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A SIMPLE THIN LAYER CHROMATOGRAPHY METHOD FOR SEPARATION OF SELECTED NATURAL STEROID HORMONES

(Kaedah Kromatografi Lapisan Nipis yang Mudah bagi Pemisahan Hormon Steroids Semulajadi Terpilih)

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Abstract

Chromatographic properties of seven steroids: estrogens (β-estradiol and estrone), androgens (testosterone, methyltestosterone, trans-androsterone), progesterone and cholesterol have been studied by planar chromatography with usage of High Performance Thin Layer Chromatography (HPTLC) and Thin Layer Chromatography (TLC) plates. Normal, reversed and cyano-bonded silica stationary phases were tested with five binary mobile phases (acetonitrile-water, acetonitrile-DMSO, acetonitrile-methanol, acetone-petroleum ether, acetone-water) in which the concentration of organic modifier varied from 0 to 100*% (v/v).* This study reports the optimization of steroid hormones separation. Principal Component Analysis (PCA) based on calculated molecular descriptors quantitatively differentiating solutes was performed in order to investigate the similarity and dissimilarity between tested compounds. The separation abilities of mobile and stationary phases were compared based on separation factor α. Chromatographic retention data and possible retention mechanisms also were discussed.

Keywords: thin layer chromatography, separation factor, binary phases, steroids

Abstrak

Sifat kromatografi tujuh steroid: estrogen (β-estradiol dan estron), androgen (testosteron, metiltestosteron, trans-androsteron), progesteron dan kolesterol telah dikaji oleh kromatografi satah dengan pengunaan plat kromatografi lapisan nipis (TLC) dan kromatografi lapisan nipis berprestasi tinggi (HPTLC). Fasa pegun normal, terbalik dan silika terikat siano diuji dengan lima fasa bergerak binari (asetonitril-air, asetonitril-DMSO, asetonitril-metanol, petroleum-aseton eter, aseton-air) di mana kepekatan pengubahsuai organik diubah daripada 0 hingga 100% (*v/v*). Kajian ini melaporkan keadaan optimum bagi permisahan hormon steriod. Analisis komponen prinsipal (PCA) berasaskan kiraan kuantitatif molekul yang membezakan bahan larut dilakukan untuk menentukan persamaan dan perbezaan di antara sebatian yang diuji. Keupayaan pemisahan fasa bergerak dan pegun dibandingkan berdasarkan kepada faktor pemisahan α. Data tambatan kromatografi dan kebarangkalian mekanisma tambatan juga turut dibincang.

Kata kunci: Kromatografi lapisan nipis, faktor pemisahan, fasa binari, steroid

Introduction

Steroids play a vital role in many biological processes and have also numerous therapeutic applications. Steroid hormones regulate metabolism, water - mineral balance and control the development and functioning of the sexual organs, as well as other biological differences between the sexes. Steroid drugs are used as anti-inflammatory drugs, anti-asthmatic drugs, synthetic hormones, potassium sparing diuretic or contraceptive drugs. Moreover, some

synthetic steroids (nandrolone, dromostanolone and etc) are often used illegally as doping agents in sport, since they stimulate protein synthesis and muscle-building action [1].

The core of steroids is composed of seventeen carbon atoms bonded together. They form skeleton with three cyclohexane rings and one cyclopentane ring, generally arranged in a 6-6-6-5 fashion. The great diversity of steroids structures and their wide range of polarities present crucial problems for the simultaneous analysis of different classes of steroids. Therefore, analysis of these compounds requires a well-equipped laboratory where such chromatography techniques as gas chromatography, high performance liquid chromatography, thin layer chromatography and even supercritical liquid chromatography are readily available [1-3].

Thin-layer chromatography [TLC) continues to be an important method for qualitative analysis of steroids because of its inherent advantages - numerous samples can be analyzed simultaneously and quickly, and multiple separation techniques and detection procedures can be applied [3]. A variety of samples including steroids such as biological [4, 5], environmental samples [6], plants [7,8] and pharmaceutical formulations [9] were analyzed by TLC. Both Normal-Phase TLC (NP-TLC) [10] and Reversed-Phase TLC (RP-TLC) [11-12] were successfully applied in steroids separation. Moreover, many modified stationary phases were also tested in the analysis of steroids, such as NH_2 layers [13-14], diol plates [14] and silica impregnated with silver nitrate [15].

