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A simple TLC and HPTLC method for separation of selected steroid drugs

Research Article

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Abstract: Chromatographic properties of five steroid drugs: cortisone, hydrocortisone, methylprednisolone, prednisolone and norgestrel have been studied by normal-, reversed-phase and hydrophilic neutral cyano-bonded silica stationary phase with five binary mobile phases (acetonitrile-water, acetonitrile-DMSO, acetonitrile-methanol, acetone-petroleum ether, acetone-water) in which the concentration of organic modifier was varied from 0 to 100% (v/v). This study reports the optimization of steroid hormones separation. Chromatographic retention data and possible retention mechanisms are discussed. Separation abilities of mobile and stationary phases were studied using the principal component analysis method. The best separation of methylprednisolone and prednisolone is with a chromatographic system included silica gel as stationary phase and mixture of acetonitrile and DMSO (10:90 v/v). These two anti-inflammatory drugs can be fast separated from norgestrel when CN is used as stationary phase and acetone and water (40:60 v/v) as mobile phase. The highest values of the parameter $\Delta(\Delta G^{o})$ and alfa for cortisone and hydrocortisone was observed in case of using CN as stationary phase and water-acetonitryle (40:60 v/v) as mobile phase.

Keywords: *Principal component analysis • Separation factor • Standard (Gibbs) free energy change of partition • Steroid drugs • Thin-layer chromatography*

1. Introduction

Chemical structure of Steroids includes skeleton of three hexagonal and one pentagonal carbon ring which are generally arranged in a 6-6-6-5 structure, into which various functional groups and side chains are attached [1]. Steroids are widely used both internally and externally as medication due to their variety of biological activities. They are essential for regulation of large number physiological processes such as regulation of water-mineral balance, metabolism, inflammatory process and regulation of immune functions. Steroids have also an impact on development of sexual characteristics, menstrual cycle, and maintenance of pregnancy. The steroid hormones and synthetic compounds have many therapeutic applications. On the basis of their clinical efficacy and potency, these drugs have been divided into different classes. One important group is anti-inflammatory steroid drugs. These drugs are still in use today particularly in the case of patients with severe asthma [2] or prevention of transplant rejection. Dermocorticosteroids are particularly effective in numerous inflammatory cutaneous pathologies such as scalp psoriasis and dermatitis. They are formally contraindicated in skin infections, diaper rash, acne and rosacea [3]. Glucocorticoids are compounds widely used in veterinary medicine. The improper or illegal use of corticosteroidal hormones as veterinary drugs may result in unwanted residues in food products derived from livestock breeding. To protect consumers' health, the

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Table 1. Determination of steroids in different matrices.

Steroid	Matrix	Analytical system	Ref.
Hydrocortisone, methylprednisolone	Stock solutions	TLC	[5]
Cortisone, hydrocortisone, methylprednisolone,prednisolone,	Cosmetic products	TLC HPLC-UV	[6]
Cortisone , hydrocortisone, methylprednisolone	Liquid herbal medicines	HPLC-UV	[7]
Cortisone, hydrocortisone	Human fetal cord blood	HPLC-UV	[8]
Cortisone, prednisolone	Stock solutions	HPLC-LC	[9]
Cortisone, hydrocortisone	Plasma	HPLC-Chip/ESI-MS	[10]
Methylprednisolone, prednisone	Cattle tissue and hair	LC–MS^2	[11]
Norgestrel, prednisone, methylprednisolone, cortisone	Surface water	LC–MS^2	[12]
Cortisone, hydrocortisone, prednisone	Plasma	LC–MS^2	[13]
Cortisone, hydrocortisone, prednisone, methylprednisolone	Urine	LC–MS^2	[14]
Cortisone, prednisone, methylprednisolone	Bovine liver	LC–MS^2	[15]
Cortisone, hydrocortisone	Saliva	LC–MS^2	[16]
Cortisone, hydrocortisone	Serum	LC–MS^2	[17]
Cortisone, hydrocortisone	Mouse liver and adipose tissue; Human adipose tissue	LC–MS^2	[18]
Hydrocortisone, prednisone, methylprednisolone	Muscle of swine, cattle, and sheep	LC–MS^2	[19]
Methylprednisolone, prednisone	Hematopoietic stem cell transplantation	LC–MS^2	[20]
Methylprednisolone, prednisone	Bovine liver	LC–MS^2	[21]
Methylprednisolone, prednisone	Equine plasma	LC–MS^2	[22]
Hydrocortisone, methylprednisolone	Human adipose tissue	LC–MS^2	[23]
Cortisone, hydrocortisone, prednisone, methylprednisolone	Hair	LC- ESI-MS	[24]
Methylprednisolone, prednisone	Urine	LC- ESI-MS	[25]
Norgestrel, hydrocortisone, cortisone, prednisone, methylprednisolone	Muscle (pork, beef, shrimp), milk and pig liver	LC–ESI-MS^2	[26]
Cortisone, hydrocortisone	Stock solutions in acetonitrile	UHPLC-UV	[27]
Cortisone, hydrocortisone	Plasma, urine, saliva	UHPLC-MS^2	[28]
Methylprednisolone, prednisone	Liver (bovine, porcine, ovine, equine)	UHPLC–MS^2	[29]
Hydrocortisone, prednisone, methylprednisolone	Urine	UHPLC-MS^2	[30]
Cortisone, hydrocortisone, prednisone, methylprednisolone	Sewage and river waters	UHPLC–ESI –MS^2	[31]

Continued **Table 1.** Determination of steroids in different matrices.

