Evaluation of the separation of steroids in combined forms by RP HPLC with UV-detection and gas chromatography

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The aim of the current study is the evaluation of the separation of steroids from accompanying substances in drug products as follows: estradiol hemihydrate from didrogesterone (in Femoston tabl.) and estradiol valerate from levonorgestrel (in Climonorm tabl.) and ciproterone acetate (in Climen tabl.). Reversed phase (RP) HPLC with UV-detection and gas chromatography were applied. For the RP HPLC method with UV-detection the following conditions were used: a) column: Nova Pack C_{18}; isocratic elution with mobile phase: acetonitrile : methanol : water = 40 : 5 : 55 v/v/v; flow rate: 1 ml/min; UV-detection at $\lambda = 204$ nm; b) column Nova Pack C_{18}; gradient elution with: 0-10 min: acetonitrile : methanol : water = 35 : 5 : 60 v/v/v; 10-20 min; acetonitrile : methanol : water = 70 : 5 : 25 v/v/v, flow rate: 1 ml/min, UV-detection at $\lambda = 230$ nm.

Although RP-HPLC separation at isocratic conditions allows determination of estradiol hemihydrate with high reproducibility with the highest sensitivity at $\lambda = 204$ nm, the analysis in medicinal products requires additional time for elution of components more non-polar than estradiol hemihydrate, which are present in the sample: in Femoston tabl.: $t_R = 13.42$ min for didrogesterone; $t_R = 4.85$ min for estradiol hemihydrate. The experimental results showed that RP HPLC separation with gradient elution is characterized by higher selectivity. It is found that the detection wavelength $\lambda = 230$ nm is optimal for the achievement of high sensitivity and it is universal for the identification of other active principles and for obtaning of a stable base line with gradient elution.

For the GC method: a column HP-35 (30 m × 0.25 mm × 0.25 μm), temperature program from 100 ºC to 330 °C and mass detection were used. Degradation of analytes at high temperature, their different degree of ionization and the different sensitivity of their detection lead to uncertainty in the GC/MS analysis, therefore, HPLC is the more suitable method for analysis of steroid components.

Keywords: Estradiol, RP HPLC, UV-detection, GC, combined forms.

INTRODUCTION

Osteoporosis is a skeletal disease which is more common in women than men: 50 % of women [1] and 25 % of men aged over 50 have fractures [2]. Osteoporosis in postmenopausal women is caused by the reduced estrogen levels [3, 4] leading to an imbalance between the activity of osteoblasts and osteoclasts [5]. The imbalance between bone resorption and formation is due to the extension of the life cycle of osteoclasts and shortening the cycle of osteoblasts [6]. In postmenopause, bone resorption rate sharply increases. Estrogens bind to estrogen receptors in the cells. Estrogens inhibit the degradation of bone tissue and suppress bone resorption by regulating the expression of RANK-receptor activator of nuclear factor kB (NFkB) and osteoprotegrin in osteoblasts [7].

Hormones reduce the production of interleukin 1, interleukin 6, tumor necrosis factor α [8] and macrophage colony stimulating factor, which mediate osteoclastogenesis [9]. Hormone therapy reduces the estrogen deficiency [10], decreases the risk of osteoporotic fractures, suppresses biochemical markers of bone resorption, improves bone mineral density [11] and reduces bone loss [12]. Estrone stimulates the development of osteoblasts in women and testosterone is the hormone responsible for bone health in men [13]. Hormone therapy includes: estradiol (Climara); danazol (Danocrine); medroxy-progesterone acetate (Provera); nandrolone (Retabolil); noresthisterone acetate (Primolut); oxandrolone (Anavar); testosterone; tibolone (Livial); stanozolol (Winstrol). Combination of estrogens and bisphophonates alendronate [14], risedronate [15] and zoledronate [16], has additive effects on the reduction of bone resorption markers. In postmenopausal women the combination estrogen/alendronate increases bone mineral density [17].

In combined drug products for the treatment of menopausal symptoms in postmenopause are

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applied: 1) estradiol hemihydrate with didrogesterone 10 mg (Femoston tabl.) and norethisterone acetate 1 mg (Trisequense tabl.); 2) estradiol valerate with: ciproterone acetate 1 mg (Climen tabl.) [18]; dienogest 2 mg (Climodien tabl.) [19] and Levonorgestrel 0.15 mg (Climonorm tabl.) [20].

