VALIDATION OF TLC – DENSITOMETRIC METHOD FOR IDENTIFICATION AND DETERMINATION OF ESTRADIOL

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Abstract: The aim of current study was the validation of TLC densitometric method for identification and determination of Estradiol hemihydrate in dosage forms. The TLC conditions were: glass plates with Silicagel $G_{60}F_{254}$; mobile phase: chloroform : water = 90 : 10 v/v. Linearity accordance between the concentration and spot area in range: 5.10^{-4} g/ml ÷ 5.10^{-3} g/ml is proved by the regression equation: y = 53256970.x – 7007. The results of the recovery studies presented in R [%] ± RSD [%] suit respective confidence interval: 1) $RC_{1.5}$: 98.75 % ÷ 102.13 % (SD = 1.01); 2) RC_2: 98.73 % ÷ 102.93 % (SD = 1.25); 3) $RC_{2.5}$: 98.29 % ÷ 103.03 % (SD = 1.4). All data for the obtained quantity of Estradiol hemihydrate correspond to the confidence interval: 1.95 mg ÷ 2.09 mg (SD = 0.05). The proposed validated TLC densitometric method is appropriate for quality control of Estradiol hemihydrate in commercially available tablets.

Key words: TLC densitometry, Estradiol hemihydrate, validation

Introduction

Osteoporosis is characterized by a systemic impairment of bone mass [1] and is associated with large increases in bone resorption caused by enhanced osteoclasts formation and activity and reduced osteclasts apoptosis [2]. Estrogen deficiency increases the risk of osteoporosis [3]. Hormone replacement therapy for prevenition of bone loss includes a combination of **E**stradiol and Progesterone [4].

Several methods have been reported for determination of 17 β-Estradiol: 1) high performance liquid chromatography [5]; 2) gas chromatography with flame ionization detector in plasma [6]; 3) TLC on Silicagel $G_{60}F_{254}$, mobile phase: benzene : methanol = 9 : 1 v/v [7]; 4) first derivative zero-crossing spectrophotometry at λ = 208 nm for 17β-Estradiol and at λ = 282 nm for Drospirenone [8]; 5) chemiluminescent method [9]; 6) voltametry [10]; 7) electrochemiluminescence immunoassay in human serum and plasma [11]; 8) enzyme-linked immunosorbent assay in plasma [12] and immunoassays in aquatic environment [13].

Literature review reveals that HPLC methods are very often applied for the estimation of Estradiol hemihydrate [14]. A normal phase HPLC-MS/MS method with atmospheric pressure photoionization for simultaneous determination of several estrogenic steroid hormones is implemented [15]. A reverse-phase HPLC for simultaneous determination of 17β-Estradiol and Drospirenone is based on the separation of analytes on a C_{18} column with a mobile phase: acetonitrile : water = 70 : 30 v/v, UV-detection at λ = 279 nm [8].

For the simultaneous quantitation of Estradiol and Estrone after solid-phase extraction from serum with a Sep Pak C_{18}, a combination of HPLC with radioimmunoassay is developed [16]. For the identification and quantification of 17β-Estradiol and 17α-Ethinylestradiol in surface waters Ultra HPLC coupled to MS/MS is proposed [17].

For analysis of Estradiol are reported electrochemical methods by: 1) linear sweep voltammetry with reduced graphene oxide/dihexadecylphosphate electrode [18]; 2) electropolymerized pyrrole on gold nanoparticles-graphene modified glassy carbon electrode [19]; 3) poly(L-serine)-modified electrode [20].

The aim of current study was the validation of TLC densitometric method for analysis of Estradiol hemihydrate in dosage forms.

Materials.

1. Substances: Estradiol hemihydrate reference substance batch N: D00 166536.
2. Reagents: chloroform (Sigma Aldrich, N: SZBD 074SV UN 1888); 99.98 % ethanol (Sigma Aldrich, N: SZBD 0500V UN 1170), distilled water.
3. TLC developing solvent: chloroform : distilled water = 90 : 20 v/v.

Reference substances and reagents used were of analytical grade quality.

Method: TLC densitometry.

I. Instrumentation.

1. Densitometer – VILBER LOURMAT CN-15.LC Serial : 16263
2. Sample applicator – 10 μl micropipette (Hamilton, Bonaduz, Switzerland, N : 18005 701)
3. TLC chamber (outer dimensions: 22 cm x 12 cm x 22 cm).
4. TLC glass plates precoated with Silicagel $G_{60}F_{254}$, 20 cm x 20 cm (Sigma Aldrich, N: 2364681).