Steroid	Matrix	Analytical system	Ref.
Cortisone, hydrocortisone, prednisone, methylprednisolone	Urine	UPLC-TOF-MS	[32]
Norgestrel, hydrocortisone, cortisone, prednisone	Surface water, wastewater and sludge	RRLC–MS^2	[33]
Hydrocortisone, prednisone	Plasma	GC-MS	[34]
Cortisone , hydrocortisone	Urine	GC-MS	[35]
Cortisone, hydrocortisone	Plasma	GC-MS	[36]
Cortisone, hydrocortisone	Stock solutions in 1:1 (v/v) water:acetonitrile	SALDI TOF-MS; MALDI	[37]
Cortisone, hydrocortisone	Stock solutions in methanol	SALDI -MS	[38]
Cortisone, hydrocortisone	Stock solution in ethanol	PF-MEKC–ESI-MS	[39]
Hydrocortisone, prednisone	Stock solutions	MEKC-ESI-MS MEKC-DA-APPI-MS ITMS	[40]
Cortisone, hydrocortisone	Stock solutions in methanol	IEC	[41]
Cortisone, hydrocortisone, prednisone	stock solutions in methanol	SFC-UV	[42]
Cortisone, hydrocortisone	Stock solutions	CSI-MS (PFG) NMR X-ray	[43]
Cortisone, prednisone	Stock solutions in acetonitrile	SOSLC	[44]

TLC - thin-layer chromatography
HPLC-UV – high performance liquid chromatography - ultraviolet spectrophotometry detection
HPLC-LC - high performance liquid chromatography with chemiluminiscence system
HPLC-Chip-ESI-MS - microfluidic-based liquid chromatography-electrospray ionization/mass spectrometric system
LC-MS - liquid chromatography - mass spektrometry detection
LC–ESI-MS- - liquid chromatography–electrospray-tandem mass spektrometry detection
UHPLC-UV - ultra high performance liquid chromatography ultraviolet spectrophotometry detection
UHPLC-MS^2 - ultra high performance liquid chromatography–tandem mass spectrometry
UHPLC-ESI-MS^2 - ultra high performance liquid chromatography– electrospray-tandem mass spektrometry detection
UPLC-TOF-MS - ultra performance liquid chromatography - time-of-flight mass spectrometry detection
RRLC–MS-MS - rapid resolution liquid chromatography–tandem mass spectrometry detection
GC-MS - gas chromatography – mass spectrometry detection
SALDI TOF-MS - surface-assisted laser desorption ionisation time-of-flight mass spectrometry detection
MALDI - matrix assisted laser desorption ionisation
SALDI -MS - surface-assisted laser desorption ionization- mass spectrometry detection
PF-MEKC–ESI-MS - partial filling micellar electrokinetic chromatography - elektrospray mass spectrometry
MEKC-ESI-MS - micellar electrokinetic chromatography - electrospray ionisation mass spectrometry detection
MEKC-DA-APPI-MS-ITMS - dopant-assisted atmospheric pressure photoionization – electrospray ionization for the coupling of micellar electrokinetic chromatography with ion trap mass spectrometry detection
IEC - ion-exchange chromatography
SFC-UV - supercritical fluid chromatography- ultraviolet spectrophotometry detection
CSI-MS- PFG-NMR - cold-spray ionization mass spectrometry – pulsed field gradient – nuclear magnetic resonance spektrometry
SOSLC - stationary phase optimized selectivity liquid chromatography

European Union has outlawed the use of corticosteroidal hormones in livestock breeding and aquaculture [4].

The determinations of studied steroids in different matrices are shown in Table 1.

HPLC was used to separate steroids. Nowadays, this is most popular technique of separation. It allows you to separate effectively and obtain results which are reproducible. The advantage of HPLC is the ability to use different types of stationary phases and great opportunities in modification of the mobile phase. Different types of detection are used. The best is use of a mass detector. The spectrum of a mass detector confirms the identity of the compound. The UV and luminescence spectrometer is also in use. One of the most sensitive and selective detection techniques for liquid chromatography is chemiluminescence (CL), in which the emission of electromagnetic radiation (ultraviolet, visible or infrared) is produced by a chemical

reaction based on the decay of an excited species to the electronic ground-state. The methods developed include derivatization procedures for corticosteroids prior to the chemiluminescence (CL) reaction. UPLC as a good separation method is presented, but nowadays this method is not widespread. The increased resolution obtained in shorter time in this method can generate more information faster by using higher pressure and smaller particles. In GC, steroid drugs must be converted to a volatile derivative, with the use of the special enzymes.

