Original Article

Application of TLC-Densitometry for Analysis of Estradiol Hemihydrate in Dosage Forms

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ABSTRACT

The aim of current study was the application of validated TLC-densitometric method for identification and determination of Estradiol hemihydrate in dosage forms. The applied TLC conditions were: Silicagel $G_{60}F_{254}$ glass plates; mobile phase: chloroform : acetone = 90 : 10 v/v, migration distance of mobile phase: 120 mm, UV-detection at λ = 254 nm. All of the experimental results for the content of Estradiol hemihydrate correspond to the respective confidence interval: Estrofem *table*: 1.78 mg ÷ 2.12 mg; Femoston F1 *table*: 1.88 mg ÷ 2.2 mg; Femoston F2 *table*: 1.99 mg ÷ 2.19 mg; Trisequens T1 *table*: 1.78 mg ÷ 2.18 mg; Trisequens T2 *table*: 1.92 mg ÷ 2.12 mg; Trisequens T3 *table*: 0.97 mg ÷ 1.17 mg. The proposed validated TLC-densitometric method is appropriate for quality control of Estradiol hemihydrate in commercially available tablets.

Key words: Estradiol hemihydrate, TLC, Densitometry, Tablets, analysis, Determination.

INTRODUCTION

Osteoporosis is designated as the third socially significant disease in the world, after cardiovascular and oncological diseases and the forecast is to take second place in 2020.[1] Osteoporosis is caused by the reduced levels of estrogen, which in postmenopausal women are 1/10 of the levels in premenopausal women.[2] Lowered levels of estrogen lead to: 1) increase of oxidative stress; 2) apoptosis of osteoblasts; 3) rapid loss of bone mass due to increased rate of degradation of the bone tissue by osteoclasts; 4) a long life of osteoclasts compared to osteoblasts; 5) reduction of the absorption and utilization of calcium in bones.[3] Most pharmacological agents used in the prevention and treatment of osteoporosis reduce bone resorption or delay the total rate of bone turnover. For the prevention of fractures are applied: inhibitors of the activity of osteoclasts – bisphosphonates Rizedronate and Zoledronate,[4] selective estrogen receptor modulators Bazedoxifene[5] and Lasofoxifene[6] parathyroid hormone,[7] Strontium ranelate[8] and anti-resorptive agent Denosumab – a human monoclonal antibody IgG_2.[9] Bisphosphonates cause adverse effects on gastrointestinal tract[10]: nausea, heartburn, scleritis and iritis (Alendronate).[11] Selective estrogen receptor modulators Bazedoxifene[12] and Raloxifene[13] reduce resorption activity by inhibition of the production of interleukin 6, tumor necrosis factor α and the number of osteoclasts. Preclinical studies indicate that Strontium ranelate has a dual mechanism of action: 1) induces the bone formation by synthesis of bone collagen, alkaline phosphatase and osteocalcin; 2) inhibits the osteoclastogenesis by suppressing the differentiation and activity of osteoclasts.[14]

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Hormonal therapy reduces the risk of osteoporotic fractures and improves the bone mineral density.[15,16]

Several methods have been reported for the determination of 17β-Estradiol: UV-spectrophotometry, high performance liquid chromatography (HPLC)[17] thin layer chromatography (TLC)[18] and voltametry.[19] Literature review reveals that HPLC methods are very often applied for the estimation of Estradiol hemihydrate.[17]

For the quantification of 17β-Estradiol alone or in combinations in different pharmaceutical preparations are developed the following methods: I) TLC on Silicagel $G_{60}F_{254}$, mobile phase: benzol : methanol = 9 : 1 v/v:[18] 17β-Estradiol; II) RP-HPLC with UV-detection: 1) column Phenomenex C_{18} (4.6 mm x 250 mm x 5 μm), mobile phase: methanol : water = 70 : 30 v/v, flow rate: 1.0 ml/min., λ = 281 nm,[17] 2) 17β-Estradiol, Estriol and Estrone: column C_{18} micro Bondapak, isocratic mode, mobile phase: acetonitrile : water = 50 : 50 v/v, flow rate: 1 ml/min., column temperature: 30 °C, λ = 205 nm,[20] 3) 17β-Estradiol and its degradation products in Estrogel: column Zorbax SB-CN (4.6 mm x 150 mm x 5 μm), mobile phase: acetonitrile : 0.085% phosphoric acid : tetrahydrofurane = 27 : 63 : 10 v/v, flow rate: 1.0 ml/min., λ = 225 nm,[21] 4) 17β-Estradiol and Estrone in Estrogel: column Supelco Discovery C_{18} (250 mm x 3.0 mm), mobile phase: acetonitrile : methanol : water = 23 : 24 : 53 v/v, flow rate: 0.9 ml/min., λ = 225 nm,[22] III) HPLC with fluorimetric detection: 17β-Estradiol and Estriol at λexcitation = 280 nm and λemission = 312 nm,[23] IV) NP-HPLC with mas detection with atmospheric pressure photoionization,[24] V) fluorimetry after derivatization reaction with dansylchloride,[25] VI) electrochemical methods with: 1) glass carbon electrode, modified with electropolymerized pyrrole,[26] 2) poly(L-serine)-film-modified electrode,[27] 3) linear sweep voltammetry.[28]In our previous work TLC-densitometric method was validated in accordance with analytical parameters: linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy and precission (repeatability).[29] The aim of current study was the application of the validated TLC-densitometric method for identification and determination of Estradiol hemihydrate in dosage forms.