For the determination of 17β-estradiol and estradiol valerate in combination with other components in the tablets the following methods have been developed: I) RP HPLC with UV-detection: 1) 17β-estradiol/estriol/estrone: column C_{18} micro Bondapak (250 mm × 4.6 mm × 10 μm) in isocratic mode, mobile phase: acetonitrile : water = 50 : 50 v/v, flow rate: 1 ml/min, column temperature: 30 °C, λ = 205 nm [21]; 2) 17β-estradiol/drospirenone: column Waters Symmetry C_{18} (250 mm × 4.6 mm × 5 μm), mobile phase: acetonitrile : water = 70 : 30 v/v, λ = 279 nm [22]; 3) estradiol valerate/dienogest: column ACE C_8 (250 mm × 4.6 mm × 5 μm), mobile phase: ammonium nitrate : acetonitrile = 30 : 70 v/v, flow rate: 2 ml/min, λ = 280 nm, internal standard cyproterone acetate [23]; II) gas chromatography with mass detection (GC/MS): 17β-estradiol [24] and estradiol valerate/medroxyprogesterone acetate [25].

The advantage of these methods is good selectivity, obtained by using columns with length 250 mm and diameter size of particles 5-10 μm.

The aim of the current study is the estimation of the influence of lower column length (150 mm) and lower diameter size of particles (3.5-4 μm) on the isocratic and gradient RP-HPLC separation of steroids from accompanying substances in drug products.

MATERIALS AND METHODS

Materials

1) Reference substances:

Estradiol hemihydrate N: D00 166 536, purity > 99 %

Estradiol valerate, purity > 99 %

Didrogesterone, purity > 99 %

Levonorgestrel, purity > 99 %

Ciproterone acetate, purity > 99 %

2) Solvents with pharmacopoeial purity: acetonitrile for HPLC (Sigma Aldrich, N: SZBD 150 SV UN 1648); methanol (Sigma Aldrich, N: SZBD 063 AV UN 1230); ultra pure water.

3) drug products: Femoston tabl. (estradiol hemihydrate 2 mg/didrogesterone 10 mg) (N: 341141 Abbott, Netherland); Climonorm tabl. estradiol valerate 2 mg/levonorgestrel 0.15 mg (N: WEKSBH Bayer (Germany); Climen tabl.: estradiol valerate 2 mg/ciproterone acetate 1 mg (N: 344418, Bayer (Germany).

Methods

I. Instrumentation.

1) HPLC 200 chromatograph (Perkin Elmer, USA) with: spectrophotometric detector LC-785A (Bioanalytical systems, USA); autosampler 200 series; thermostat (Perkin Elmer, Waltham, MA, USA); columns: Nova Pack C_{18} (150 mm × 4.6 mm × 4 μm) (Waters USA,WAT 044375); Li Chrospher C_{18} (125 mm × 4 mm × 4 μm) (Phenomenex, USA, 00E-3043-D0); Zorbax Eclipse XDB C_8 (150 mm × 4.6 mm × 3.5 μm) (Agilent, USA, 993967-906).

2) Gas chromatograph "Trace" with mass spectral detector TSQ ("Thermo Fisher Scientific", Waltham, MA, USA); "split/splitless" injector; capillary column HP-35 (30 m × 0.25 mm × 0.25 μm) ("Agilent", Santa Clara, CA, USA).

3) Ultrasonic bath (Branson Wilmington, NC, USA).

4) Apparatus for ultra pure water "Milli-Q", "Millipore" (Bedford, MA, USA) and "Elga" (VWR International, Randor, PA, USA).

II. Chromatographic conditions.

1) RP HPLC – columns: Nova Pack C_{18}, Li Chrospher C_{18}, Zorbax Eclipse XDB C_8, isocratic elution with mobile phase: acetonitrile : methanol : water = 40 : 5 : 55 v/v/v, flow rate: 1 ml/min, UV-detection at an analytical wavelength λ = 204 nm.

2) RP HPLC – columns: Nova Pack C_{18}, Li Chrospher C_{18}, Zorbax Eclipse XDB C_8, gradient elution with: 0-10 min: acetonitrile : methanol : water = 35 : 5 : 60 v/v/v; 10-20 min: acetonitrile : methanol : water = 70 : 5 : 25 v/v/v, flow rate: 1 ml/min, UV-detection at λ = 230 nm.