New separation techniques are supercritical fluid chromatography (SFC), ion-exchange chromatography (IEC) and similar were used in studies of stock solutions. Testing new expensive and complicated methods proves that, separation of steroid drugs is a considerable analytical problem.

TLC can be applied as screening method. This method has some advantages over the other chromatographic methods: it is rapid and relatively simple, it is a low cost method and the laboratory does not require expensive equipment. Additionally, TLC requires small amounts of substances in analysis which can be detected by chemical reactions.

2. Theoretical details

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 the chemical structure of the analytes and also types and properties of both phases. With the assumption that mixture is completely selected, selectivity of the system might be described as distance between the maximum of adjacent chromatographic peaks. Relative retention reflects differences in influence of both ingredients with mobile and stationary phases and is defined by equation [45]:

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

in which: k_1, k_2 – retention factors of substances 1 and 2, K_1 i K_2 – distribution coefficients.

Eq. 1 shows that separation factor is equal to relation of partition coefficients in balanced state or factors of both substances' retention. When α = 1, it means that the resolving power equals zero which means that both chromatographed substances are not different in the thermodynamic aspect and the selection could not be achieved.

Selectivity is a measure of thermodynamic differences in partition behavior between components 1 and 2:

$$\Delta\left(\Delta G^0\right) = -RT \ln \alpha \quad (2)$$

in which: $\Delta(\Delta G^o)$ is a standard (Gibbs) free energy change of partition [45-47].

In order to separate components 1 and 2, there have to be some differences in thermodynamics of their partition. Larger amount of substances are characterized by correlations $R_{M\,1}$ *vs.* $R_{M\,2}$. Efficiency of solvent might be calculated using relative retention while efficiency of stationary phase is described by retention factor.

Principal component analysis (PCA) is central to the study of multivariate data. PCA is widely used in chemistry and has recently been used for mobile phase optimization in thin layer chromatography [48-50]. PCA allows maximum use of the information contained in the data matrices. The parameter R_M was the basis for the principal components analysis. As it is apparent from the formula log:

$$R_M = \log\left(\frac{1}{R_f} - 1\right) \quad (3)$$

that it is impossible to calculate log *k,* where the R_f parameter values are equal to 0 and 1. Thus, in order to conduct statistical analysis it has been assumed that for R_f = 0, R_M = 2, which may suggest a very strong analyte-adsorbent interaction. R_f = 1 corresponds to the R_M = -2, which also implies a strong interaction of the test compound with the eluent. All statistical calculations were performed using STATISTICA 9.1.

The purpose of the work discussed in this paper was: - to study the retention and separation of selected steroids by TLC and HPTLC with a variety of aqueous and non-aqueous mobile phases, and a variety of adsorbents; to determine the effect of the solvents used as mobile phases, and adsorbents used as stationary phases on the chromatographic behavior of the compounds; and to evaluate the usefulness of particular chromatographic systems for analysis of steroids. Furthermore, the optimization study resulted in a better understanding of the structure-retention relationship of steroids. PCA was used to assess the suitability of different chromatographic systems for the separation of a mixture of steroids.

3. Experimental procedure

3.1. Solvents and chemicals

The glucocorticoids (cortisone and hydrocortisone) were purchased from Sigma-Aldrich (UK). All reagents

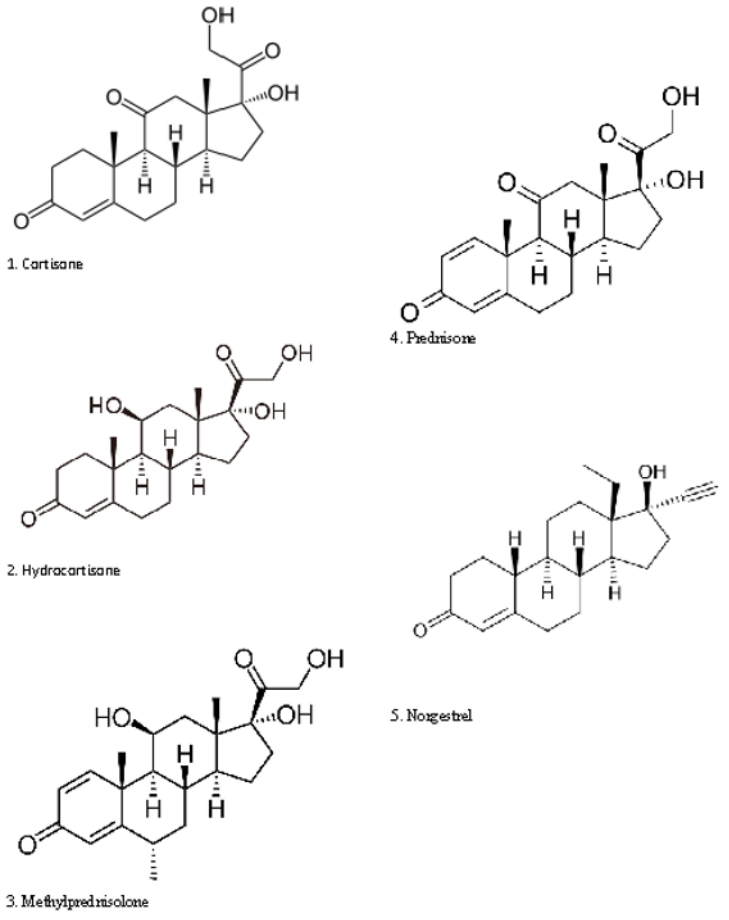

Figure 1. Structure of selected steroids.