MATERIALS

Drug products tablets

Estrofem *table* (EF70064, Nono Nordisk, Danemark): Estradiol hemihydrate 2 mg

Femoston F1 *table* (341141, Abbott, Netherlands): Estradiol hemihydrate 2 mg

Femoston F2 *table* (341141, Abbott, Netherlands): Estradiol hemihydrate 2 mg; Didrogesterone 10 mg

Trisequence T1 *table* (DF 70298, Nono Nordisk, Danemark): Estradiol hemihydrate 2 mg

Trisequence T2 *table* (DF 70298, Nono Nordisk, Danemark): Estradiol hemihydrate 2 mg; Noretisterone acetate 1 mg

Trisequence T3 *table* (DF 70298, Nono Nordisk, Danemark): Estradiol hemihydrate 1 mg

Reference standard: Estradiol hemihydrate substance, batch N: D00 166536.

Reagents with analytical grade quality: chloroform (Sigma Aldrich, N: SZBD 074SV UN 1888); acetone (Sigma Aldrich, N: SZBC 1861 SV); 99.98 % ethanol (Sigma Aldrich, N: SZBD 0500V UN 1170), distilled water.

METHODS

TLC-densitometry

Instrumentation: Densitometer VILBER LOURMAT CN-15.LC Serial: 16263; sample applicator 10 ml micropipette (Hamilton, Bonaduz, Switzerland, N:18005701); TLC glass chamber (22 cm x 12 cm x 22 cm); stationary phase: TLC glass plates, 20 cm x 20 cm (Sigma Aldrich, N: 2364681) were used.

Chromatographic conditions: stationary phase: TLC glass plates precoated with Silicagel $G_{60}F_{254}$, mobile phase: chloroform : acetone = 90 : 10 v/v, migration distance of mobile phase: 120 mm, UV-detection at λ = 254 nm.

Preparation of test solutions from dosage formulations (tablets), containing Estradiol hemihydrate for the investigation of analytical parameter precision (repeatability)

Separately from every drug formulation 20 tablets were weighed accurately. From homogenous powdered tablets from every drug preparation on an analytical balance accurately were weighed 6 samples, containing an amount equivalent respectively to 10 mg (Trisequence T3 *table*) and 20 mg (Estrofem *table*, Femoston F1 *table*, Femoston F2 *table*, Trisequence T1 *table*, Trisequence T2 *table*) Estradiol hemihydrate. Every sample was transferred separately to a 10.0 ml volumetric flask and was diluted with 99.98% ethanol. The solutions were filtered through a blue band filter and were analyzed by the densitometric method described.

Chromatographic procedure: Chromatographic analysis was achieved by using glass TLC plates. From every solution separatelly were spotted aliquot parts of 10 μl onto glass plates Silicagel $G_{60}F_{254}$, keeping 10 mm

distance between bands. The plate was developed about at 25 ± 1°C in ascending vertical manner in glass chromatographic chamber, previously presaturated for 1 h with mobile phase: chloroform: acetone = 90 : 10 v/v. The migration distance of the mobile phase in all experiments was 120 mm. The developed plates were dried on air. Densitometric scanning was performed on scanner VILBER LOURMAT CN-15 LC, operated in the absorbance mode at λ = 254 nm.

RESULTS

In recent work the validated TLC-densitometric method was applied for identification and determination of Estradiol hemihydrate in dosage pharmaceutical formulations–tablets. The obtained by TLC-densitometric method chromatograms of tablets were presented as follows: Estrofem *table*, Femoston F1 *table*, Femoston F2 *table* (Figure 1), Trisequens T1 *table* and Trisequens T2 *table* (Figure 2).

