy, Medical University of Silesia, 4 Jagiellońska Street, PL-41-200 Sosnowiec, Poland. E-mail: apyka@sum.edu.pl

in the identification of hydrocortisone, estradiol, testosterone, and cholesterol. Comparison and characterization of chromatographic spots of examined compounds on the basis of resolution (R_S), separation factor (α), constant of the pair separation (R_F^{α}), and ΔR_F values were discussed. It was stated, that the resolution R_s values are similar at the wavelengths 250, 275, 300, 325, 375, and 400 nm for the particular separated compounds analyzed after the use of sulphuric acid solution as visualizing reagent.

Keywords: Adsorption HPTLC, Cholesterol, Densitometric analysis, Estradiol, Hydrocortisone, Separation factors, Spectrodenitometric analysis, Spot visualization, Sulphuric acid, Testosterone

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 cholesterol and its derivatives, including steroid hormones and 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]

Steroids are compounds having a four ringed carbon skeleton derived from 1,2-cyclopentanoperhydrophenanthrene. Many steroids are present in plants and animals. Cholesterol is the most important sterol representative of animal origin. 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.[5–8]

The separated substances on a thin layer can be detected by the following methods: physical (individual colour of substance or fluorescence; of substance in UV light); chemical (colored reactions of separated substances with visualizing reagents); physicochemical (e.g., the application of isotopes as visualizing reagent); and biological (the application of biodetectors).

The visualizing reagents have the special significance to detect separated compounds on thin layers. In view of the detection mechanism of the compound, the visualizing reagents can be sorted as follows: conservative reagents, which do not destroy separated substances; destructive reagents, which destroy or change the structures of separated substances.[1,9]

The aim of this study was to compare the spectrodensitograms and densitograms of estradiol (E), hydrocortisone (H), testosterone (T), and cholesterol (CH) on Diol plates without the use of a visualizing reagent and after the use of sulphuric acid as visualizing reagent.

EXPERIMENTAL

Chemicals

Chloroform (POCh, Gliwice, Poland, pure p.a.) was as a mobile phase used for the adsorption TLC analysis. The commercial samples of H, E, (E. Merck, Germany), T (Fluka, Switzerland), CH (St. Louis, Sigma Company, USA), were used as test solutes. Methanol, chloroform, acetone (POCh, Gliwice, Poland, pure p.a.), and ethanol (96%, POCh, Gliwice, Poland; pure p.a.) were used for the preparation of steroid compound solutions. Sulphuric acid, 95% (Chempur, Piekary Śląskie, Poland) was used to prepare the visualizing reagents.

Sample Preparation

The following solutions of studied compounds were prepared:

5 mg of testosterone in 1 mL of ethanol;
5 mg of estradiol in 1 mL of methanol;
5 mg of hydrocortisone in 1 mL of the mixture of chloroform and acetone in volume composition 7:3;
5 mg of cholesterol in 1 mL of chloroform.

Next, the above mentioned solutions were mixed. Mixed solution was in the concentration 1.25 mg/ 1 mL of each compound.

Thin Layer Chromatography

Adsorption HPTLC was performed on 10×10 cm glass plates precoated with DiolF_{254} (E. Merck, #1.05636, lot:OB 006288). The plates were prewashed with methanol and dried for 24 h at room temperature ($18 \pm 1°C$).

Before use the plates were activated at 120°C for 20 min. Micropipettes (Camag, Switzerland) were used to apply the standard solutions to the plates. Mixed solutions of the standard steroids were manually spotted on a chromatographic plate in the quantity 1.25 μg of each standard in 1 μL ethanol. Chloroform as mobile phase (50 mL) was placed in a classical chamber (Camag, Switzerland) and, after saturation of the chamber with the mobile phase vapor for 20 min, the plates were developed vertically at room temperature (18 ± 1°C), to a distance 7.5 cm. The plates were then dried at room temperature (18 ± 1°C) in a fume cupboard.

Visualizing Reagent

The investigated compounds were evaluated on the plates using the solution of: sulphuric acid in methanol in different volume compositions (1:10, 1:11, 1:12, 1:13, 1:14, 1:15, 1:16, 1:17, 1:18, 1:19, and 1:20) as visualizing reagents. The dried plates were dipped in particular sulphuric acid solutions for 5, 10, 15, 20, 25, and 30 s. They were then heated at temperatures from 80 to 140°C for times ranging from 5 to 30 min.