II. Preparation of standard solutions of Estradiol hemihydrate for validation of TLC method for analytical parameter linearity.

For validation of method for analytical parameter linearity an accurately weighed quantity of reference standard Estradiol hemihydrate: 5 mg, 10 mg, 15 mg, 20 mg, 25 mg, 30 mg, 35 mg, 40 mg, 45 mg, 50 mg was dissolved in separate volumetric flask of 10.0 ml in 99.98 % ethanol to obtain solutions with concentration correspondingly: 5.10^{-4} g/ml; 1.10^{-3} g/ml; $1.5.10^{-3}$ g/ml, 2.10^{-3} g/ml, $2.5.10^{-3}$ g/ml, 3.10^{-3} g/ml, $3.5.10^{-3}$ g/ml, 4.10^{-3} g/ml, $4.5.10^{-3}$ g/ml, 5.10^{-3} g/ml.

III. Preparation of model mixtures for validation of TLC method for analytical parameter accuracy.

An accurately weighed quantity equivalent respectively to 15 mg, 20 mg, 25 mg of reference standard Estradiol hemihydrate was dissolved in separate volumetric flask of 10.0 ml in 99.98 % ethanol to obtain for 3 samples from 3 different model mixtures with content of Estradiol hemihydrate respectively equivalent to level of 80 % (1.5 mg/ml, I), 100 % (2 mg/ml, II) and 120 % (2.5 mg/ml, III) of theoretical concentration in tablets (2 mg).

IV. Preparation of model mixtures for validation of TLC method for analytical parameter precision (repeatability).

An accurately weighed quantity equivalent to 20 mg of reference standard Estradiol hemihydrate was dissolved in 6 separate volumetric flask of 10.0 ml in 99.98 % ethanol to obtain 6 model mixtures with content of Estradiol hemihydrate equivalent to level of 100 % (2 mg/ml) of theoretical concentration in tablets (2 mg).

V. Chromatographic procedure.

Densitometric scanning was performed on scanner VILBER LOURMAT CN-15 LC Serial: 16263, operated in the absorbance mode at $\lambda = 254$ nm. From all solutions 10 μl aliquot parts were spotted onto Silicagel $G_{60}F_{254}$ plates keeping 10 mm distance between bands. The migration distance of the mobile phase in all experiments was 12 mm. The plate was developed about 1 h min at 25 ± 1°C in ascending vertical manner in glass chamber, previously presaturated for 1 h with mobile phase: chloroform : destilled water = 90 : 10 v/v. The developed plates were dried on air.

Results and discussion.

The TLC densitometric method was validated in accordance with analytical parameters: selectivity, linearity, limit of detection, limit of quantitation, accuracy and precision as per ICH guidelines [21].

I. Selectivity.

In the same manner like the standard solution, placebo solution, without the active substance Estradiol hemihydrate was prepared. By the fact that on chromatogram with placebo preparation did not exist spot with Rf, corresponding to Rf of Estradiol hemihydrate (0.64), the selectivity of the applied method was confirmed.

II. Linearity.

For the investigation of analytical parameter linearity solutions with increasing concentration of reference standard Estradiol hemihydrate were prepared. Solution were analyzed separately by the written TLC densitometric method.

On Fig. 1. is illustrated the chromatogram for linearity for Estradiol hemihydrate.

The data for value of the spot area for every concentration (C) in g/ml were measured and were plotted against the corresponding concentration. Linear regression analysis was performed. The regression calibration curve was built. The proportional accordance between corresponding concentration and spot area in range: 5.10^{-4} g/ml ÷ 5.10^{-3} g/ml is proved by the obtained regression equation $y = 53256970.x - 7007$. The calculated correlation coefficient (R^2) was 0.994.

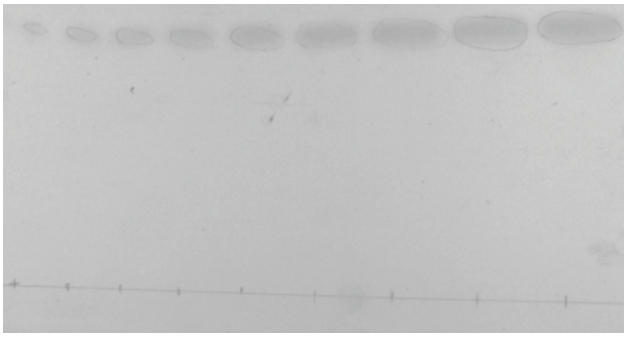

Fig. 1. Chromatogram for linearity for Estradiol hemihydrate.