On Table 1 are presented the data for the values of Rf and radius r [cm] for Estradiol hemihydrate in Estrofem *table*, Femoston *table* and Trisequens *table*. On Table 2 are summarized the results for spot area (A) and Chauvenet's criterion for spot area (UA) and quantity (UC) of Estradiol hemihydrate in Estrofem *table*, Femoston *table* and Trisequens *table*. The amount of Estradiol hemihydrate in Estrofem *table* (C), Femoston F1 *table* (C_{F1}), Femoston F2 *table* (C_{F2}), Trisequens T1 *table* (C_{T1}), Trisequens T2 *table* (C_{T2}) and Trisequens T3 *table* (C_{T3}) (Table 3) was determined by method of calibration curve.

DISCUSSION

In our previous work a TLC-densitometric method was validated in accordance with International Conference on Harmonization guidelines for validation of analytical procedures for analytical parameters: limearity, LOD, LOQ, accuracy and precission (repeatability).[30] Placebo solution, containing as supplement starch, without the active substance Estradiol hemihydrate was prepared. The selectivity of the applied method was confirmed by the fact that on chromatogram with placebo preparation did not exist spot with Rf, corresponding to Rf of Estradiol hemihydrate (0.66) in reference standard solution. This fact confirms that there was no interference from the commonly present in tablets excipient starch. The calibration curve was obtained by using the data for different concentrations of standard solutions of Estradiol hemihydrate and was generated by plotting the sample concentration versus the mean peak area. Linear regression analysis was performed. Linearity accordance between the concentration and spot area in range: 5.10^{-4} g/ml ÷ 5.10^{-3} g/ml was proved by the regression equation: y = 53256970.x – 7007 (y – peak area, x – concentration of analyte). The least squares regression yielded a correlation coefficient R = 0.994. Limit of detection and limit of quantitation were determined based on the standard deviation of the response and the slope of the regression equation for the calibration curve. The limit of detection, defined the concentration giving a signal with signal to noise ratio of 3, was $3.91.10^{-4}$ g/ml. The limit of quantitation, defined the concentration giving a signal with signal to noise ratio of 10, was $1.18.10^{-3}$ g/ml. For the estimation of analytical parameter accuracy the recovery study was carried out by application of the method in triplicate to every to 3 different model mixtures, containing known amount of Estradiol hemihydrate: 75%, (1.5 mg, $C_{1.5}$); 100% (2 mg, C_2); 125% (2.5 mg, $C_{2.5}$). The content of drug in model mixtures was determined by method of calibration curve using the regression equation. For the assessment of accuracy and precision was calculated sample 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] The results for accuracy at P = 90 % (t = 2.92), presented by the degree recovery 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). For the estimation of an analytical parameter precision (repeatability) was used the uncertainty of the result, which was determined by: standard deviation (SD), relative standard deviation (RSD) and confidence range. All data for the obtained quantity of Estradiol hemihydrate correspond to the confidence interval: 1.95 mg ÷ 2.09 mg (SD = 0.05).[29]

In current study the indentity of Estradiol hemihydrate in analysed drug formulations was proved by the correspondence between the retardation factors of drug in samples and reference standard Rf = 0.65. (Table 1).

For the assessment of the need for the removal of sharply differing data is used the criterion of Chauvent. From Table 2. it is obvious that for all of the analysed tablets the calculated Chauvenet's criterion for the area of the spots (UA) and for the quantities (UC) of Estradiol hemihydrate are lower than the maximum value of the criterion (Umax = 1.73; N = 6), which proves that the results suit to the requirements of the criterion for the analysis of 6 separate samples from Estrofem *table*, Femoston F1 *table*, Femoston F2 *table*, Trisequens T1 *table*, Trisequens T2 *table* and Trisequens T3 *table*.

On Table 3. are presented the data for: N – number of individual measurements (1 ÷ 6); $\bar{X}$ – mean arithmetic

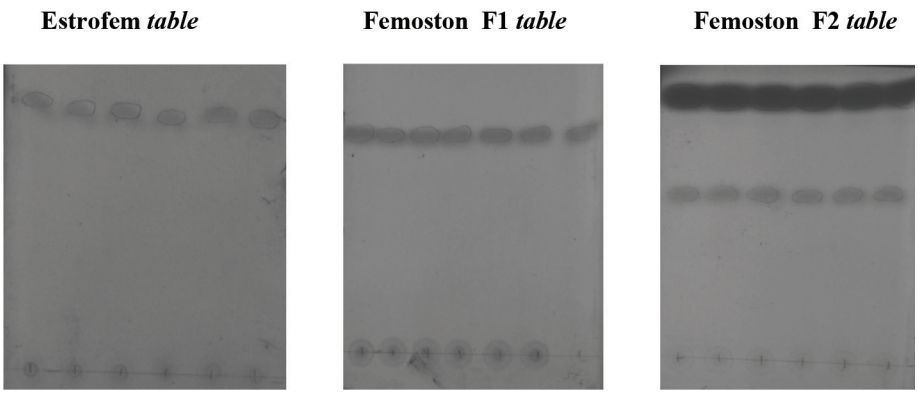

Figure 1: Chromatograms of Estradiol hemihydrate in Estrofem *table*, Femoston F1 *table* and Femoston F2 *table.*

