Bulgarian Chemical Communications, Volume 49, Number 2, (pp. 384 – 389) 2017

Estimation of linearity and precision of the HPLC-HILIC method for analysis of estradiol hemihydrate

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Received March 28, 201; Revised December 2, 2016

The aim of the present study was the estimation of linearity and precision of an isocratic HPLC-HILIC method with UV-detection for identification and determination of estradiol hemihydrate in pharmaceutical dosage forms. Linear regression analysis was performed. The regression calibration curve was built. Linearity accordance between concentration and peak area in the range: 3.10^{-6} g/ml ÷ 4.10^{-5} g/ml was proved by the regression equation: y = 2698.99 x – 2307.98. The least squares regression yielded a correlation coefficient R^2 = 0.999. LOD = 8.10^{-7} g/ml, LOQ = 8.10^{-6} g/ml. The results for the accuracy at P = 99 % (t = 4.03) were presented by the percent recovery R [%] within the confidence interval: RC: 97.16 % ÷ 101.84 % (RSD = 1.42).

Precision was estimated by standard deviation, relative standard deviation and confidence interval. All data for the obtained quantity of estradiol hemihydrate correspond to the confidence interval: 1.96 mg/100 ml ÷ 2.02 mg/100 ml (SD = 0.03; RSD = 1.51).

The high selectivity and efficiency of separation by HPLC-HILIC with UV-detection at λ = 230 nm in an Amino-column and the elution of non-polar analytes before the polar ones (estradiol) shortens the time for analysis and leads to high repeatability.

Keywords: HPLC-HILIC, estradiol hemihydrate, linearity, precision.

INTRODUCTION

Osteoporosis is a widespread disease throughout the world and is considered as a major risk factor for public health [1]. In osteoporosis, the imbalance between bone resorption and formation is due to the increased life cycle of osteoclasts and shortened cycle of osteoblasts [2].

Estrogens bound to the estrogen receptors in the cells suppress the degradation of bone tissue and inhibit bone resorption by regulation of the expression of RANK-receptor activator of nuclear factor kB and osteoprotegrin in osteoblasts [3].

In postmenopausal women bone resorption rate is sharply increased. The low levels of estrogen at menopause reduce the ability for absorption and utilization of calcium in the bones, stimulate osteoclasts and inhibit the function of osteoblasts [4].

Stimulators of apoptosis of osteoclasts are bisphosphonates and calcitonin [5]. The mechanism of action of bisphosphonates on bone is complex and involves a decrease in the production and activity of osteoclasts, increase of osteoclast apoptosis, resulting in specific inhibition of farnesyl pyrophosphate synthetase – an enzyme that regulates biosynthesis of malonate, cholesterol and regulatory proteins (rab, rho, rac), which mediate the osteoclast activity. By affecting the osteoclasts, bisphosphonates inhibit bone resorption and decrease vertebral and non-vertebral fracture risk [6].

Adverse effects of bisphosphonates are esophageal cancer, nausea, vomiting [7] and atrial fibrillation (zoledronate) [8].

The molecular mechanism of action of selective estrogen receptor modulators (SERM) like raloxifene [9] include selective binding to α and β estrogen receptors.

Strontium ranelate inhibits osteoclastogenesis by suppression of the differentiation and activity of osteoclasts [10].

Hormonal therapy with estradiol [11] suppresses bone resorption and reduces the loss of bone mass [12]. Combined therapy leads to a greater reduction of fractures than single agent. The administration of bisphosphonate with raloxifene shows greater improvement in body mass density [13].

Hydrophilic interaction chromatography (HILIC-HPLC) has been described by Andrew Alpert in 1990 [14] as HPLC with hydrophilic

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stationary phase and reversed-phase type eluents [15]. The HILIC mode of separation currently has been successfully applied for separation of some organic compounds and biomolecules [16]. It is suitable for analysis of carbohydrates, peptides and polar pharmaceuticals [17] and for quality assurance of glycoproteins in biological products [18].

Through HPLC with hydrophilic interaction a 10-fold increase in sensitivity in comparison with RP-HPLC is achieved [15].

Literature review reveals that for the determination of 17β-estradiol alone or in combinations with other drugs the most often applied methods are UV-spectrophotometry [19], derivative spectrophotometry [20], HPLC with normal phases [21] and HPLC with reversed phases and UV-detection [19], whereas for 17β-estradiol and drospirenone: a C_{18} column, mobile phase: acetonitrile : water = 70 : 30 v/v, λ = 279 nm [20].