3) GC: column HP-35 (30 m × 0.25 mm × 0.25 μm), split/splitless injection at 260 °C, temperature program from 100 ºC to 330 °C and mass detection by electron impact ionisation mode and monitoring of positive ions.

III. Preparation of stock standard solution of estradiol hemihydrate and estradiol valerate.

Accurately weighed quantities (0.05 g) of the reference substances estradiol hemihydrate and estradiol valerate were separately dissolved in 15 ml acetonitrile under sonication in ultrasonic bath. After dilution with acetonitrile in a volumetric flask of 50.0 ml solutions with a concentration of steroids 1.0 mg/ml were obtained.

IV. Preparation of standard solution of estradiol hemihydrate for RP HPLC.

Standard solution was prepared by dilution of 200 μl of the stock standard solution of estradiol

hemihydrate (1.0 mg/ml) in a volumetric flask of 10.0 ml with acetonitrile. The resulting solution was with concentration of estradiol hemihydrate 2.10^{-5} g/ml (20 µg/ml). The solution was filtered through a membrane filter 0.45 µm and analyzed by the described RP HPLC method.

V. Preparation of standard solution of estradiol hemihydrate for GC/MS.

The standard solution was prepared by dilution of 1 ml of the stock standard solution of estradiol hemihydrate (1.0 mg/ml) in a volumetric flask of 10.0 ml with acetonitrile. The resulting solution was with concentration of estradiol hemihydrate: 1.10^{-4} g/ml (100 µg/ml). The solution was filtered through a membrane filter 0.45 µm and analyzed by the described RP HPLC method.

VI. 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 samples were sonicated for 5 min in an ultrasonic bath with 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 for 30 min in the dark place for precipitation. An aliquot part was filtered through a membrane filter 0.45 µm and analyzed by the described RP HPLC method.

RESULTS AND DISCUSSION

I, Analysis of estradiol hemihydrate and estradiol valerate by RP-HPLC with UV-detection.

HPLC with UV-detection was carried out in order to find out the more suitable stationary phase for separation of estradiol hemihydrate and estradiol valerate from potential related ingredients in: Femoston tabl. (esteradiol hemihydrate/didrogesterone), Climonorm tabl. (estradiol valerate/levonorgestrel) and Climen tabl. (estradiol valerate/ciproterone acetate).

Columms Nova Pack C_{18} (150 mm × 4.6 mm × 4 µm), Li Chrospher C_{18} (125 mm × 4 mm × 4 µm) and Zorbax HDB C_8 (150 mm × 4.6 mm × 3.6 µm) were investigated. The evaluation of the various columns operating in reversed mode shows that Nova Pack C_{18} (150 mm × 4.6 mm × 4 µm) displays the highest parameters (efficiency and selectivity) for estradiol hemihydrate and estradiol valerate and accompanying substances in Femoston tabl., Climonorm tabl. and Climen tabl. In isocratic RP-HPLC with columm Nova Pack C_{18} in Femoston tabl. for didrogesterone $t_R = 13.42$ min was obtained in comparison with didrogesterone $t_R = 8.5$ min obtained with Li Chrospher C_{18}.

On Fig. 1. are illustrated: chromatogram of standard solution of estradiol hemihydrate and chromatogram of 10 µl of solution of Femoston tabl. in 100 ml of acetonitrile.

The chromatograms are obtained under conditions of the isocratic mode, column Nova Pack C_{18}, mobile phase: acetonitrile : methanol : water = 40 : 5 : 55 v/v/v, flow rate: 1 ml/min and UV-detection at λ = 204 nm.
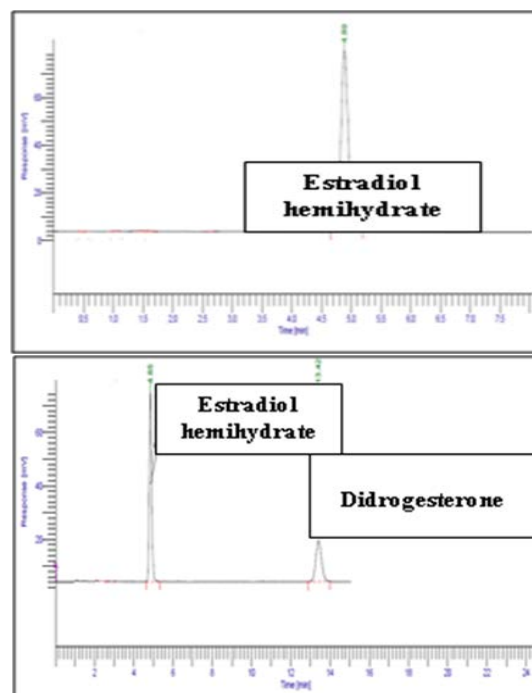
Fig. 1. Chromatograms of 20 µl of 20 µg/ml standard solution of estradiol hemihydrate and of Femoston tabl: isocratic RP-HPLC, flow rate: 1 ml/min, λ = 204 nm.