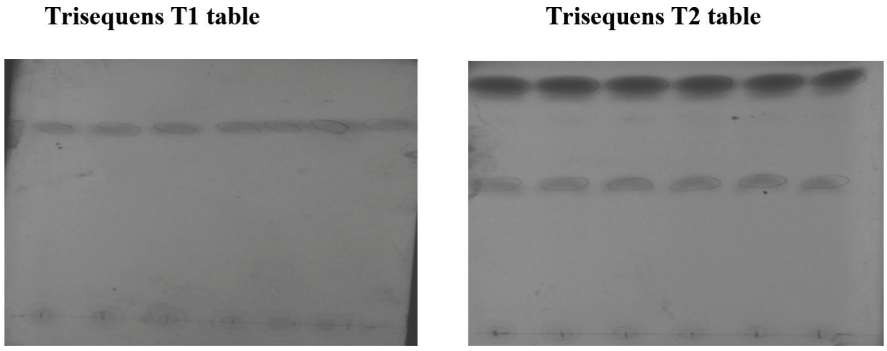

Figure 2: Chromatograms of Estradiol hemihydrate in Trisequens T1 *table* and Trisequens T2 *table.*

Table 1: Results for Rf and for Estradiol hemihydrate in Estrofem *table*, Femoston *table* and Trisequens *table*

N:	Estrofem *table*		Femoston F1 *table*		Femoston F2 *table*	
	Rf	r [cm]	Rf	r [cm]	Rf	r [cm]
1.	0.64	0.23	0.65	0.255	0.65	0.25
2.	0.64	0.23	0.64	0.23	0.66	0.26
3.	0.65	0.25	0.65	0.255	0.66	0.26
4.	0.65	0.25	0.65	0.25	0.66	0.25
5.	0.64	0.24	0.65	0.25	0.66	0.26
6.	0.65	0.25	0.64	0.24	0.65	0.25
N:	Trisequens T1 *table*		Trisequens T2 *table*		Trisequens T3 *table*	
	Rf	r [cm]	Rf	r [cm]	Rf	r [cm]
1.	0.64	0.23	0.65	0.25	0.65	0.15
2.	0.65	0.25	0.65	0.25	0.64	0.14
3.	0.63	0.225	0.64	0.24	0.65	0.15
4.	0.65	0.25	0.65	0.25	0.66	0.16
5.	0.64	0.24	0.65	0.25	0.64	0.14
6.	0.65	0.25	0.65	0.25	0.65	0.15

Table 2: Spot area and Chauvenet's criterion for the spot area (UA) and quantity (UC) of Estradiol hemihydrate in Estrofem *table*, Femoston *table* and Trisequens *table*

N:	Estrofem table			Femoston F1 table			Femoston F2 table		
	A	UA	UC	A	UA	UC	A	UA	UC
1.	88000	1.44	1.42	106200	0.81	0.82	99100	1.24	1.25
2.	91800	0.84	0.75	92900	1.45	1.45	107500	0.70	0.75
3.	100500	0.54	0.58	107300	1.0	1.0	108400	0.91	1.0
4.	102400	0.84	0.83	102700	0.22	0.18	100600	0.89	0.88
5.	95900	0.19	0.17	95500	1.01	1.09	109000	1.04	1.13
6.	104000	1.09	1.08	104000	0.44	0.36	102200	0.52	0.5
$\bar{X}$	97100			101433			104467		
SD	6318			5888			4339		
RSD [%]	6.51			5.80			4.15		
N:	**Trisequens T1 *table***			**Trisequens T2 *table***			**Trisequens T3 *table***		
	A	**UA**	**UC**	**A**	**UA**	**UC**	**A**	**UA**	**UC**
1.	93000	0.8	0.83	98100	0.83	0.83	50200	0.06	0
2.	101700	0.55	0.5	100800	0.005	0	45200	1.46	1.5
3.	88500	1.5	1.58	95900	1.52	1.5	51700	0.51	0.5
4.	103300	0.8	0.75	102400	0.5	0.5	53500	1.05	1.17
5.	97400	0.12	0.17	103200	0.75	0.83	46900	0.94	1.0
6.	105100	1.08	1.08	104300	1.09	1.17	52600	0.78	0.83
$\bar{X}$	98167			100783			50017		
SD	6444			3221			3304		
RSD [%]	6.56			3.2			6.61		

Table 3: Content of Estradiol hemihydrate in Estrofem *table* (C), Femoston *table* (C_{F1}, C_{F2}) and Trisequens *table* (C_{T1}, C_{T2}, C_{T3}).