Separation factor α – also known as selectivity factor or relative retention - is a measure of chromatographic system's selectivity. Selectivity of chromatographic system refers to mutual influence of substances selected with mobile and stationary phases. This parameter depends mainly on chemical structure of analytes and also on types and properties of both phases. The selectivity of chromatographic system might be described as distance between maximum of adjacent chromatographic peaks. Relative retention reflects differences in influence of both ingredients with mobile and stationary phases and is defined by equation 1 [16]:

$$\alpha = \frac{k_2}{k_1} = \frac{K_2}{K_1} \qquad (1)$$

in which k_1, k_2 is the retention factors of substances 1 and 2, K_1 and K_2 is the distribution coefficients. An equation 1 shows that separation factor is equal to relation of partition coefficients in balanced state or factors of both substances' retention. When $\alpha = 1$, separation has not been achieved for those substances.

The objectives of our investigations were to study the separation of selected steroids by various TLC and HPTLC systems. The novelty in this study is the use of cyano-bonded silica gel (CN). Results obtained on CN were compared with silica gel and C_{18} bonded silica gel. Different aqueous and non-aqueous mobile phases were also tested. Finally the best chromatographic system for the separation of tested compounds, have been proposed as well as possible retention mechanisms on CN-TLC were discussed.

Materials and Methods

Reagents and Mobile Phases

The solutes (β-estradiol, estrone, testosterone, methyl testosterone, trans-androsterone, progesterone, cholesterol) were purchased from Sigma-Aldrich (UK). All reagents used as mobile phases (acetonitrile, acetone, methanol, DMSO and petroleum ether) were HPLC – reagent grade from Sigma-Aldrich (Steinheim, Germany). Sulphuric acid (POCh, Poland) was used to prepare the visualizing reagents. Solutions of the steroids (1 mgL^{-1}) were prepared by dissolving appropriately weighed amounts in methanol. Binary phases (acetonitrile-water, acetonitrile-DMSO, acetonitrile-methanol, acetone-petroleum ether, acetone-water) were executed by mixing appropriate quantities of pure organic solvents in the proportions from 0 to 100*% v/v*.

Chromatography

Chromatography was performed on 5 cm × 10 cm Kieselgel 60 WF_{254s} TLC plates and Kieselgel 60 F_{254}, Kieselgel 60 CN F_{254s}, RP-18 F_{254s}, RP-18 WF_{254s} HPTLC plates manufactured by Merck (Darmstadt, Germany). The chromatographic chambers (7 x 11 cm) were previously saturated with mobile-phase vapour. When using water –

organic modification mixture as mobile phase, Kieselgel 60 WF_{254s} TLC plates and RP-18 WF_{254s} HPTLC plates dedicated on water--containing mobile phases were used. Chromatograms were developed to a distance of 8 cm at room temperature (20 ± 2°C). The substances were separated as a mixture and as individual standards. They were visualized by spraying the plates with a 1:4 *(v/v)* mixture of concentrated sulphuric acid and methanol and then heated in an incubator at 120°C for approximately 10 min, and then the compounds were detected in both UV light and visible light as coloured spots. All studies were repeated three times and the calculated average was used in further studies. Significantly different results were rejected.

Molecular Modeling

HyperChem 8.08 software with ChemPlus Extension (Hypercube, Waterloo, Canada) was used for the calculation of a large number of molecular descriptors. Before this calculation, the structures were constructed using the OpenBabel software. The structures were first optimized using the molecular mechanic calculations (MM+). The molecular modeling structural descriptors (energetic parameters) were computed using the semi-empirical calculation method Austin Model 1 (AM1). Finally, numerous molecular descriptors quantitatively differentiating solutes were calculated such as molecular bulkiness-related descriptors (Total Energy, Binding Energy, Isolated Atomic Energy, Electronic Energy, Core-Core Interaction, Heat of Formation, Surface Area, Volume, Volume Van der Wals, Hydration Energy, cLog P, Refractivity, Polarizability, Mass), molecular polarity-related descriptors (Energy HOMO and LUMO, δ_{max}, δ_{min}, μ). The number of H-Bond donor and acceptor can be found in the PubChem database (http://pubchem.ncbi.nlm.nih.gov/).