used as mobile phases (acetonitrile, acetone, methanol, DMSO and petroleum ether) were HPLC – reagent grade from Sigma-Aldrich (Steinheim, Germany). Sulfuric acid (POCh, Poland) was used to prepare the visualizing reagents. Water was double distilled.

3.1.1. Tablets

The following available tablets were used in the present investigation: Medrol (declared amount per tablet 16.0 mg methylprednisolone, Pfizer Manufacturing Belgium N.V.), Encorton (declared amount per tablet 10.0 mg prednisone, Pabianickie Zakłady Farmaceutyczne POLFA, Poland) and Prempak-C (declared amount per tablet 0.15 mg norgestrel, Wyeth, U.S.). These tablets were obtained commercially. Chemical structures of the hormones are depicted in Fig. 1.

3.2. Sample preparation

Solutions of cortisone and hydrocortisone (1 mg L^{-1}) were prepared by dissolving appropriate weighed amounts in methanol. Methanol was chosen as the modifier as it was found to be a proper solvent for the analytes.

Tablets were crushed into a fine powder and appropriate amounts of each one were dissolved in methanol diluent using an ultrasonic bath. The solution was filtered through a disposable syringe filter Chromafil® PET-45/25 (Macherey-Nagel). An aliquot of the filtrate solution was taken in the volumetric flask and diluted to obtain standard stock solutions of 1 mg L^{-1}. This solution was used as the working standard for the analysis of all samples.

3.3. Mobile phase

Binary phases (acetonitrile-water, acetonitrile-DMSO, acetonitrile-methanol, acetone-petroleum ether, acetone-water) were prepared by mixing appropriate quantities of pure organic solvents in the proportions from 0 to 100% *(v/v)*.

3.4. 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), in chromatographic chambers (7×11 cm) previously saturated with mobile-phase vapour. The chromatographic chamber was saturated 20 minutes before analysis. In the case of using water – organic modification mixture as mobile phase was used Kieselgel 60 WF_{254s} TLC plates and RP-18 WF_{254s} HPTLC plates. Chromatograms were developed to a distance of 8 cm at room temperature (20±2°C). The steroids were visualized by spraying the plates with a 1:4 (v/v) mixture of concentrated sulphuric acid and methanol. After spraying, the plates were heated for 10 min at 120°C .

4. Results and discussion

The largest achieved values of separation factors α of cortisone and hydrocortisone (Table 2) and prednisone, methylprednisolone and norgestrel (Table 2) on silica, octadecylsilane and cyanopropyl adsorbent (CN-silica) were compared. RP-18 plates gained the maximum value of parameter α for cortisone and hydrocortisone after usage of acetonitrile-methanol 90:10 (v/v) binary mobile phase. An example of silica gel, parameter α reached maximum value with usage of acetone in water 80:20 (v/v) while in case of CN adsorbent with usage of acetonitryle in water 60:40 (v/v). Therefore, the addition of water into the organic mobile phase significantly improved separation of cortisone and hydrocortisone on plates covered by silica gel and cyanopropyl phase. It leads to considerations on the hypothesis of retention mechanism's similarity on both adsorbents. In this case, chromatographic processes in such a system of stationary and mobile phases are based on the effect of adsorption.

While analyzing the results gained in studies of prednisone, methylpredisolone and norgestrel, similarities of chromatographic behavior of prednisone and methylopredisolone on silica and cyanopropyl adsorbents (with use of eluents containing acetonitryle and small addition of DMSO) were observed. In such chosen chromatographic systems, values of parameter α for these compounds gained a maximum value. Probably interrelations between hormones and cyanopropyl phase are similar to these in normal phase systems which means that the chromatographic process has adsorption character, like in case of cortisone and hydrocortisone, but in the non-water environment. In the reversed-phase, a maximum value of parameter α was gained for prednisone and methylpredisolone with use of a binary mobile phase acetone-water 30:70 (v/v). After usage of acetone in water 40:60 (v/v) on the cyanopropyl adsorbent, maximum values of parameter α were gained for pairs: prednisone/norgestrel and methylpredisolone/norgestrel with values of α = 10.02 and α = 6.00 respectively. In normal- and reversed-phases the best separation of both pairs took place after usage of non-water eluents.

Data related to differences of easy separation energy of two compounds $\Delta(\Delta G^o)$ were shown in Table 3. They somehow confirm previous conclusions related to satisfying separation of analyzed steroids.