On Fig. 2. is presented the calibration curve for linearity.

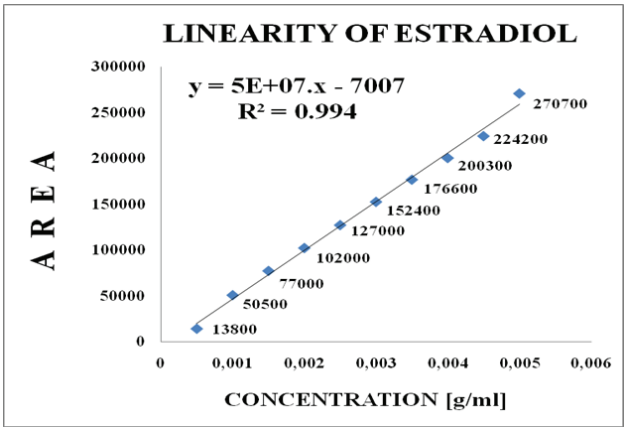

Fig. 2. Calibration curve for linearity for Estradiol hemihydrate.

III. Limit of detection (LOD) and limit of quantitation (LOQ).

The limit of detection (LOD) and limit of quantification (LOQ) were obtained by calculating using the standard formula as per the ICH guidelines. LOD is the smallest concentration of the analyte that gives the measurable response and was calculated as a ratio between SD of the response (6309) and the slope of calibration curve (53256970).

$$LOD = \frac{3.3x6309}{53256970} = 3.91.10^{-4}\, g/ml$$

LOQ is the smallest concentration of the analyte, which gives response that can be accurately quantified.

$$LOQ = \frac{10x6309}{53256970} = 1.18.10^{-3}\, g/ml$$

IV. Accuracy for model mixtures with reference standard Estradiol hemihydrate.

For the estimation of analytical parameter accuracy the recovery study was carried out by applying the method in triplicate to every to 3 different model mixtures containing known amount of Estradiol hemihydrate added separately by the standard addition method respectively at level of 80 %, 100 % and 120 % of labeled content in tablets. On Fig. 3. is shown the chromatogram for accuracy of Estradiol hemihydrate.

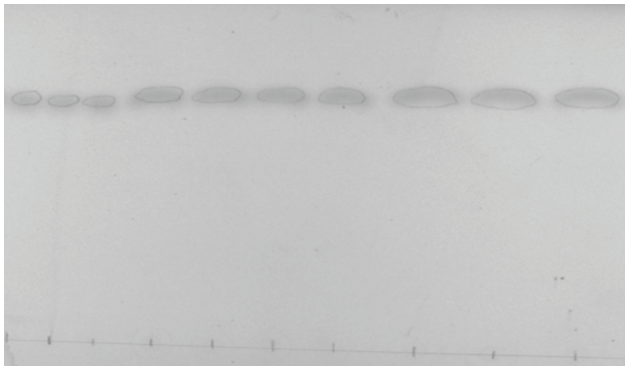

Fig. 3. Chromatogram for accuracy of Estradiol hemihydrate.

On Table 1. for model mixtures are summarized data for spot area (A) and Chauvenet's criterion for A (U_A).

For all of the obtained results for spot area is necessary to estimate the Chauvenet's criterion (U), because when U for one value is higher than the relevant standard criterion (USt), the result must be removed as unexpected. The relations: U < 1.68 (Table 1.) show, that all experimental results for U_A are lower, than standard requirement: Umax = 1.68 (n = 3) and it isn't necessary to remove data for spot area.