N:	Estrofem table	Femoston table		Trisequens table		
	C	C_{F1}	C_{F2}	C_{T1}	C_{T2}	C_{T3}
1.	1.78	2.13	1.99	1.88	1.97	1.07
2.	1.86	1.88	2.15	2.04	2.02	0.98
3.	2.02	2.15	2.17	1.79	1.93	1.10
4.	2.05	2.06	2.02	2.07	2.05	1.14
5.	1.93	1.92	2.18	1.96	2.07	1.01
6.	2.08	2.08	2.05	2.11	2.09	1.12
$\bar{X} \pm$ SD	1.95 ± 0.12	2.04 ± 0.11	2.09 ± 0.08	1.98 ± 0.12	2.02 ± 0.06	1.07 ± 0.06
SD	0.12	0.11	0.08	0.12	0.06	0.06
RSD [%]	0.15	5.39	3.83	6.06	2.97	5.61
$S_{\bar{X}}$	0.05	0.04	0.03	0.05	0.024	0.024
P [%]	98.0	99.0	98.0	99.0	99.0	99.0
t	3.37	4.03	3.37	4.03	4.03	4.03
$t.S_{\bar{X}}$	0.17	0.16	0.1	0.2	0.1	0.1
$\bar{X} - t.S_{\bar{X}}$; $\bar{X} + t.S_{\bar{X}}$	1.78 ÷ 2.12	1.88 ÷ 2.2	1.99 ÷ 2.19	1.78 ÷ 2.18	1.92 ÷ 2.12	0.97 ÷ 1.17
E [%]	2.56	1.96	1.44	2.53	1.19	2.24

error; SD – standard deviation; RSD – relative standard deviation (%); $S\overline{X}$ – mean square error; $\overline{X} \pm t.S\overline{X} = \overline{X} - t.S\overline{X} + t.S\overline{X}$ – confidence interval; E (%) – relative error. The used values for confidence possibility was P = 95 % and for coefficient of Student: t = 2.57.

CONCLUSION

The validated TLC-densitometric method was applied for the identification and determination of Estradiol hemihydrate in tablets. The analytical parameter repeatability for the content of Estradiol hemihydrate in tablets was characterized with SD and RSD. The results showed that all of the obtained by method of calibration curve experimental results for the content of Estradiol hemihydrate correspond to the respective confidence interval. The proposed validated TLC-densitometric method is appropriate for quality control of Estradiol hemihydrate in commercially available tablets.

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CONFLICT OF INTEREST

The authors contributing to this study and manuscript have no conflict of interests

SUMMARY

- The aim of current study was the application of validated TLC-densitometric method for identification and determination of Estradiol hemihydrate in dosage forms.
- The applied TLC conditions were: Silicagel $G_{60}F_{254}$ glass plates; mobile phase: chloroform : acetone = 90 : 10 v/v, migration distance of mobile phase: 120 mm, UV-detection at λ = 254 nm.
- The indentity of Estradiol hemihydrate in analysed drug formulations was proved by the correspondence between the retardation factors of drug in samples and reference standard.
- The content of Estradiol hemihydrate in tablets was determined by method of calibration curve using the regression equation.
- For the estimation of an analytical parameter precision (repeatability) was used the uncertainty of the result, which was determined by: standard deviation (SD), relative standard deviation (RSD) and confidence range.
- All of the experimental results for the content of Estradiol hemihydrate correspond to the respective confidence interval: Estrofem table: 1.78 mg ÷ 2.12 mg; Femoston F1 table: 1.88 mg ÷ 2.2 mg; Femoston F2 table: 1.99 mg ÷ 2.19 mg; Trisequens T1 table: 1.78 mg ÷ 2.18 mg; Trisequens T2 table: 1.92 mg ÷ 2.12 mg; Trisequens T3 table: 0.97 mg ÷ 1.17 mg.
- The proposed validated TLC-densitometric method is appropriate for quality control of Estradiol hemihydrate in commercially available tablets.

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