Spectrodenstiometric and Densitometric Analysis

The spectrum was recorded using Camag Scanner TLC 3, operated in absorbance mode and controlled by winCATS 1.4.2 software. 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. The start wavelength was 200 nm and end wavelength was 700 nm. The slit dimensions were 8.00 × 0.90 mm, Macro; 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 remission; and the measurement mode was absorption; the optical filter was second order.

Densitometric scanning was then performed at: 200 and 248 nm for the chromatogram without the use of a visualizing reagent; and multi wavelength in the range of 250 to 400 nm, at change of wavelength at every step of 25 nm for the chromatogram with the use of sulphuric acid as visualizing reagent. 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 to 800 nm. The slit dimensions were 8.00 × 0.90 mm, Macro; the optimized 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.

Separation Factors

The chromatograms were done in triplicate and each track was scanned three times, and the mean of the R_F values were calculated. The separation factors, namely: $\triangle R_F$ values, selectivity (α),[10] and constant of the pair separation (R_F^{α})[11] were calculated for all the densitograms.

$\triangle R_F$ was calculated according to the formula:

$$\Delta R_{F(1,2)} = R_{F1} - R_{F2} \tag{1}$$

where R_{F1} and R_{F2} are the R_F values of two adjacent peaks on the densitogram; and $R_{F1} > R_{F2}$.

The selectivity (α) was calculated using the equation:

$$\alpha = \frac{1/R_{F1} - 1}{1/R_{F2} - 1} \tag{2}$$

where R_{F1} and R_{F2} are the R_F values of two adjacent peaks on the densitogram; and $R_{F1} < R_{F2}$.

The constant of the pair separation (R_F^{α}) was calculated for the investigated compounds as the ratio of the R_F values of the two adjacent peaks on the densitogram:

$$R^{\alpha}_{F(1,2)} = \frac{R_{F1}}{R_{F2}} \tag{3}$$

where R_{F1} and R_{F2} are the R_F values of two adjacent peaks on the densitogram; and $R_{F1} > R_{F2}$.

Resolution Factor

Densitometric Method of R_S Calculation

The peak resolution ($R_{S(b)}$) was calculated using the equation:[12,13]

$$R_{s(b)} = \frac{2d}{W_{b1} + W_{b2}} \tag{4}$$

where d is the distance between the centers of two adjacent peaks on the densitogram, whereas w_{b1} and w_{b2} are the peaks width at the base.

RESULTS AND DISCUSSION

The separation of hydrocortisone (H), estradiol (E), testosterone (T), and cholesterol (CH) were preformed on DiolF_{254} plates using chloroform as

mobile phase. The dried plates were densitometric analyzed without the use of a visualizing reagent and after the use of sulphuric acid solutions as visualizing reagents. The solutions of sulphuric acid in methanol in different volume compositions were used to detect investigated compounds. Chromatographic plates with separated compounds were dipped from 5 to 30 s in particular sulphuric acid solutions and then heated at temperature from 80°C to 140°C for times ranging from 5 to 30 min. The best detection conditions for high signal intensity [AU] of the separated steroid spots were determined. Particularly robust and sensitive detection of investigated compounds separated was observed by dipping the plate for 15 s in the solution of sulphuric acid in methanol in the volume composition 1:19 and for temperature equal to 120°C and heating for at 10 min. The spectrodensitometric characteristic of separated compounds on $DiolF_{254}$ plates without the use of a visualizing reagent and after the application of methanolic solution of sulphuric acid in the volume composition 1:19 and heating at 120°C for 10 min are presented in Table 1. Without the use of a visualizing reagent the fundamental

Table 1. Spectrodensitogram characteristics of investigated compounds without using visualizing reagents and after using sulphuric acid as visualizing reagents

