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Short communication

# Analysis of some steroids by thin-layer chromatography using optimum mobile phases

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**Abstract**

The aim of this paper was the analysis of five androstane isomers by thin-layer chromatography (TLC). The choice of proper mobile phase and the optimization of the mobile phase composition are very important because the chromatographic separation is difficult to achieve. In the first step, the proper mobile phase system was chosen from seven elution systems presented in the literature using numerical taxonomy method. The proper solvent system was found to be the mixture of chloroform, acetone, and petroleum ether. In the second step the composition of mobile phase was optimized by "simplex" method and "prisma" method. The optimum TLC system can be applied for the separation of androstane isomers from real samples such as drug formulation, biological and natural sources.
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*Keywords:* Thin-layer chromatography; Androstane isomers; Numerical taxonomy; "Simplex" method; "Prisma" method

## 1. Introduction

Steroids are a class of compounds that have a cyclopentanoperhydrophenanthrene skeleton and that occur in nature and in synthetic products. The bile acids, androgens, estrogens, corticosteroids, ecdysteroids, sterols and vitamin D are compounds included in the class of steroids.

Steroids and their metabolites are analyzed by thin-layer chromatography (TLC) in a variety of samples such as biological samples or plants and pharmaceutical formulations. TLC continues to be an important method for the determination of steroids because of its advantages. Many samples can be analyzed simultaneously and quickly at relatively low cost, multiple separation techniques and detection procedures can be applied and the detection limits are often in the low nanogram range and quantitative densitometric methods are accurate. The importance of steroid analysis is evidenced by many papers and chapters of books [1–3].

Many chromatographic systems have been applied for the TLC of steroids. Silica gel TLC and HPTLC layers are the most frequently used. C-8 and C-18 modified silica gel [4], alumina [5], silica gel with 3–10% silver nitrate [6], cellulose unimpregnated or impregnated with 1,2-propanediol [2] and kieselgur [3] have been used as stationary phases. As regarding the detection of steroids new reagents are updated biennially in the review of planar chromatography written by Sherma in the ACS Journal of Analytical Chemistry [7]. Most of the reagents used for detecting steroid spots contain sulfuric acid. Antimony trichloride, molybdophosphoric acid, chlorosulphonic acid, acetic acid and phosphoric acid have been used as destructive reagents for steroids detection.

The aim of this paper was the analysis of five androstane isomers by TLC. The androstane isomers are part of steroid class and they have almost the same structures differing by the number and position of hydroxyl radical. The choice of proper mobile phase and the optimization of the mobile phase composition are very important because the chromatographic separation is difficult to achieve [2]. In the first step, the proper mobile phase system was chosen from seven elution systems presented in the literature using the numerical taxonomy method. The proper solvent system was found to be the mixture of chloroform, acetone, and petroleum ether. In the second step the composition of mobile phase was optimized by "simplex" method and "prisma" method.

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## 2. Experimental

The separations of androstane derivatives by thin-layer chromatography (TLC) on silica gel 60 plates (5 cm × 10 cm, Merck) were performed. The chromatographic plates were developed at room temperature in a saturated N-chamber, by the ascending technique using different mobile phases.

The solutions (0.1%) of the compounds 1–5 (5α-androstan-3β-ol, 5α-androstan-3α-ol, 5α-androstan-17β-ol, 5β-androstan-3α,17β-diol, 5α-androstan-3β,17β-ol) were prepared in methanol. The solutions of standards and the mixture of these compounds were applied with a micropipette as spots on the plate.

The detection was made by spraying the dried plates with 5% ammonium molybdate and 5% sulfuric acid in water and heated at 80 °C [8]. The components appear as dark blue spots on light blue background. The plates were scanned using a flat-bed scanner, canoscan-lide20 type, with 600 × 1200 dpi resolution. The obtained images were processed using a computer program, which selected the zone of interest from plate image and the densitogram was extracted in three spectral fields: red, green and blue. The chromatogram was obtained by smoothing and baseline substraction.

## 3. Results and discussions

In order to separate the androstane isomers the choice of proper mobile phase and the optimization of its composition were made. The structural formulas of androstane isomers are presented in Fig. 1.

In the first step, the numerical taxonomy method was applied for the choice of proper mobile phase system from seven elution systems (Table 1) presented in the literature [3].