The content of Estradiol hemihydrate in model mixtures is determined by method of calibration curve using the regression equation. On Table 2. are indicated: C – obtained quantity of Estradiol hemihydrate ($\mathbf{C}_{1.5}$, $\mathbf{C}_2$, $\mathbf{C}_{2.5}$) in model mixtures after application of densitometric method; R (%) – degree of recovery ($R\mathbf{C}_{1.5}$, $R\mathbf{C}_2$, $R\mathbf{C}_{2.5}$); UC – Chauvenet's criterion for obtained quantity Estradiol hemihydrate ($U\mathbf{C}_{1.5}$, $U\mathbf{C}_2$, $U\mathbf{C}_{2.5}$); N – number of the individual measurements (1 ÷ 3); $\bar{X}$ – arithmetical mean; SD – standard deviation; RSD – relative standard deviation (%); $S\bar{X}$ – mean quadratic error; P – confidence possibility (%); t – coefficient of Student; $\bar{X} \pm t.S\bar{X}$ –

Table 1. *Spot area and Chauvenet's criterion for spot area for model mixtures with reference standard Estradiol hemihydrate.*

N:	Model mixture I Estradiol hemihydrate 1.5 mg			Model mixture II Estradiol hemihydrate 2 mg			Model mixture III Estradiol hemihydrate 2.5 mg		
	Added quantity [mg]	A	U_A	Added quantity [mg]	A	U_A	Added quantity [mg]	A	U_A
1.	1.49	71800	1.01	1.99	98500	0.97	2.48	124200	1.02
2.	1.50	73500	0.02	2.00	100400	0.06	2.50	127100	0.05
3.	1.52	75100	0.99	2.02	102700	1.03	2.52	129600	0.97
$\bar{X}$		73467			100533			126967	
SD		1650			2103			2702	
RSD [%]		2.25			2.09			2.13	

confidence interval); E (%) – relative error [21].

The relation UC < 1.68 shows, that all experimental results for UC are lower, than standard requirement: Umax = 1.68 (n = 3).

For the assessment of accuracy is calculated sample standard deviation (SD) and relative standard deviation (SD), by the applying of the Bessel's correction, in which the denominator N − 1 (degrees of freedom) is used instead of N and in this case $(S)^2$ is an unbiased estimator for $(SD)^2$.

Analytical parameter accuracy is presented by the degree recovery R (%) ± RSD (%) [21]. Results from Table 2. showed that at confidence possibility P = 90.0 % (t = 2.92), all data for R are included in respective confidence interval: 1) $RC_{1.5}$: 98.75 % ÷ 102.13 % (SD = 1.01, RSD = 1.01 %); 2) RC_2: 98.73 % ÷ 102.93 % (SD = 1.25, RSD = 1.24 %); 3) $RC_{2.5}$: 98.29 % ÷ 103.03 % (SD = 1.4, RSD = 1.39 %). For all model mixtures values for SD and RSD are lower than 1.4 and mean quadratic error and relarive error are lower than 1.

V. Precision for model mixtures with reference standard Estradiol hemihydrate.

For the validation the TLC method in accordance with analytical parameter precision, the method was applied for 6 model mixtures containing known amount of Estradiol hemihydrates, respectively equivalent to level of 100 % (2 mg/ml) of theoretical concentration Estradiol hemihydrate in tablets (2 mg). Representative chromatogram for precision is given on Fig. 4.

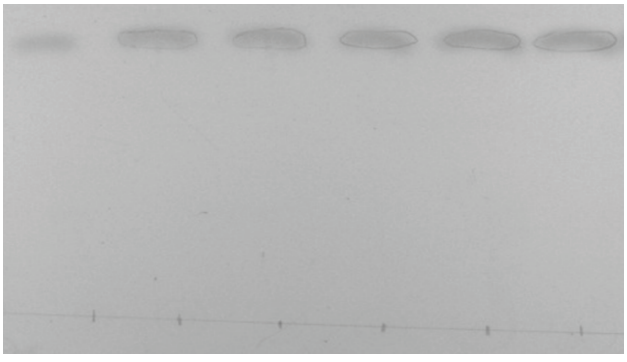

Fig. 4. Chromatogram for precision for Estradiol hemihydrate.