	Spectrodensitogram characteristic					
	Without using visualizing reagent			After application of sulphuric acid as visualizing reagent		
	Fundamental absorption band	Remaining absorption bands		Fundamental absorption band	Remaining absorption bands	
Compound	λ_{max} (nm)[a]	λ (nm)	Intensity (AU)	λ_{max} (nm)[1]	λ (nm)	Intensity (AU)
Hydrocortisone	248	–	–	252	307	81.6
					371	71.0
		221	33.1			
Estradiol	200	282	19.5	273	293	94.3
		399	3.5		541	91.4
Testosterone	248	–	–	251	373	84.8
					210	82.5
					246	93.4
Cholesterol	200	399	3.5	399	273	88.2
					452	73.0
					543	64.7

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

absorption bands of estradiol, and cholesterol occur at the wavelength equal to 200 nm. However, the hydrocortisone, and testosterone have the fundamental absorption bands at the wavelength equal to 248 nm. After the application of sulphuric acid in methanol (1:19, v/v; the plate was immersed in dipping the solution of sulphuric acid for 15 s, and it was then heated to 120°C for 10 min) as visualizing reagent, the fundamental absorption bands of hydrocortisone, estradiol, testosterone, and cholesterol occur at the wavelengths equal to 252, 273, 251, and 399, respectively. It was found that the spectrodensitograms of hydrocortisone, estradiol, testosterone, and cholesterol on DiolF_{254} plates and by the use of sulphuric acid as visualizing reagents are different than the spectrodensitograms obtained on the plate without the use of a visualizing reagent. The obtained spectrodensitograms of investigated compounds after the detection with the use of sulphuric acid as visualizing reagent differ in the number and intensity of additional absorption bands. This fact has analytical and pharmaceutical significance in the identification of hydrocortisone, estradiol, testosterone, and cholesterol. The colors of chromatographic spots for the investigated compounds and background colors, without the use of a visualizing reagent and after the detection with sulphuric acid in the optimal conditions, are presented in Table 2. It was stated, that studied compounds without the use of a visualizing reagent are invisible on the chromatogram in visible light. The spots of investigated compounds after the detection with the use of sulphuric acid as visualizing reagent are visible on the chromatograms. All obtained chromatographic spots of investigated compounds after the application of sulphuric acid as visualizing reagent were durable and visible for over 2 weeks. The retention

Table 2. The separation factors of studied compounds[a] and the colors of spots after the application of sulphuric acid[b] as visualizing reagent

	Separation factors				Color of spot on light beige background
	R_F	ΔR_F	$R_F{}^{\alpha}$	α	
Hydrocortisone	0.02				beige
Estradiol	0.18	0.16	9.00	10.76	reddish-violet
Testosterone	0.28	0.10	1.56	1.77	light brown
Cholesterol	0.46	0.18	1.64	2.19	beige

[a]mobile phase: chloroform.
[b]Sulphuric acid in methanol in volume composition 1:19; the plate was immersed in dipping solution of sulphuric acid for 15 s, and it was then heated to 120°C for 10 min.

parameter R_F, and separation parameters ΔR_F, α, and R_F^{α} are also presented in Table 2.

In a further part of this work, densitometric analysis of the examined compounds without the use of a visualizing reagent and after the use of sulphuric acid as visualizing reagent were performed. Densitograms of investigated compounds (H – hydrocortisone, E – estradiol, T – testosterone, CH – cholesterol), at the wavelengths 200, and 248 nm after their separation using a chloroform as mobile phase and without the use of a visualizing reagent are presented in Figure 1. On the densitogram obtained at the wavelength 248 nm occur three bands from hydrocortisone (H), estradiol (E), and testosterone (T). This denstiogram has no band of cholesterol. It results from the fact, that the spectrodensitogram of cholesterol is extremely scanty. However, the densitometric band of estradiol has weak intensity. The densitogram bands of all investigated