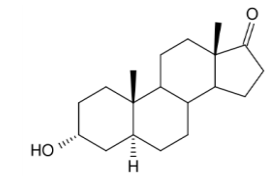
Principal Component Analysis

The data was mean‑centered and scaled to unit variance (Z-score scaling) before PCA analysis in order to prevent highly abundant components dominating in the final result over the components present in much smaller quantities. The PCA procedure was performed on the data matrix, where the rows (cases) correspond to the steroids hormones, whereas the columns (variables) correspond to the molecular descriptors. All statistical calculations were performed using STATISTICA 9.1 (StatSoft, Tulsa, Oklahoma, USA).

Results and Discussion

Tested group included seven steroids: estrogens (β-estradiol and estrone), androgens (testosterone, methyltestosterone, trans-androsterone), progesterone and cholesterol. Figure 1 was presented chemical structures of tested compounds.

In order to investigate the similarity and dissimilarity between tested compounds the PCA was performed. This technique is a very efficient way of multivariate data analysis. Its goal is to extract the important information from the table, to represent it as a set of new orthogonal variables called principal components. The performed PCA based on calculated molecular descriptors quantitatively differentiating solutes such as molecular bulkiness-related descriptors, molecular polarity-related descriptors and the number of H-Bond donor and acceptor. The first three components account for about 94.01% of the total variance. The score plot presented in Figure 2 demonstrates the similarities and dissimilarities between tested substances. As might have been expected slight differences between androgens were observed. Value of first principal component (PC1) differentiates compounds into two groups: androgens and estrogen, as well as two outliners: cholesterol and progesterone. The values of PC1 located progesterone near androgens. Structurally progesterone is similar to testosterone; the only difference between these two compounds is functional group in position C-17. The hydroxyl group in testosterone is in this position, whereas in the molecule of progesterone in the same position acetyl group is placed. The second (PC2) and third principal component (PC3) differentiates progesterone on androgens. Interestingly, cholesterol has similar values of PC2 and PC3 as β-estradiol and estrone.



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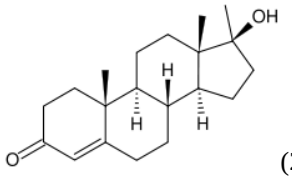

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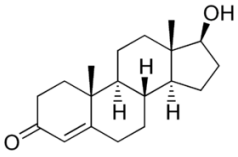

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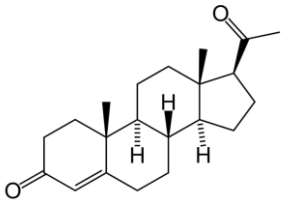

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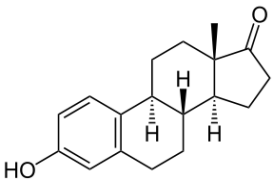

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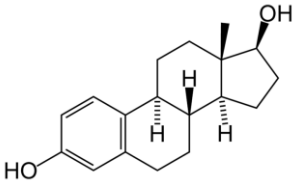

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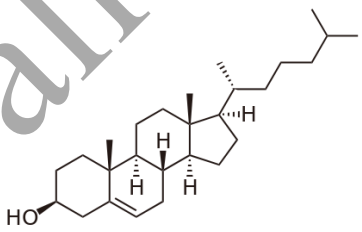

(7)

1. Trans-androsterone, 2. Methyltestosterone, 3. Testosterone, 4. Progesterone, 5. Estrone, 6. β-Estradiol, 7. Cholesterol