Identification of estradiol hemihydrate in Femoston tabl. is proven by the equal data for the chromatographic parameter retention time ($t_R = 4.86$ min) in the tablets and in the standard of estradiol hemihydrate. The equal results for $t_R = 13.42$ min in the tabl. and in the standard confirm the identification of didrogesterone in Femoston tabl.

RP HPLC separation in isocratic conditions allows determination of estradiol hemihydrate with high reproducibility. Sensitivity is highest at λ = 204 nm. Inspite of this benefit, the disadvantage is that the determination under isocratic conditions in medicinal products requires additional time for elution of components more non-polar than estradiol hemihydrate present in the sample: in Femoston tabl.: $t_R = 13.42$ min for didrogesterone; $t_R = 4.85$ min for estradiol hemihydrate.

In order to optimize the conditions for RP HPLC analysis of estradiol hemihydrate in the presence of related components the RP HPLC with gradient elution was applied for analysis in tablets in the

column: Nova Pack C_{18}. After isocratic elution the time for cleaning of the column take more time (1.5 h) than after gradient elution (1 h), which is the reason for using the gradient mode of analysis.

Chromatograms are illustrated on Fig. 2. (Femoston tabl.), Fig. 3. (Climonorm tabl.) and Fig. 4. (Climen tabl.).

The equal data for $t_R = 4.86$ min in the tabl. and in the standard of estradiol hemihydrate prove the identification of estradiol hemihydrate in Femoston tabl. Identification of didrogesterone in Femoston tabl. is confirmed by the equal results for $t_R = 8.5$ min in the tablets and in the standard of didrogesterone.

Identification of estradiol valerate in Climonorm tabl. and Climen tabl. is proven by the equal values of $t_R = 11.46$ min in tabl. and in standard of estradiol valerate. The equal results for $t_R = 8$ min in Climonorm tabl. and in the respective standard confirm the identification of levonorgestrel in tabl. In Climen tabl. ciproterone acetate is identified by the equal data for $t_R = 8.29$ min in the tablets and in the standard of ciproterone acetate.

In gradient RP-HPLC analysis of estradiol hemihydrate in the presence of related components, the gradient elution with detection at $\lambda = 230$ nm is optimal for the achievement of high sensitivity and is universal for the identification of other active principles and for obtaining of stable base line. From Figs. 2, 3 and 4 it is obvious that in RP gradient elution the active principles of the examined products were separated for 10 min, but the presence of other non-polar ingredients requires additional 10 min for elution and this leads to increasing of the time of analysis to about 20 min. The methods can be used for the routine analysis of estradiol hemihydrate and estradiol valerate in combined pharmaceutical products.

II. Identification of estradiol hemihydrate and estradiol valerate by gas chromatography with mass spectral detection.

On Fig. 5. a chromatogram of the standard solution of estradiol hemihydrate with concentration of 100 μg/ml and the mass spectrum of the peak with a retention time of $t_R = 6.2$ min, obtained by electron impact ionisation mode and monitoring of positive ions are illustrated.
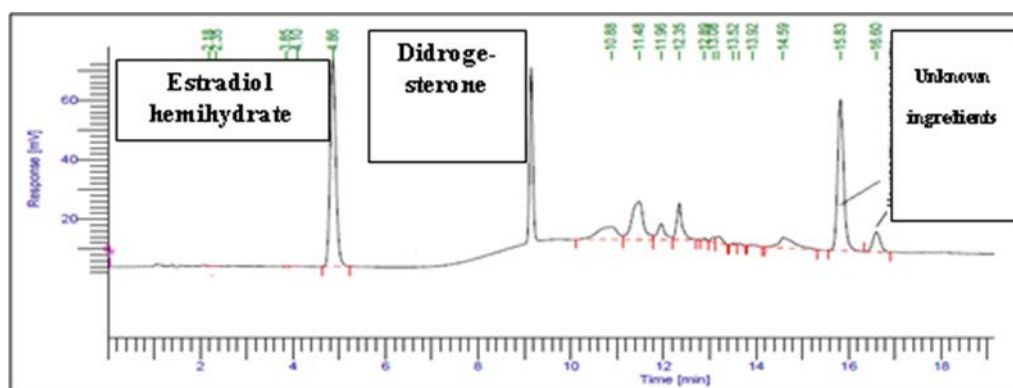