Retention of selected steroids was analyzed on polar adsorbents: bounded phase (CN-silica phase type), silica gel and on non-polar octadecylsilane adsorbent. Parameters of correlation lines calculated from equations $R_{M\,CN} = a + b\,R_{M\,NP}$ and $R_{M\,CN} = a + b\,R_{MRP}$ (R_M CN - parameters R_M calculated on cyanopropyl phase, R_M NP - parameters R_M calculated in normal-phase, R_M RP - parameters R_M calculated in reversed-phase) give an opportunity to compare mechanisms of steroids retention in analyzed chromatographic systems (Table 4). Interrelation $R_{M\,I}$ *vs.* $R_{M\,II}$ which means comparison of retention parameters on plates covered by cyanopropyl phase (I), octadecysilane (II) and silica gel (II) allows to present two important conclusions. Firstly, it allows quite easy interpretation of relations above as chemism and distribution of active centers on silica gel surface are commonly known which allows for easy identification of cyanopropyl phase's active centers. Secondly, such interrelations allows you to choose the most selective systems in relation to specific group of compounds. Linear correlations $R_{M\,I}$ vs. $R_{M\,II}$ for analyzed steroids were observed which might suggest the fact that active centers which decide on adsorption of this group's substances are similar. The slope of the correlation lines larger than 1 showed a good division of compounds.

Low factors of straight lines correlation do mean individual differences in selectivity of separation. Large correlation factors of line $R_{M\,I}$ vs. $R_{M\,II}$ confirm theory of co- adsorption of chromatographed substances on monolayer of this polar dissolvent on silica gel and cyanopropyl surface which makes two surfaces similar and cause that mechanism of interrelations in both systems is also similar. Additionally, high values of correlation lines inclination show high adsorption energy of analyzed compounds and strong solvation effect of compounds by polar dissolvent and strong elution with silica gel in some eluents systems.

Linear correlation between R_M values and the concentration of organic solvent in the eluents was calculated (Table 5). The high correlation coefficients (r), the significance levels (p), and small values of the standard deviation indicated that all the equations

Table 2. The highest values of the parameter α fo r prednisone, methylprednisolone, norgestrel and for cortisone and hydrocortisone, using different mobile phases (v/v).

Adsorbent	Eluent (v/v)	Methylprednisolone/ prednisone	Prednisone/ norgestrel	Methylprednisolone/ norgestrel	Eluent (v/v)	Cortisone / hydrocortisone
Kieselgel 60 CN	ACN-MeOH 40:60	1.19	1.56	1.85	ACN-MeOH 30:70	1.13
	ACN-H_2O 40:60	1.08	1.38	1.27	ACN-H_2O 60:40	1.58
	ACN-DMSO 80:20	1.23	2.31	1.89	ACN-DMSO 80:20	1.07
	DMK-H_2O 40:60	1.14	10.02	6	DMK-H_2O 80:20	1.4
	DMK-PET 30:70	1.15	5.25	4.32	DMK-PET 20:80	1.48
RP-18	ACN-MeOH 90:10	1.35	2.73	2.02	ACN-MeOH 90:10	1.36
	ACN-H_2O 30:70	1.44	2.37	1.65	ACN-H_2O 70:30	1.15
	ACN-DMSO 90:10	1.08	2.92	2.7	ACN-DMSO 30:70	1.18
	DMK-H_2O 30:70	1.99	1.18	1.64	DMK-H_2O 60:40	1.24
	DMK-PET 30:70	1.13	2.27	73	DMK-PET 70:30	1.22
Kieselgel 60	ACN-MeOH 20:80	1.2	1.39	1.16	ACN-MeOH 90:10	1.23
	ACN-H_2O 80:20	1.38	2.14	2.95	ACN-H_2O 60:40	1.36
	ACN-DMSO 90:10	2.53	1.57	3.96	ACN-DMSO 80:20	1.23
	DMK-H_2O 80:20	1.08	2.34	2.53	DMK-H_2O 80:20	1.48
	DMK-PET 40:60	1.24	3.31	2.35	DMK-PET 20:80	1.21

Table 3. The highest values of the parameter $\Delta(\Delta G^{\circ})$ for prednisone, methylprednisolone, norgestrel and for cortisone and hydrocortisone, using different mobile phases (v/v).