Figure 1. Chemical structures of seven steroids

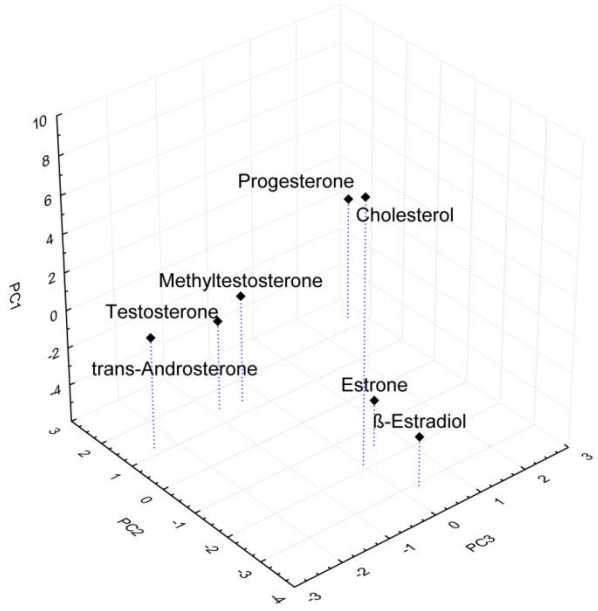

Figure 2. The score plot of the first, second and third principal components

The values of separation factors α between analyzed androgens and progesterone were presented in Table 1. A separation can be regarded effective when α is higher as 1,5. Generally, the highest values of separation factor between progesterone and tested androgens were observed when water-containing mobile phases were used. Small differences of this role occurred when silica gel was used. The best separation between testosterone and progesterone was observed on RP-TLC in particular when acetonitrile-water (40:60) as eluent was used. The lowest values of separation factors were obtained between methyl testosterone and progesterone. Methyl testosterone (clog P 3,91) is more lipophilic that testosterone (clog P 3,84), as it has additional methyl group. The same situation occurred when the retention between trans-androsterone (clog P 4,25) and progesterone (clog P 4,63) were compared. The separation factors have decreased in RP-TLC and CN-TLC. In RP-TLC the chromatographic process is described by partition and as might have been expected the separation is reduced when the difference on the lipophilicity between solutes is smaller. The cyano-bonded silica gel has intermediate properties between reserved and normal phases material (17). On this stationary phases were observed a strong dipole and moderate hydrophobic interaction. The decrease on separation factor α can suggest that the hydrophobic interaction determined the steroids - stationary phases interaction on CN bonded silica gel when water- water-containing mobile phases were used.

Better separation was observed on silica gel. From a practical point of view, acetone-water (20:80) can be recommended in order to separate methyl testosterone from progesterone. On the other hand, using binary mobile phase consisting 30% v/v acetonitrile in water, the highest value of selectivity factor was obtained between trans-androsterone and progesterone. Only in two chromatographic systems a significant separation between methyl testosterone and testosterone was observed, on RP-TLC with acetone-petroleum ether (10:90) as mobile phase and NP-TLC with acetone-water (40:60). The highest value of separation factor α was calculated in chromatographic systems including silica gel as stationary phases.

Significant difference was observed in the ability to separate methyl testosterone or testosterone from trans-androsterone. CN-TLC can separate testosterone from trans-androsterone with water-containing mobile phases whereas the effective separation of more lipophilic methylo testosterone from trans-androsterone was impossible. Regarding obtained results on RP-TLC the same conclusions can be drawn. On silica gel the separation factor α between methyl testosterone or testosterone and trans-androsterone was high (2,27), which means that they could be easily separated, when mixture of acetone-water (20:80) as mobile phases was used. For both compounds the values α are the same, as they have the same retention factor in this chromatographic systems.

Table 1. The highest values of the parameter α between tested androgens and progesterone using different mobile phases (v/v).

Adsorbent	**Eluent (v/v)**	**Testosterone /Progesterone**	**Methyltestosterone /Progesterone**	**trans-Androsterone /Progesterone**
CN	ACN-MeOH	1,33[L]	1,27[L]	1,29[A]
	ACN-H_2O	2,15[E]	1,97[E]	1,54[F]
	ACN-DMSO	1,33[L]	1,41[K]	1,19[K]
	DMK-H_2O	2,48[E]	2,00[E]	1,72[F]
	DMK-PET	1,49[C]	1,36[C]	1,26[J]
C_{18}	ACN-MeOH	1,40[L]	1,48[L]	1,34[E]
	ACN-H_2O	3,27[E]	2,37[E]	2,37[E]
	ACN-DMSO	1,63[E]	1,48[L]	1,27[L]
	DMK-H_2O	2,59[E]	1,88[E]	1,88[E]
	DMK-PET	1,73[B]	1,59[C]	1,67[B]
Si	ACN-MeOH	1,47[K]	1,47[K]	1,47[K]
	ACN-H_2O	1,71[C]	1,51[C]	2,50[C]
	ACN-DMSO	1,74[J]	1,83[J]	1,71[J]
	DMK-H_2O	2,46[C]	2,46[C]	1,38[D]
	DMK-PET	1,74[B]	1,55[B]	1,55[B]

Concentration of mobile phases: [A]-0:100 [B]-10:90 [C]-20:80 [D]-30:70 [E]-40:60 [F]-50:50 [G]-40:60 [H]-30:70 [J]-20:80 [K]-10:90 [L]-0:100

Table 1 (Cont'd). The highest values of the parameter α between tested androgens and progesterone using different mobile phases (v/v).