The aim of the present study was the validation of the isocratic HPLC-HILIC method with UV-detection in accordance with the International Conference on Harmonization Guidelines [22] for the analytical parameters: limearity, LOD, LOQ. precision (repeatability) and accuracy for identification and determination of estradiol hemihydrate in dosage preparation.

MATERIALS AND METHODS

Materials

I) Reference standard: estradiol hemihydrate: D00 166536 with purity > 99 %.

II) Reagents with analytical grade quality: acetonitrile for HPLC (Sigma Aldrich, N: SZBD 150 SV UN 1648), distilled water.

III) Tablets

Trisequens tabl. (2 mg estradiol hemihydrate/1 mg norethisterone acetate) (DF 70298 Novo Nordisk, Netherlands)

Climonorm tabl. (2 mg estradiol valerate/0.15 mg levonorgestrel) (WEKSBH Bayer, Germany)

Climen tabl. (2 mg estradiol valerate/10 mg ciproterone acetate) (344418, Bayer, Germany).

Methods: HPLC-HILIC method

Instrumentation

HPLC 200 (Perkin Elmer, USA) with: spectrophotometric detector LC-785A (Bioanalytical systems, USA); thermostat (Perkin Elmer, Waltham, MA, USA); ultrasonic bath (Branson Wilmington, NC, USA); apparatus for ultra pure water: "Milli-Q", "Milipore" (Bedford, MA, USA) and " Elga" (VWR International, Randor, PA, USA).

Chromatographic conditions: isocratic HPLC-HILIC elution with: stationary phase: column Spherisorb Amino (250 mm × 4.6 mm × 5 μm), mobile phase: acetonitrile : water = 55 : 45 v/v, flow rate: 2 ml/min and UV-detection at λ = 230 nm.

Preparation of solutions of the reference substance estradiol hemihydrate for validation of the HPLC-HILIC-method for analytical parameter linearity.

Preparation of stock standard solution of estradiol hemihydrate

An accurately weighed quantity (0.05 g) of the reference substance estradiol hemihydrate was dissolved in 15 ml of acetonitrile under sonication in a ultrasonic bath. After dilution with acetonitrile in a volumetric flask of 50.0 ml a solution with a concentration of estradiol hemihydrate of 1.0 mg/ml was obtained.

Preparation of standard solutions of estradiol hemihydrate

For the calibration curve for HPLC-HILIC-method a series of standard solutions were prepared by dilution of 30 μl, 50 μl, 100 μl, 200 μl, 300 μl and 400 μl of the stock standard solution of estradiol hemihydrate (1.0 mg/ml) in volumetric flasks of 10.0 ml with the mobile phase acetonitrile : water = 55 : 45 v/v. The resulting solutions were with concentrations of estradiol hemihydrate: 3.10^{-6} g/ml, 5.10^{-6} g/ml, 1.10^{-5} g/ml, 2.10^{-5} g/ml, 3.10^{-5} g/ml and 4.10^{-5} g/ml, respectively. The solutions were filtered through a membrane filter 0.45 μm and analyzed by the described HPLC-HILIC-method.

Preparation of solutions from tablets

From the stirred tablet mass an amount equivalent to 2 mg estradiol hemihydrate was weighed, 10 ml of acetonitrile were added and the samples were sonicated for 5 min in an ultrasonic bath under periodical stirring. The resulting suspension was diluted in a volumetric flask of 100.0 ml with acetonitrile, sonicated for 10 min in an ultrasonic bath, and placed in the dark place for 30 min for precipitation. An aliquot was filtered through a membrane filter 0.45 μm and analyzed by the described HPLC-HILIC-method.

RESULTS AND DISCUSSION

In order to search for opportunities to shorten the time of analysis, other options for chromatographic separation were used. Due to the

presence in the analyzed compounds of mainly less polar components than estradiol hemihydrate and estradiol valerate, the possibilities for their elution in reversed order by HPLC-HILIC distribution mechanism were tested.

The chromatograms from the isocratic HPLC-HILIC elution of the components of tablets are illustrated for: trisequens tabl. (estradiol hemihydrate/norethisterone acetate) (Fig. 1.); climonorm tabl. (estradiol valerate/levonorgestrel) (Fig. 2.) and climen tabl. (estradiol valerate/ciproterone acetate) (Fig. 3.). The optimized conditions were: column Spherisorb Amino (250 mm × 4.6 mm × 5 μm), mobile phase: acetonitrile : water = 55 : 45: v/v, flow rate: 2 ml/min, UV-detection at λ = 230 nm.