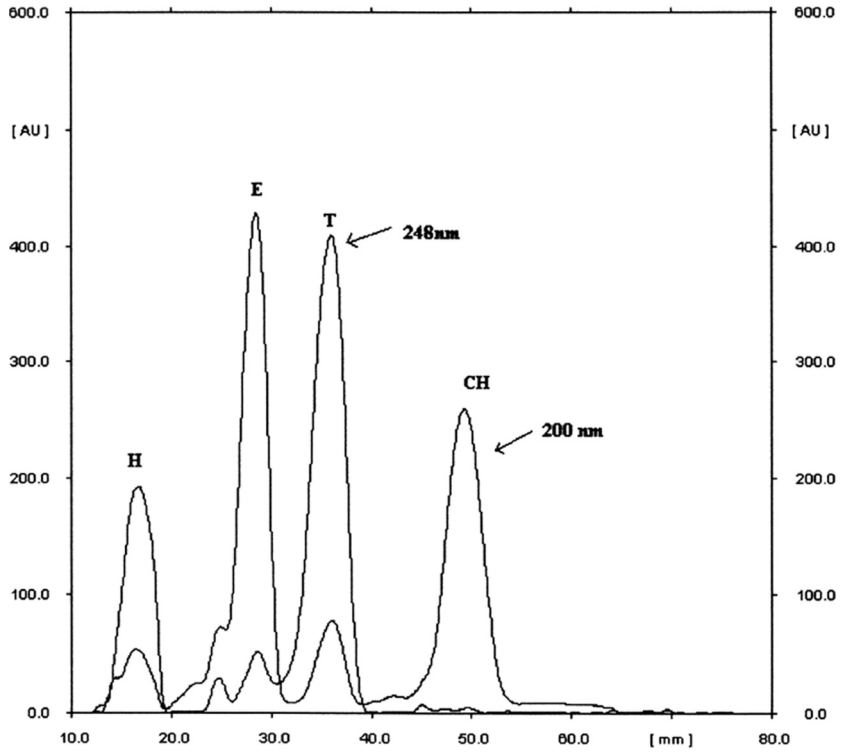

Figure 1. Densitograms of investigated compounds (H – hydrocortisone, E – estradiol, T – testosterone, CH – cholesterol) at the wavelengths 200, and 248 nm after their separation using a chloroform as mobile phase and without the use of a visualizing reagent.

compounds were obtained (without the use of a visualizing reagent) at the wavelength 200 nm. A three dimensional densitogram of investigated compounds at the wavelengths 250, 275, 300, 325, 350, 375, and 400 nm after their separation using a chloroform as mobile phase and after the application of sulphuric acid in methanol (1:19, v/v; the plate was immersed in the dipping solution of sulphuric acid for 15 s, and it was then heated to 120°C for 10 min) as visualizing reagent is presented in Figure 2.

The characteristics of the obtained densitometric bands are presented in Tables 3 and 4. Characteristics of the chromatographic band were realized using the densitometric method by the determination of peak height (h), peak area (A), and the angle (β) between the tangents at the inflection points to the curves of the densitometric peak. The heights and areas of chromatographic bands obtained at different wavelengths have differentiated values. The densitometric bands with the largest area and height, as well as the smallest angle, were obtained

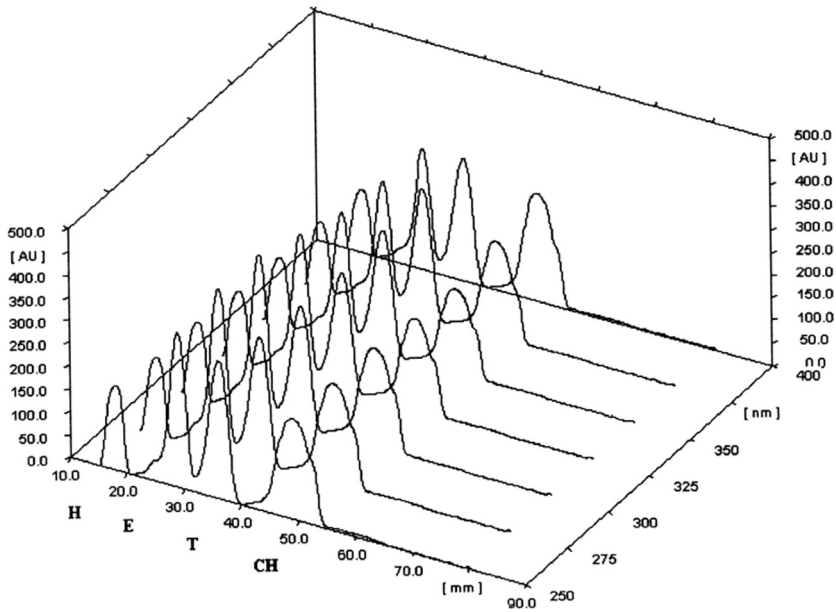

Figure 2. 3D densitograms of investigated compounds (H – hydrocortisone, E – estradiol, T – testosterone, CH – cholesterol) at the wavelengths 250, 275, 300, 325, 350, 375, and 400 nm after their separation using a chloroform as mobile phase and after the application of sulphuric acid in methanol (1:19, v/v; the plate was immersed in dipping the solution of sulphuric acid for 15 s, and it was then heated to 120°C for 10 min) as visualizing reagent.