Adsorbent	Eluent (v/v)	Testosterone/ Methyltestosterone	Testosterone/ trans-Androsterone	trans-Androsterone /Methyltestosterone
CN	ACN-MeOH	1,06[H]	1,26[C]	1,26[C]
	ACN-H_2O	1,13[F]	1,53[E]	1,40[E]
	ACN-DMSO	1,05[L]	1,15[L]	1,18[K]
	DMK-H_2O	1,24[E]	1,55[D]	1,36[C]
	DMK-PET	1,17[D]	1,32[D]	1,14[C]
C_{18}	ACN-MeOH	1,23[A]	1,13[G]	1,29[D]
	ACN-H_2O	1,43[F]	1,43[F]	1,17[L]
	ACN-DMSO	1,38[E]	1,81[E]	1,31[E]
	DMK-H_2O	1,38[E]	1,70[D]	1,35[C]
	DMK-PET	1,54[B]	1,63[B]	1,08[E]
Si	ACN-MeOH	1,12[C]	1,27[L]	1,19[C]
	ACN-H_2O	1,99[E]	1,46[C]	1,99[E]
	ACN-DMSO	1,08[L]	1,27[L]	1,18[L]
	DMK-H_2O	1,23[D]	2,27[C]	2,27[C]
	DMK-PET	1,34[B]	1,43[B]	1,09[E]

Concentration of mobile phases: [A]-0:100 [B]-10:90 [C]-20:80 [D]-30:70 [E]-40:60 [F]-50:50 [G]-40:60 [H]-30:70 [J]-20:80 [K]-10:90 [L]-0:100

Table 2 presents separation between estrogens and progesterone. As might have been expected the obtained separation factor α between both estrogens was often less than 1.5. The highest value of the parameter α (2,43) betweenβ-estradiol and estrone was observed when using RP-TLC with acetonitrile– dimethyl sulfoxide (30:70 v/v) as a mobile phase. Additionally, on silica gel and C_{18} bonded silica gel with acetone-petroleum ether satisfying separation of these two compounds took place. Excellent separation was observed between estrogens and progesterone on C_{18} bonded silica gel with acetonitrile– dimethyl sulfoxide as mobile phase.

The separation factors α between cholesterol and other solutes are summarized in Table 3. Among the analyzed compounds cholesterol is present in the largest quantities in biological samples, thus the separation of other analytes from cholesterol is really important. The effective separation was observed on C_{18} bonded silica gel, when acetonitrile-methanol was used as mobile phase. Good results were obtained in pure methanol, with exception of estrone. Better separation of this compound was achieved with the addition of acetonitrile (40:60). Excellent separation was observed on silica gel when acetone-water was used as mobile phase. In this chromatographic systems cholesterol migrated around the front of the mobile phase whereas tested hormones were eluted slightly. In comparison with previous study on diol plates [29], the value of separation factor α between steroid hormones (estradiol, testosterone) and cholesterol was higher when silica gel with mixtures of acetone and water was used.

Table 2. The highest values of the parameter α between tested estrogens and progesterone using different mobile phases (v/v).

Adsorbent	Eluent (v/v)	β -Estradiol/ Estrone	β-Estradiol/ Progesterone	Estrone/ Progesterone
CN	ACN-MeOH	1,35^{K}	1,48^{F}	1,42^{K}
	ACN-H_2O	1,71^{D}	1,74^{F}	1,55^{C}
	ACN-DMSO	1,26^{K}	1,38^{H}	1,57^{J}
	DMK-H_2O	1,50^{F}	2,11^{J}	1,44^{J}
	DMK-PET	1,65^{B}	2,22^{B}	1,42^{D}
C_{18}	ACN-MeOH	1,64^{L}	2,55^{K}	3,61^{K}
	ACN-H_2O	1,43^{E}	4,24^{E}	3,78^{K}
	ACN-DMSO	2,43^{D}	14,53^{F}	14,53^{F}
	DMK-H_2O	1,28^{K}	2,39^{F}	2,63^{F}
	DMK-PET	1,94^{B}	3,07^{D}	3,38^{D}
Si	ACN-MeOH	1,40^{K}	1,38^{D}	1,40^{K}
	ACN-H_2O	1,35^{L}	1,79^{C}	2,26^{C}
	ACN-DMSO	1,64^{J}	1,90^{J}	3,12^{J}
	DMK-H_2O	1,28^{D}	1,99^{D}	2,56^{D}
	DMK-PET	1,99^{C}	3,20^{B}	1,88^{B}

Concentration of mobile phases: ^{A}-0:100 ^{B}-10:90 ^{C}-20:80 ^{D}-30:70 ^{E}-40:60 ^{F}-50:50 ^{G}-40:60 ^{H}-30:70 ^{J}-20:80 ^{K}-10:90 ^{L}-0:100

Table 3. The highest values of the parameter α between tested steroids and cholesterol using different mobile phases (v/v).