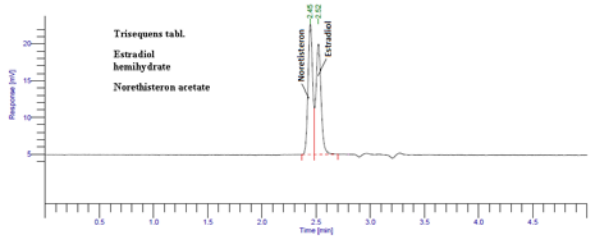

Fig. 1. Chromatogram of trisequens tabl. (estradiol hemihydrate/norethisterone acetate) at isocratic HPLC-HILIC, flow rate: 2 ml/min, UV-detection at λ = 230 nm.

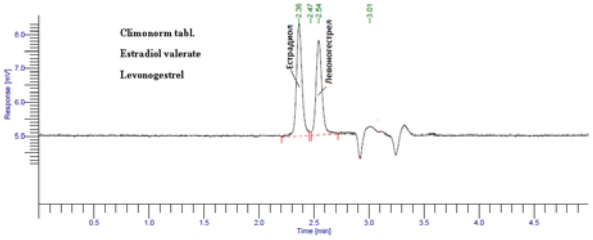

Fig. 2. Chromatogram of climonorm tabl. (estradiol valerate/levonorgestrel) at isocratic HPLC-HILIC, flow rate: 2 ml/min, UV-detection at λ = 230 nm.

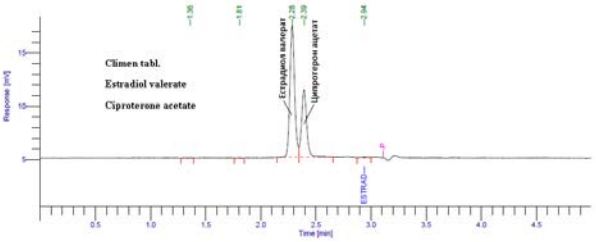

Fig. 3. Chromatogram of climen tabl. (estradiol valerate/ciproterone acetate) at isocratic HPLC-HILIC, flow rate: 2 ml/min, UV-detection at λ = 230 nm.

From Fig. 1, Fig. 2 and Fig. 3. it is obvious that the components of trisequens tabl., climonorm tabl. and climen tabl. are separated by HPLC-HILIC mechanism using Amino column in less than 4 min.

Validation of the isocratic HPLC-HILIC-method with UV-detection for analysis of estradiol hemihydrate

HPLC-HILIC-analytical method for estradiol hemihydrate was validated in terms of the analytical parameters: selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), precision (repeatability) and accuracy.

Selectivity

Placebo solution, containing starch as a supplement, without the active substance estradiol hemihydrate was prepared. The selectivity of the applied method was confirmed by the fact that on the chromatogram with placebo preparation there was no peak with t_R, corresponding to t_R of estradiol hemihydrate (t_R = 2.87) in standard solution. This fact confirms the lack of interference from the excipient starch commonly present in tablets [22].

Study of the analytical parameter linearity

For estimation of the linearity, the HPLC-HILIC-method was applied under chromatographic conditions: column Spherisorb Amino (250 mm × 4.6 mm × 5 μm), mobile phase acetonitrile : water = 55 : 45 v/v, flow rate: 2 ml min, UV-detection at λ = 230 nm.

Data for the concentration (C) and the peak area (A) for solutions with estradiol hemihydrate are presented in Table 1.

Table 1. Concentration (C) and peak area (A) for linearity for estradiol hemihydrate.

N:	C [g/ml]	A
1.	3.10^{-6}	5904.8
2.	5.10^{-6}	9872.6
3.	1.10^{-5}	22932.9
4.	2.10^{-5}	51561.5
5.	3.10^{-5}	79069.4
6.	4.10^{-5}	105994.0

The dependence of chromatographic peak area on the concentration of estradiol hemihydrate was evaluated by obtaining the chromatograms of standard solutions with concentrations: 3.10^{-6} g/ml, 5.10^{-6} g/ml, 1.10^{-5} g/ml, 2.10^{-5} g/ml, 3.10^{-5} g/ml, 4.10^{-5} g/ml. The experimental results for the peak areas obtained for solutions with increasing concentration, were subjected to linear regression analysis to obtain the regression equation: y = ax + b (a – slope, b – interept).