Adsorbent	Eluent (v/v)	Methylprednisolone/ prednisone	Prednisone/ norgestrel	Methylprednisolone/ norgestrel	Eluent (v/v)	Cortisone / hydrocortisone
Kieselgel 60 CN	ACN-MeOH 40:60	2.95	3.87	4.59	ACN-MeOH 30:70	2.8
	ACN-H_2O 40:60	2.68	3.42	3.15	ACN-H_2O 60:40	3.92
	ACN-DMSO 80:20	3.05	5.73	469	ACN-DMSO 80:20	2.65
	DMK-H_2O 40:60	2.83	24.84	14.87	DMK-H_2O 80:20	3.47
	DMK-PET 30:70	2.85	13.01	10.71	DMK-PET 20:80	3.67
RP-18	ACN-MeOH 90:10	3.35	6.77	5.01	ACN-MeOH 90:10	3.37
	ACN-H_2O 30:70	3.57	5.88	4.09	ACN-H_2O 70:30	2.85
	ACN-DMSO 90:10	2.68	7.24	6.69	ACN-DMSO 30:70	2.93
	DMK-H_2O 30:70	4.93	2.93	4.07	DMK-H_2O 60:40	3.07
	DMK-PET 30:70	2.8	5.63	6.77	DMK-PET 70:30	3.02
Kieselgel 60	ACN-MeOH 20:80	2.97	3.45	2.88	ACN-MeOH 90:10	3.05
	ACN-H_2O 80:20	3.42	5.31	7.31	ACN-H_2O 60:40	3.37
	ACN-DMSO 90:10	6.27	3.89	9.82	ACN-DMSO 80:20	3.05
	DMK-H_2O 80:20	2.68	5.80	6.27	DMK-H_2O 80:20	3.67
	DMK-PET 40:60	3.07	8.21	5.83	DMK-PET 20:80	3.00

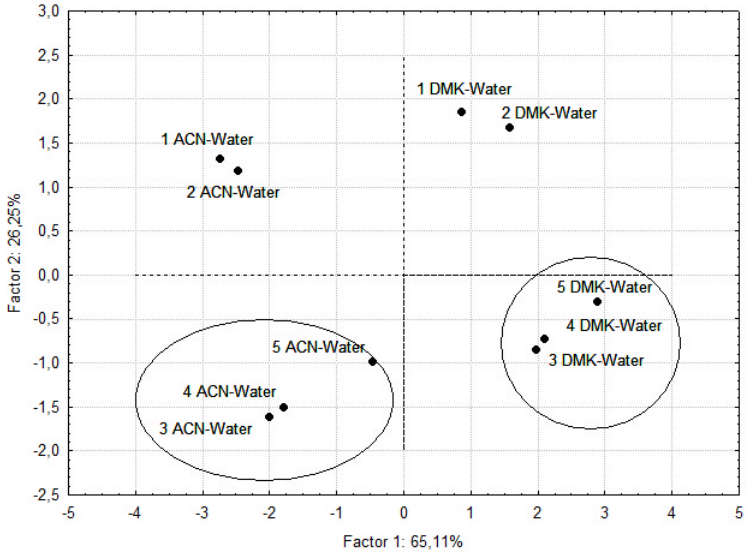

Figure 2. Principal component analysis 1 shows structure-retention relationships of studied steroids. The numbers on the graph correspond to the hormone respectively: 1 – cortisone, 2 – hydrocortisone, 3 – methylprednisolone, 4 – prednisone and 5 – norgestrel. Data incorporated into the matrix consisted of retention data (R_M) of the studied steroids using silica gel adsorbent as a stationary phase and a mixture of acetonitrile-water and acetone-water in a wide range of concentrations (20-80% v/v) as the mobile phase. The matrix consists of 10 cases and 7 variables.

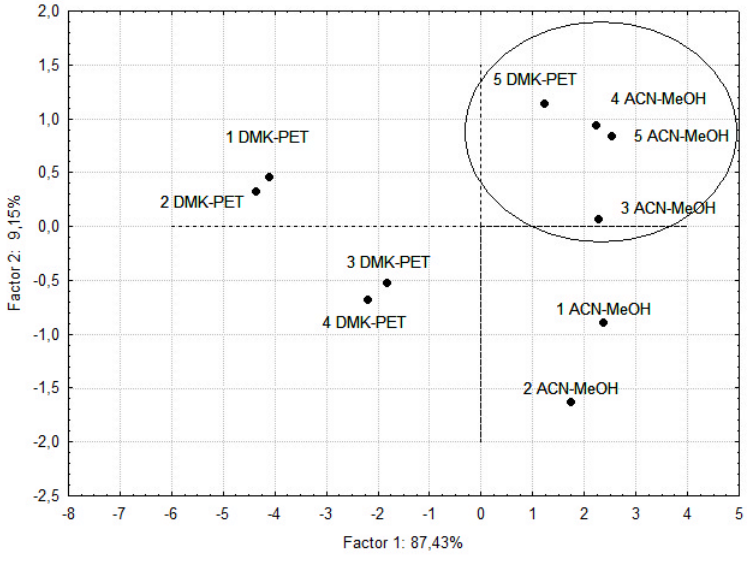

Figure 3. Principal component analysis 2 was performed with retention data (R_M) of studied steroids using acetonitrile-methanol and acetone-petroleum ether (10-90% v/v) as mobile phases and silica gel as a stationary phase. The numbers on the graph correspond to the hormone respectively: 1 – cortisone, 2 – hydrocortisone, 3 – methylprednisolone, 4 – prednisone and 5 – norgestrel. The matrix consists of 10 cases and 9 variables.

obtained were highly significant. Most frequently, in the case of using stationary phase RP 18 and CN correlation between RM values and the concentration of organic solvent in mobile phase were linear.