Table 3. Characteristic of densitometric band[a] of investigated compounds separeted using chloroform as mobile phase and on the basis of densitometric analysis at wavelengths equal to 200 and 248 nm without using visualizing reagent

	200 nm			248 nm		
	h (AU)	A (AU)	β (°)	h (AU)	A (AU)	β (°)
Hydrocortisone	53	1937	48.0	194	6741	11.0
Estradiol	425	13140	5.5	52	1474	42.0
Testosterone	78	2653	30.5	413	16126	7.0
Cholesterol	258	11588	12.0	–	–	–

[a]h – high of densitometric band; A – area of densitometric band; β - the angle between the tangents at the inflection points to the curves of the densitometric band, formulated in degrees (°).

at wavelengths in the neighborhood of absorption maximum (λ_{max}) for the particular substances investigated.

The resolution R_s values after the separation of investigated compounds using a chloroform as mobile phase and without the use of a visualizing reagent, as well as after using the sulphuric acid solution as visualizing reagent, are presented in Table 5. It was stated, that the resolution R_s values are similar at the wavelengths 250, 275, 300, 325, 375,

Table 4. Characteristic of densitometric bands[a] of investigated compounds separeted using chloroform as mobile phase and after using sulphuric acid[b] as visualizing reagent

	Hydrocortisone			Estradiol			Testosterone			Cholesterol		
λ (nm)	h (AU)	A (AU)	β (°)	h (AU)	A (AU)	β (°)	h (AU)	A (AU)	β (°)	h (AU)	A (AU)	β (°)
250	190	5924	7.0	345	12732	6.0	307	12183	7.0	225	15610	13.0
275	164	5110	8.5	353	13268	5.5	270	10432	8.5	214	14516	15.5
300	160	4989	8.0	345	13182	5.5	257	9841	9.0	210	14238	16.0
325	153	4764	8.5	317	12024	6.0	258	9962	8.5	199	13397	16.5
350	145	4519	9.0	282	10548	7.0	268	10433	8.5	185	12191	18.5
375	149	4632	9.0	279	10446	7.0	288	11441	8.0	216	13826	16.0
400	136	4227	8.0	262	9807	7.0	267	10518	7.0	235	14841	12.0

[a]h – high of densitometric band; A – area of densitometric band; β – the angle between the tangents at the inflection points to the curves of the densitometric band, formulated in degrees (°).

[b]Sulphuric acid in methanol in volume composition 1:19; the plate was immersed in dipping solution of sulphuric acid for 15 s, and it was then heated to 120°C for 10 min.

Table 5. Resolution R_s values after the separation of investigated compounds using a chloroform as mobile phase obtained without the use a visualizing reagent and after using the sulphuric acid solution[a] as visualizing reagent

	Without using visualizing reagent		After using sulphuric acid as visualizing reagent						
	Resolution factor R_S at the wavelength equal to (nm)								
	200	248	250	275	300	325	350	375	400
$R_{S(H,E)}$	2.09	2.35	2.11	2.03	2.03	2.03	2.03	1.98	1.90
$R_{S(E,T)}$	1.41	1.37	1.22	1.22	1.22	1.22	1.18	1.18	1.18
$R_{S(T,CH)}$	2.15	–	1.40	1.46	1.47	1.51	1.47	1.47	1.51

[b]Sulphuric acid in methanol in volume composition 1:19; the plate was immersed in dipping solution of sulphuric acid for 15 s, and it was then heated to 120°C for 10 min.

and 400 nm for the particular separated compounds analyzed after the use of sulphuric acid solution as visualizing reagent.

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