Linearity accordance between concentration and peak area in the range: 3.10^{-6} g/ml ÷ 4.10^{-5} g/ml was proved by the regression equation: y = 2698.99.x – 2307.98, with calculated correlation coefficient R^2

= 0.9999. The linear relationship between peak area (A) and concentration (C) [g/ml] is shown in Fig. 4.

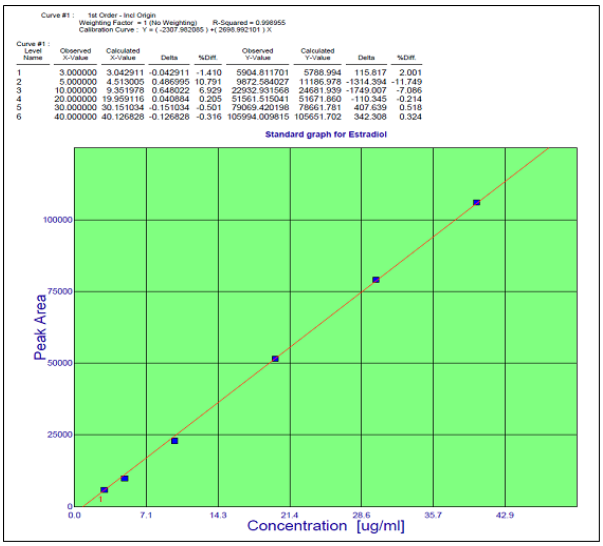

Fig. 4. Linearity for estradiol hemihydrate of the HPLC-HILIC-method with UV-detection at λ = 230 nm and flow rate 2 ml/min

Study of the analytical parameters LOD and LOQ.

The sensitivity of the HPLC-HILIC-method using chromatographic conditions was defined as LOD (concentration, at which the obtained ratio of signal to noise is 3:1) and LOQ (concentration, at which the obtained ratio of signal to noise is 10:1). From the chromatogram of 10 µl standard estradiol hemihydrate solution (C = 3.10^{-6} g/ml) were calculated: LOD = 8.10^{-7} g/ml; LOQ = 8.10^{-6} g/ml.

Estimation of the analytical parameters accuracy and precision (repeatability) of the HPLC-HILIC-method.

For the estimation of analytical parameters accuracy and precision (repeatability), the descibed HPLC-HILIC-method was applied to 6 standard solutions containing known amounts of estradiol hemihydrate: 100 % (2 mg/100 ml).

On Fig. 5. the chromatogram of a standard solution of estradiol hemihydrate is presented.

The quantity of drug was calculated by the method of calibration curve using the regression equation. In Table 2. are summarized data for: N – number of individual measurements (N = 6); A – peak area; UA – Chauvenet criterion for peak area; C – quantity of estradiol hemihydrate obtained by the method of calibration curve; UC – Chauvenet criterion for obtained content; RC – degree of recovery [%]; $\overline{X}$ – arithmetical mean; SD – standard deviation; RSD [%] – relative standard deviation; $S\overline{X}$ – mean quadratic error; P – confidence possibility [%]; t – coefficient of Student; $\overline{X} \div t.S\overline{X}$ – confidence interval; E – relative error [%].

Table 2. Validation of the HPLC-HILIC method for accuracy and precision (repeatability) of estradiol hemihydrate.

N:	A	U A	C [mg/100 ml]	UC	RC [%]
1.	50287	1.38	1.95	1.33	97.5
2.	50522	1.09	1.96	1.0	98
3.	51688	0.36	2.00	0.33	100
4.	51697	0.37	2.00	0.33	100
5.	51899	0.62	2.01	0.67	100.5
6.	52312	1.13	2.02	1.0	101
$\overline{X}$ ± SD	51401 ± 808		1.99 ± 0.03		
$\bar{R}$ [%] ± RSD [%]					99.5 ± 1.42
SD	808		0.03		1.41
RSD [%]	1.57		1.51		1.42
$S\overline{X}$			0.01		0.58
P [%]			95.0		99.0
t			2.57		4.03
t. $S\overline{X}$			0.03		2.34
$\overline{X} \pm t.S\overline{X}$			1.96 ÷ 2.02		97.16 ÷ 101.84
E [%]			0.5		0.58