PCA was carried out by taking into consideration the R_M values of steroid hormones. Cases and variables for both PCA analyses are listed in Table 6. Greater effect on the retention of the test substances resulted in the presence or lack of a double bond between C1 and C2 atoms, as suggested in Table 2. Therefore, it is interesting that the change of substituents on the atoms C11, C13, and C17 of sterane molecule changes the retention norgestrel, which, despite the lack of a double bond in ring A is grouped with prednisone and methylprednisolone. This situation occurs when both aqueous (Fig. 2) and nonaqueous (Fig. 3) were used as mobile phases and

Table 4. Parameters of equation $R_{M\,CN} = a + b\,R_{M\,NP}$ and $R_{M\,CN} = a + b\,R_{M\,RP}$ for steroid drugs with binary mobile phases (*NP* – parameters R_M on normal-phase, *RP* – parameters R_M on reversed-phase, *CN* - parameters R_M on cyano-bonded silica stationary phase).

Eluent Adsorbent	Steroid	a	δ a	b	δ b	N	Concentration range % (v/v)	r
DMK-PET Silica gel (NP)	Cortisone	0.88	0.081	-0.30	0.050	7	20-80	0.980
	Hydrocortisone	1.01	0.099	-0.41	0.061	8	20-90	0.972
	Methylprednisolone	2.10	0.399	-0.26	0.055	6	20-80	0.935
	Prednisone	1.38	0.045	-0.30	0.008	4	40-70	0.999
DMK-H_2O RP-18	Cortisone	1.00	0.039	-0.10	0.027	9	10-90	0.995
	Hydrocortisone	1.07	0.057	-0.21	0.039	9	10-100	0.989
	Methylprednisolone	0.66	0.050	-0.12	0.026	8	30-100	0.983
	Prednisone	0.68	0.107	-0.07	0.049	5	30-70	0.965
	Norgestrel	1.76	0.162	0.41	0.067	7	40-100	0.979
ACN-H_2O Silica gel (NP)	Cortisone	1.09	0.185	-0.19	0.050	7	40-100	0.935
	Hydrocortisone	1.05	0.198	0.29	0.094	7	40-100	0.921
	Methylprednisolone	0.69	0.111	-0.19	0.068	9	20-100	0.920
	Prednisone	0.96	0.066	-0.21	0.034	8	20-90	0.986
	Norgestrel	0.21	0.036	-0.53	0.014	5	50-90	0.957

δ - standard deviation

Table 5. Regression coefficients (*a*, *b*) and correlation coefficient (*r*) for the regression equation $R_M = a\,\%S + b$ for chromatography of steroids; $p < 0.05$.

Kieselgel 60 CN Eluent	Steroid	a	δ a	b	δ b	N	Concentration range % (v/v)	r	p
ACN-H_2O	Cortisone	-0.02	0.002	1.15	0.131	10	10-100	0.958	0.000
	Hydrocortisone	-0.02	0.003	1.05	0.150	10	10-100	0.942	0.000
	Methylprednisolone	-0.02	0.003	0.96	0.182	8	20-100	0.933	0.001
	Prednisone	-0.02	0.003	0.96	0.184	8	20-100	0.932	0.001
	Norgestrel	-0.02	0.003	0.73	0.180	8	20-100	0.914	0.002
ACN- DMSO	Cortisone	0.02	0.002	-1.95	0.129	4	70-100	0.991	0.009
	Hydrocortisone	0.02	0.002	-1.98	0.173	4	70-100	0.985	0.015
	Norgestrel	0.01	0.001	-1.78	0.096	5	60-100	0.987	0.002
DMK-H_2O	Cortisone	-0.03	0.001	1.29	0.075	11	0-100	0.989	0.000
	Hydrocortisone	-0.03	0.002	1.23	0.095	11	0-100	0.982	0.000
	Methylprednisolone	-0.02	0.002	1.22	0.121	10	10-100	0.961	0.000
	Prednisone	-0.02	0.002	1.07	0.130	10	10-100	0.950	0.000
	Norgestrel	-0.03	0.003	2.46	0.249	7	40-100	0.975	0.000
DMK-PET	Cortisone	-0.02	0.001	1.27	0.084	10	10-100	0.987	0.000
	Hydrocortisone	-0.02	0.001	1.30	0.085	10	10-100	0.988	0.000
	Methylprednisolone	-0.02	0.004	0.96	0.218	9	20-100	0.906	0.002
	Prednisone	-0.02	0.004	1.08	0.233	9	20-100	0.904	0.002

Continued **Table 5.** Regression coefficients (*a*, *b*) and correlation coefficient (*r*) for the regression equation $R_M = a\ \%S + b$ for chromatography of steroids; $p < 0.05$.