The numerical taxonomy used a variety of related mathematical techniques in order to classify the solvent systems into groups based on the $R_f$ values [9]. The similarity between two solvent systems was characterized by the taxonomic distance, $d_{j,k}$:

$$d_{j,k} = \sqrt{\sum_{i=1}^{n} \frac{(x_{i,j} - x_{i,k})^2}{n}} \quad (1)$$

where $x_{i,j}$ and $x_{i,k}$ represent the $R_f$ values of the component $i$ in the system $j$ and $k$ (Table 2) and $n$ is the number of analyzed compounds ($n = 5$).

The smallest $d_{j,k}$ value is selected and the solvent system $j$ and $k$ are the most similar solvent systems and they are considered to

Table 1
The composition of solvent systems and the values of amount of information, $I$ and of objective function $F_{obj}$

| Number | Solvent system composition (v/v/v) | $I$ | $F_{obj}$ |
|---|---|---|---|
| 1 | Chloroform–acetone–petroleum ether, 5:4:1 | 2.322 | 13.35 |
| 2 | Petroleum ether–ethyl ether–dichloromethane, 5:9:6 | 1.922 | 40.46 |
| 3 | Cyclohexane–ethyl acetate–ethanol, 24:16:1 | 1.922 | 18.54 |
| 4 | Petroleum ether–dichlorometane–acetonitrile, 8:2:1 | 1.522 | 68.31 |
| 5 | Chloroform–ethyl acetate–benzene, 8:1:1 | 2.322 | 13.66 |
| 6 | Toluene–acetone–chloroform, 8:2:5 | 2.322 | 13.78 |
| 7 | Dichlorometane–ethyl acetate–methanol, 14:4:1 | 2.322 | 19.94 |

Table 2
The $R_f$ values of components obtained by elution with the tested solvent systems

| Compounds | $R_{f1}$ | $R_{f2}$ | $R_{f3}$ | $R_{f4}$ | $R_{f5}$ | $R_{f6}$ | $R_{f7}$ |
|---|---|---|---|---|---|---|---|
| 1 | 0.62 | 0.60 | 0.72 | 0.14 | 0.41 | 0.49 | 0.76 |
| 2 | 0.77 | 0.74 | 0.79 | 0.20 | 0.59 | 0.61 | 0.86 |
| 3 | 0.72 | 0.73 | 0.78 | 0.18 | 0.53 | 0.56 | 0.84 |
| 4 | 0.14 | 0.18 | 0.32 | 0.02 | 0.06 | 0.15 | 0.45 |
| 5 | 0.27 | 0.28 | 0.44 | 0.03 | 0.17 | 0.28 | 0.56 |

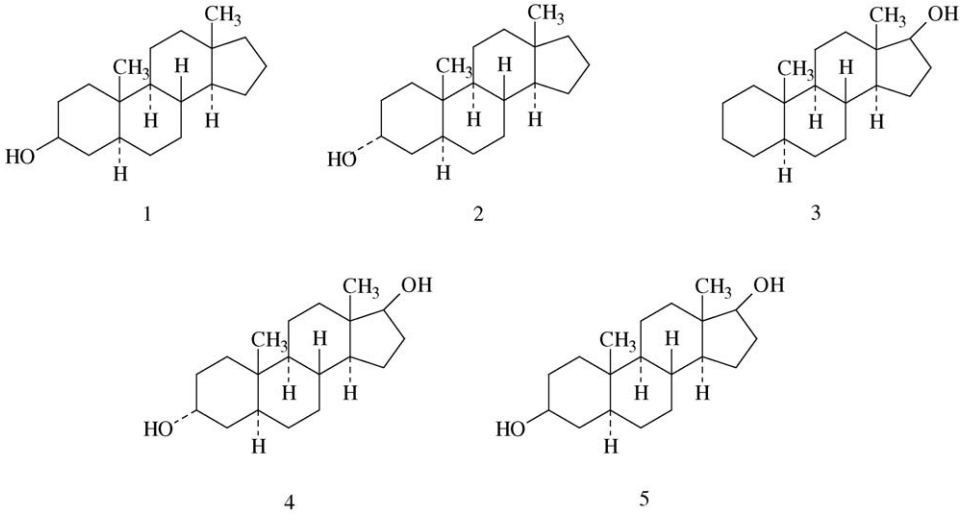


Fig. 1. (1) 5α-androstan-3β-ol; (2) 5α-androstan-3α-ol; (3) 5α-androstan-17β-ol; (4) 5β-androstan-3α,17β-diol; (5) 5β-androstan-3β,17β-diol.