Fig. 2. Chromatogram of Femoston tabl. obtained by gradient RP-HPLC with UV-detection at $\lambda = 230$ nm.
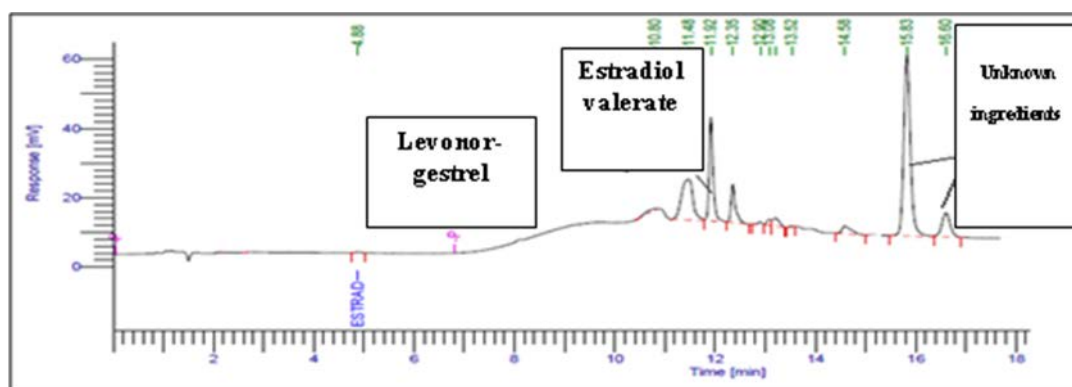

Fig. 3. Chromatogram of Climonorm tabl. obtained by gradient RP-HPLC with UV-detection at $\lambda = 230$ nm.
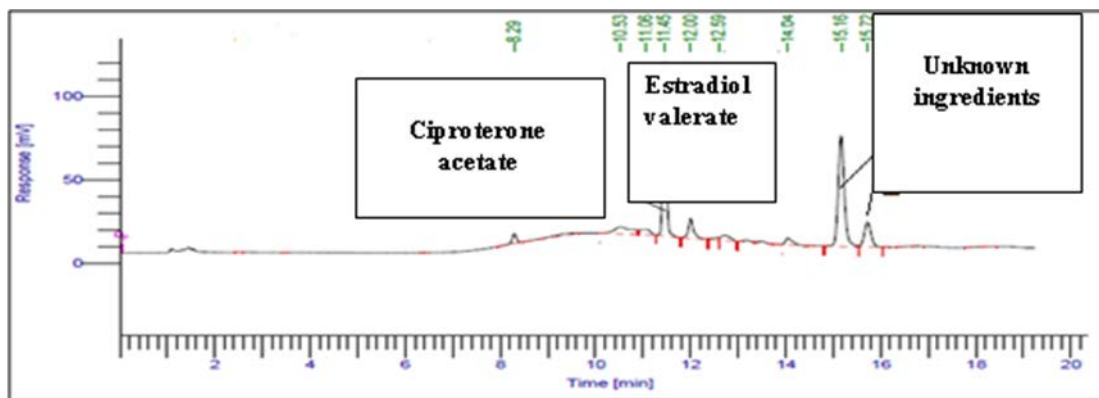
Fig. 4. Chromatogram of Climen tabl. obtained by gradient RP-HPLC with UV-detection at λ = 230 nm.
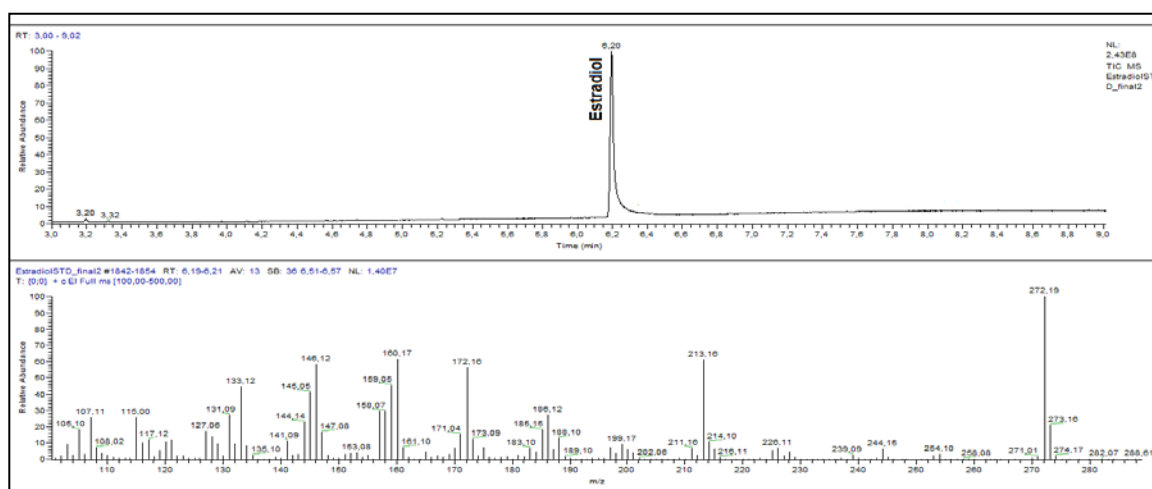