Adsorbent	Eluent (v/v)	trans-Androsterone	Methyltestosterone	Testosterone
CN	ACN-MeOH	1,83^{D}	1,73^{D}	1,73^{D}
	ACN-H_2O	9,89^{D}	7,51^{D}	6,93^{D}
	ACN-DMSO	1,93^{H}	1,93^{H}	1,93^{H}
	DMK-H_2O	4,17^{F}	4,75^{F}	5,17^{F}
	DMK-PET	4,69^{E}	5,31^{E}	5,76^{E}
C_{18}	ACN-MeOH	6,95^{L}	8,11^{L}	7,70^{L}
	ACN-H_2O	8,56^{K}	9,01^{K}	7,70^{L}
	ACN-DMSO	11,96^{E}	9,12^{E}	6,62^{E}
	DMK-H_2O	2,68^{F}	2,08^{F}	1,76^{F}
	DMK-PET	3,17^{D}	3,31^{D}	2,90^{D}
Si	ACN-MeOH	2,53^{L}	2,97^{L}	3,22^{L}
	ACN-H_2O	3,37^{D}	5,94^{D}	4,33^{D}
	ACN-DMSO	2,53^{L}	2,97^{L}	3,22^{L}
	DMK-H_2O	164,04^{B}	95,12^{B}	79,95^{B}
	DMK-PET	2,45^{E}	2,34^{B}	2,52^{C}

Concentration of mobile phases: ^{A}-0:100 ^{B}-10:90 ^{C}-20:80 ^{D}-30:70 ^{E}-40:60 ^{F}-50:50 ^{G}-40:60 ^{H}-30:70 ^{J}-20:80 ^{K}-10:90 ^{L}-0:100

Table 3 (Cont'd). The highest values of the parameter α between tested steroids and cholesterol using different mobile phases (v/v).

Adsorbent	Eluent (v/v)	Progesterone	Estrone	β-Estradiol
CN	ACN-MeOH	1,83[D]	1,63[D]	1,35[D]
	ACN-H_2O	11,00[D]	11,00[D]	6,42[D]
	ACN-DMSO	1,93[H]	1,40[H]	1,40[H]
	DMK-H_2O	2,42[F]	3,30[F]	4,96[F]
	DMK-PET	4,31[E]	5,10[E]	5,10[E]
C_{18}	ACN-MeOH	5,49[L]	1,77[G]	2,50[L]
	ACN-H_2O	6,73[K]	3,47[E]	2,50[L]
	ACN-DMSO	10,78[E]	1,70[F]	1,70[F]
	DMK-H_2O	3,61[F]	1,98[J]	1,55[J]
	DMK-PET	2,25[D]	1,85[D]	1,85[D]
Si	ACN-MeOH	2,23[L]	1,81[D]	2,33[L]
	ACN-H_2O	6,46[D]	4,54[D]	4,54[D]
	ACN-DMSO	2,23[L]	1,72[L]	2,33[L]
	DMK-H_2O	164,04[B]	114,33[B]	104,13[B]
	DMK-PET	1,69[D]	1,84[D]	4,83[B]

Concentration of mobile phases: [A]-0:100 [B]-10:90 [C]-20:80 [D]-30:70 [E]-40:60 [F]-50:50 [G]-40:60 [H]-30:70 [J]-20:80 [K]-10:90 [L]-0:100

Conclusion

The suggested HPTLC and TLC methods have proven to be simple, rapid and low cost for effective separation of the tested steroids. Excellent separation between cholesterol and other tested compounds was observed on silica gel when acetone-water was used as mobile phase. From the practical point of view, in order to separate tested hormones NP-TLC and RP-TLC can be recommended. Some of analyzed chromatographic systems can be recommended for further studies in which the biological, pharmaceutical or environmental samples would be examined. Similar chromatographic behavior of tested steroids was observed on CN-bonded plates, consequently this stationary phases does not has good ability to separate of tested steroids.

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