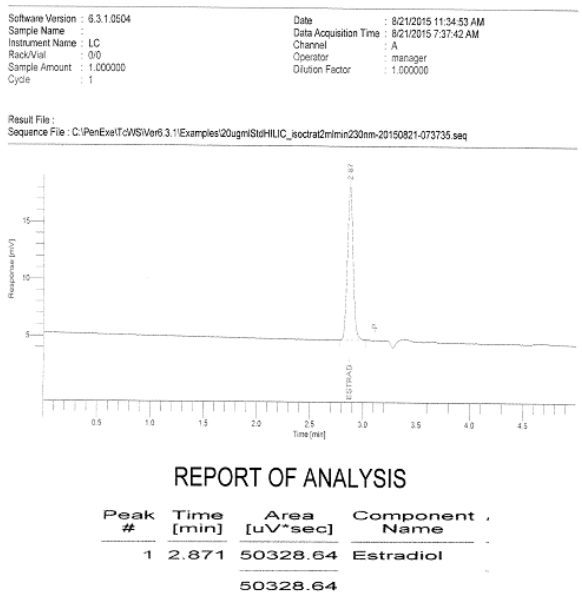

Fig. 5. Chromatogram of a standard solution of estradiol hemihydrate: HPLC-HILIC-method with UV-detection at λ = 230 nm and flow rate 2 ml/min.

For the assessment of accuracy and precision the sample standard deviation (SD) was calculated by applying 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) [22].

For the estimation of precision (repeatability) the uncertainty of the result was used, which is determined by: standard deviation (SD), relative standard deviation (RSD) and confidence range. Repeatability is expressed by SD and RSD for 6 standard solutions with added content of estradiol hemihydrate: 2 mg/100 ml. All data for the obtained concentration of estradiol hemihydrate correspond to the confidence interval: 1.96 mg/ 100 ml ÷ 2.02 mg/100 ml (SD = 0.03; RSD = 1.51).

The results for the accuracy at P = 99 % (t = 4.03), expressed by the percent recovery R [%] ± RSD [%] are within the respective confidence interval RC: 97.16 ÷ 101.84 % (RSD = 1.42).

CONCLUSIONS

The HPLC-HILIC-method is validated in terms of the analytical parameters specificity, linearity: y = 2698.99 x - 2307.98, LOD = 8.10^{-7} g/ml, LOQ = 8.10^{-6} g/ml, repeatability. The higher selectivity and efficiency of separation by HPLC-HILIC with UV-detection at λ = 230 nm in an Amino-column and elution of the less polar analytes before the more polar ones (estradiol) shortens the time for analysis and leads to better repeatability.

Acknowledgements: *This article was prepared with the financial support from Grant Project Contract N:13/2015 – DP, Medical University-Plovsiv, Bulgaria.*

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ОЦЕНКА НА ЛИНЕЙНОСТТА И ТОЧНОСТТА НА HPLC-HILIC-МЕТОД ЗА АНАЛИЗ НА ЕСТРАДИОЛ ХЕМИХИДРАТ

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Постъпила на.28 март 2016 г.;. коригирана на.21 декември 2016 г.

(Резюме)

Целта на настоящото изследване е оценката на линейността и прецизността на изократичен HPLC-HILIC метод с УВ-детекция за идентифициране и определяне на естрадиол хемихидрат във фармацевтични дозирани форми. Извършен е линеен регресионен анализ. Построена е калибрационна права. Линейната зависимост между концентрацията и площта на пиковете в диапазона: 3.10-6 g/ml ÷ 4.10-5 g/ml се доказва от регресионното уравнение: y = 2698.99 x - 2307.98. Регресията на най-малките квадрати дава коефициент на корелация R^2 = 0.999. LOD = 8.10^{-7} g/ml, LOQ = 8.10^{-6} g/ml. Резултатите за точността при P = 99 % (t = 4.03) са представени чрез аналитичния добив R [%] и отговарят на доверителния интервал: RC: 97.16 ÷ 101.84% (RSD = 1.42).

Прецизността е представена чрез стандартно отклонение, относително стандартно отклонение и доверителен интервал. Всички данни за полученото количество естрадиол хемихидрат съответстват на доверителния интервал: 1.96 mg/100 ml ÷ 2.02 mg/100 ml (SD = 0.03, RSD = 1.51).

Високата селективност и ефективност на разделянето чрез HPLC-HILIC-метода с UV-детекция при λ = 230 nm при използване на Амино-колона и елуирането на неполярните аналити преди полярните (естрадиол), съкращава времето за анализ и води до висока повторяемост.