Fig. 5. Chromatogram of a standard solution of estradiol hemihydrate and mass spectrum of the peak at t_R = 6.2 min.
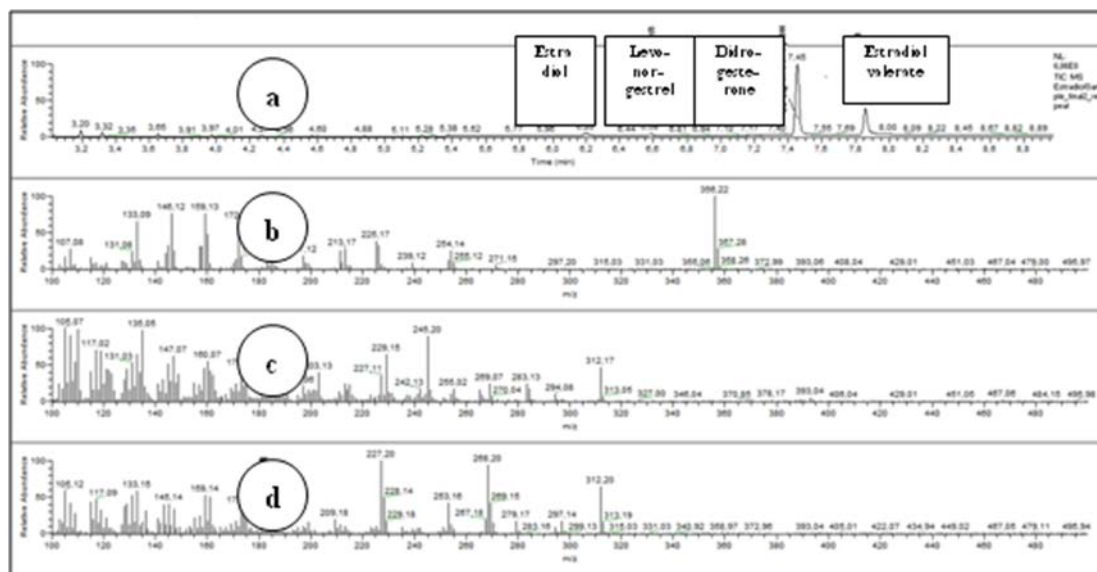

Fig. 6. Total ion chromatogram of a mixed acetonitrile solution of Femoston tabl., Climonorm tabl. and Climen tabl.

A mixed solution of Femoston tabl., Climonorm tabl. and Climen tabl. was filtered and analysed by gas chromatography. Fig. 6. shows: a) chromatogram of a mixed solution of Femoston tabl., Climonorm tabl. and Climen tabl. in acetonitrile and mass spectra of: b) estradiol valerate: t_R = 7.86 min; c) levonorgestrel: t_R = 6.58 min; d) didrogesterone: with t_R = 7.45 min.