Kieselgel 60 CN Eluent	Steroid	a	δ a	b	δ b	N	Concentration range % (v/v)	r	p
RP 18									
	Methylprednisolone	-0.02	0.002	1.46	0.141	9	20-100	0.971	0.000
ACN-H_2O	Prednisone	1.11	0.027	0.08	0.015	9	20-100	0.998	0.000
	Norgestrel	1.02	0.150	-0.47	0.086	8	30-100	0.941	0.000
	Cortisone	0.03	0.002	-2.69	0.165	7	40-100	0.981	0.000
ACN-DMSO	Hydrocortisone	0.03	0.002	-2.82	0.166	7	40-100	0.984	0.000
	Norgestrel	0.03	0.004	-3.27	0.322	4	70-100	0.987	0.013
	Cortisone	-0.02	0.002	1.32	0.134	10	10-100	0.967	0.000
	Hydrocortisone	-0.02	0.002	1.31	0.124	10	10-100	0.971	0.000
DMK-H_2O	Methylprednisolone	-0.02	0.003	1.47	0.213	8	30-100	0.942	0.000
	Prednisone	-0.02	0.003	1.11	0.220	8	30-100	0.921	0.001
	Norgestrel	-0.02	0.002	1.35	0.161	8	30-100	0.965	0.000
	Cortisone	-0.02	0.003	0.48	0.137	6	20-80	0.942	0.001
	Hydrocortisone	-0.02	0.002	0.55	0.133	6	20-80	0.955	0.001
DMK-PET	Methylprednisolone	-0.02	0.002	0.47	0.123	7	10-80	0.932	0.001
	Prednisone	-0.01	0.002	0.40	0.115	7	10-80	0.932	0.001
	Norgestrel	-0.01	0.001	0.13	0.074	7	10-80	0.929	0.001
Kieselgel 60									
	Cortisone	0.01	0.002	-0.85	0.126	7	30-100	0.906	0.005
ACN-H_2O	Hydrocortisone	0.01	0.002	-1.07	0.160	7	30-100	0.920	0.003
	Methylprednisolone	0.01	0.001	-0.78	0.102	7	30-100	0.899	0.006
DMK-H_2O	Methylprednisolone	0.01	0.001	-1.69	0.088	10	10-100	0.954	0.000
	Prednisone	0.01	0.002	-1.68	0.100	10	10-100	0.940	0.000
	Cortisone	-0.02	0.002	1.34	0.163	9	20-100	0.939	0.000
DMK-PET	Hydrocortisone	-0.02	0.003	1.39	0.165	9	20-100	0.939	0.000
	Methylprednisolone	-0.01	0.000	0.38	0.025	10	10-100	0.986	0.000
	Prednisone	-0.01	0.001	0.48	0.045	10	10-100	0.965	0.000

δ - standard deviation, %S - ratio of more polar solvent in less polar in percents

silica gel was used as the stationary phase. As shown in Fig. 2, methylprednisolone, prednisone and norgestrel are grouped in the same quadrant, depending on themobile phase used, acetonitrile-water in quadrant III (-, -), and the acetone-water in quadrant IV (-, +). The difference in retention of norgestrel between the nonaqueous mobile phases (acetonitrile-methanol and acetone-petroleum ehter) (Fig. 3) are much smaller than in aqueous mobile phases because norgestrel by applying both non-aqueous eluents grouped in quadrant I (+, +) next to methylprednisolone and prednisone using acetonitrile-methanol as eluent.

Table 6. Eigenvalues of various PCA. PCA 1 corresponds to Fig. 2 and PCA 2 corresponds to Fig. 3.

No.	Eigenvalues	
	PCA 1	PCA 2
1	4.557517	7.868750
2	1.837171	0.823641
3	0.515310	0.246317
4	0.054106	0.030869
5	0.020991	0.023247
6	0.010821	0.003359
7	0.004084	0.002541
8		0.001265
9		0.000012

5. Conclusion

Addition of water to organic mobile phases had a significant influence on the separation of analyzed steroid drugs, especially in the case of cortisone and hydrocortisone. Remaining analytes were separated in a satisfying way in a non-water environment. Similarities of interrelations between hormones and stationary cyanopropyl and silica phases were observed which leads to considerations on adsorption retention mechanisms on CN adsorbent. This thesis was confirmed by calculated differences of energy related to easy division of two compounds $\Delta(\Delta G^o)$ and comparison of correlation lines parameters.

The best separation of methylprednisolone and prednisolone is in a chromatographic system that includes silica gel as a stationary phase and a mixture of acetonitrile and DMSO (10:90 v/v). These two anti-inflammatory drugs can be fast separated from norgestrel in the case of a CN stationary phase used and a mobile phase of acetone and water (40:60 v/v). The highest values of the parameter $\Delta(\Delta G^o)$ and α for cortisone and hydrocortisone were observed in the case of a CN stationary phase used and water-acetonitrile (40:60 v/v) as a mobile phase.

PCA does not prove but mathematically indicates similarities of retention behavior of norgestrel in aqueous and non-aqueous mobile phases and silica gel as stationary phases is similar to methyloprednisone and predisone, despite the absence of double bond in the norgestrel structure.

Abbreviations

ACN – acetonitrile;
DMK – acetone;
DMSO – dimethyl sulphoxide;
MeOH – methanol;
PET – petroleum ether.

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