Table 2. *Content of Estradiol hemihydrate in model mixrutes.*

N:	Model mixture I Estradiol hemihydrate 1.5 mg			Model mixture II Estradiol hemihydrate 2 mg			Model mixture III Estradiol hemihydrate 2.5 mg		
	$C_{1,5}$ [mg]	$RC_{1.5}$ [%]	U $C_{1.5}$	C_2 [mg]	RC_2 [%]	UC_2	$C_{2/5}$ [mg]	$RC_{2/5}$ [%]	$UC_{2.5}$
1.	1.48	99.33	1	1.98	99.50	1	2.46	99.19	1.05
2.	1.51	100.67	0	2.02	101.00	0	2.52	100.80	0.10
3.	1.54	101.32	1	2.06	101.98	1	2.57	101.98	0.94
$\bar{X} \pm$ SD	1.51 ± 0.03			2.02 ± 0.04			2.52 ± 0.06		
$\bar{R}$ [%] ± RSD[%]		100.44 ± 1.01			100.83 ± 1.24			100.66 ± 1.39	
SD	0.03	1.01		0.04	1.25		0.06	1.40	
RSD [%]	1.99	1.01		1.98	1.24		2.38	1.39	
$S\bar{X}$	0.02	0.58		0.02	0.72		0.03	0.81	
P [%]	90.0	90.0		90.0	90.0		90.0	90.0	
t	2.92	2.92		2.92	2.92		2.92	2.92	
t. S $\bar{X}$	0.06	1.69		0.06	2.10		0.09	2.37	
$\bar{X} \pm$ t.S$\bar{X}$	1.45 ÷ 1.57	98.75 ÷ 102.13		1.96 ÷ 2.08	98.73 ÷ 102.93		2.43 ÷ 2.61	98.29 ÷ 103.03	
E [%]	1.32	0.58		0.99	0.71		1.19	0.80	

The content of Estradiol hemihydrate in 6 model mixtures is determined by method of calibration curve using the regression equation. On Table 3. are indicated: spot area (A), Chauvenet's criterion for A (U_A), C – obtained quantity of Estradiol hemihydrate (C_2) in model mixtures after application of densitometric method; R (%) – degree of recovery (RC_2); UC – Chauvenet′s criterion for obtained quantity Estradiol hemihydrate (UC_2); N – number of the individual measurements (1 ÷ 6); $\bar{X}$ – arithmetical mean; SD – standard deviation; RSD – relative standard deviation (%); $S\bar{X}$ – mean quadratic error; P – confidence possibility (%); t – coefficient of Student; $\bar{X}$± t.S$\bar{X}$ – confidence interval); E (%) – relative error [21].

For all of the obtained results for spot area and for content of Estradiol hemihydrate the calculated Chauvenet's criterion (U) is lower than standard Chauvenet's criterion (U = 1.73; N = 6) in analysis of 6 samples.

For the estimation of an analytical parameter precision (repeatability) is used the uncertainty of the result, which is determined by SD, RSD and confidence range [21].

From Table 3. it is obvious that at confidence possibility 90.0 % (t = 2.92), all results for obtained quantity of Estradiol hemihydrate suit respective confidence interval: C_2: 1.95 mg ÷ 2.09 mg (SD = 0.05, RDS = 2.48).

Table 3. *Content of Estradiol hemihydrate in model mixrutes for estimation of precision.*

N:	Added content [mg]	A	UA	C_2 [mg]	RC_2 [%]	UC_2
1.	1.97	97300	1.31	1.96	99.49	1.2
2.	1.98	97900	1.06	1.97	99.49	1.0
3.	2.00	100500	0.04	2.02	101.00	0
4.	2.00	101200	0.33	2.03	101.50	0.2
5.	2.02	102600	0.92	2.06	101.98	0.8
6.	2.02	103000	1.09	2.07	102.48	1.0
$\bar{X}$ ± SD		100417		2.02 ± 0.05		
$\bar{R}$ [%] ± RSD[%]					100.99 ± 1.25	
SD		2371		0.05	1.26	
RSD [%]		2.36		2.48	1.25	
S$\bar{X}$				0.02	0.51	
P [%]				98.0	98.0	
t				3.37	3.37	
t. S $\bar{X}$				0.07	1.72	
$\bar{X}$ ± t.S$\bar{X}$				1.95 ÷ 2.09	99.27 ÷ 102.71	
E [%]				0.99	0.51	

Conclusion.

The proposed validated TLC densitometric method is appropriate for routine quality control of Estradiol hemihydrate in commercially available tablets. The results for accuracy presented by the degrees of recovery are: 1) $RC_{1.5}$: 100.44 % ± 1.01; 2) RC_2: 100.83 ± 1.24; 3) $RC_{2.5}$: 100.66 ± 1.39. All data for obtained content of Estradiol hemihydrate in model mixtures for precision correspond to confidence interval: C_2: 1.95 mg ÷ 2.09 mg (SD = 0.05, RDS = 2.48).

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