From Fig. 6. it is obvious that the use of gas chromatography allows to analyze Femoston tabl., Climonorm tabl. and Climen tabl. and to identify their active principles. Mass spectral identification using specific mass spectral libraries is particularly convenient in the qualitative analysis. Steroids are very sensitive to contamination of the liner in the injector when introducing the sample at high temperature, which leads to degradation of the analytes. The electronic ionization with positive ion monitoring is the most popular variant of mass spectral detection in gas chromatography, but leads to a very different degree of ionization of the various analytes, and thus achieves a different sensitivity for detection thereof. For this reason, as shown in Fig. 7., although the concentrations of estradiol hemihydrate and didrogesterone are equal, the magnitude of their signals is clearly different and cyproterone acetate in Climen tabl. is not registered on the chromatogram. For quantitative analysis of the active ingredients in the tablets GC/MS requires the use of clean liners and a suitable internal standard.

CONCLUSION

Insipite of the fact, that RP HPLC separation with isocratic elution and UV-detection is characterized with the highest sensitivity at λ = 204 nm, the disadvantage for analysis of estradiol hemihydrate in medicinal products is that it requires additional time for elution of the more non-polar components. On the other hand, the degradation of analytes at a high temperature, their different degree of ionization and the different sensitivity of the detection lead to uncertainty of the GC/MS analysis. Due to this two reasons and because of higher selectivity, the RP HPLC method with gradient elution and UV-detection at λ = 230 nm is the most appropriate option for analysis of steroid components such as estradiol hemihydrate and estradiol valerate in dosage combined preparations.

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ОЦЕНКА НА РАЗДЕЛЯНЕТО НА СТЕРОИДИ В КОМБИНИРАНИ ФОРМИ ЧРЕЗ RP HPLC С UV-ДЕТЕКЦИЯ И ГАЗОВА ХРОМАТОГРАФИЯ

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(Резюме)

Целта на настоящото изследване е оценката на разделянето на стероиди от придружаващи вещества в лекарствени продукти: Естрадиол хемихидрат от Дидрогестерон във Femoston табл. и Естрадиол валерат от Левоноргестрел (в Climonorm табл.) и Ципротерон ацетат (в Climen табл.). Приложени са HPLC метод с обърнати фази и УВ-детекция и газова хроматография. HPLC с УВ-детекция е проведен при следните условия: а) колона: Nova Pack C_{18}, изократично елуиране с подвижна фаза: ацетонитрил : метанол : вода = 40 : 5 : 55 об/об/об, скорост на потока: 1 мл/мин, УВ-детекция при аналитична дължина на вълната λ = 204 nm; б) колона Nova Pack C_{18}, градиентно елуиране: 0-10 мин: ацетонитрил : метанол : вода = 35 : 5: 60 об/об/об; 10-20 мин: ацетонитрил : метанол : вода = 70 : 5 : 25 об/об/об, скорост на потока: 1 мл/мин, УВ-детекция при λ = 230 mn.

Въпреки че, RP-HPLC разделянето при изократични условия позволява определяне на Естрадиол хемихидрат с висока възпроизводимост с най-висока чувствителност при λ = 204 nm, анализът на лекарствени продукти изисква допълнително време за елуиране на по-неполярни от Естрадиол хемихидрат компоненти, които присъстват в пробата: във Femoston табл.: t_R = 13.42 мин за Дидрогестерон и t_R = 4.85 мин за Естрадиол хемихидрат. Експерименталните резултати показват, че HPLC методът с градиентно елуиране се характеризира с по-висока селективност и, че дължината на вълната λ = 230 nm е оптимална за постигането на висока чувствителност и е универсална за идентифицирането на други активни съставки и за получаване на стабилна базова линия при градиентно елуиране.

При газовата хроматография са използвани: колона HP-35 (30 m x 0.25 mm x 0.25 μm), температурна програма от 100 °C до 330 °C и мас-детекция. Разграждането на аналитите при висока температура, тяхната различна степен на йонизация и различната чувствителността при детекцията им са причина за несигурност при газ-хроматографския анализ, поради което, HPLC е по-подходящ метод за анализ на стероидни компоненти.