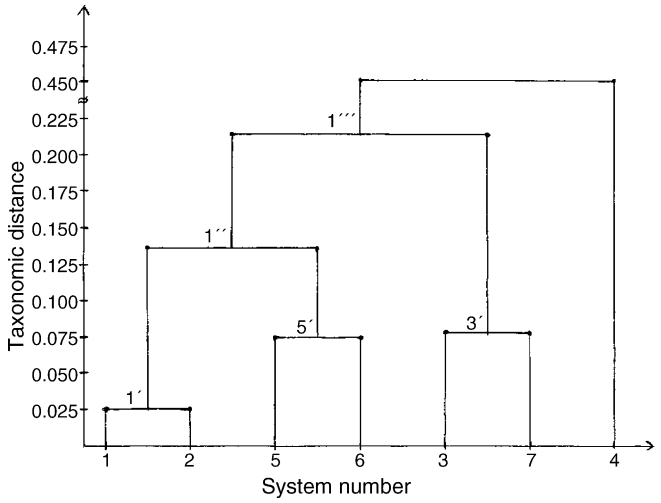


Fig. 2. Dendrogram for the tested solvent system.

form a new group $p'$. The resemblance matrix is thereby reduced by one. The distance between the new group $p'$ and all the other solvent systems, in the reduced resemblance matrix, is calculated as follows:

$$d_{j,p'} = \frac{1}{2}(d_{j,p'} + d_{j,q}) \quad (2)$$

All other $d_{j,k}$ values remain unchanged. This process is repeated until all solvent systems are brought together in one classification system consisting of a hierarchy of non-overlapping groups and subgroups. The sequence of combinations is shown in Fig. 2.

The selection of the most efficient solvent system from each group with similar separation properties was carried out according to the amount of information, $I$ [10] and objective function, $F_{obj}$ [11] (Table 1). The $F_{obj}$ reflect the quality of a chromatogram by a single number and it takes a minimum value in case of optimum.

$$I = -\sum \left(\frac{n_k}{n}\right) \log_2 \left(\frac{n_k}{n}\right) \quad (3)$$

where $n_k$ is the number of separated compounds in a group and $n$ is the number of investigated compounds.

$$F_{obj} = aI_p + \frac{b}{I} + \frac{c}{I\overline{R_s}} + \frac{d}{RRP} \quad (4)$$

where $a$, $b$, $c$, $d$ are the arbitrary weighting coefficients ($a = 1$, $b = 10$; $c = 20$ and $d = 20$)

$I_p$ is the performance index [12],

$$I_p = \sqrt{\sum (\Delta h R_{f,i} - \Delta h R_{f,t})^2 / n(n+1)}, \quad (5)$$

$\overline{R_s}$ is the medium resolution; RRP represents the relative resolution product [13].

$$RRP = \prod R_{s,i}/\overline{R_s} \quad (6)$$

It can be concluded from Fig. 2 and from the values of $I$ and of $F_{obj}$ that the proper mobile phase for the separation of androstane isomers is the solvent system 1 (chloroform–acetone–petroleum ether).

Table 3
Successive mobile phase compositions, values of $F_{obj}$ and the generated simplex

| Number | Chloroform–acetone–petroleum ether (v/v/v) | $F_{obj}$ | Simplex |
|---|---|---|---|
| 1 | 20:2:78 | 254.1 | 1–2–3 |
| 2 | 20:8:72 | 21.47 | |
| 3 | 46:5:49 | 16.38 | |
| 4 | 46:11:43 | 14.72 | 2–3–4 |
| 5 | 72:8:20 | 23.80 | 3–4–5 |
| 6 | 33:8:59 | 16.92 | 3–4–6 |
| 7 | 59:8:33 | 14.43 | 3–4–7 |
| 8 | 59:14:27 | 23.96 | 4–7–8 |
| 9 | 49.5:7.2:43.3 | 13.50 | 4–7–9 |
| 10 | 62.5:4.3:33.2 | 17.05 | 7–9–10 |
| 11 | 50.5:9.3:40.2 | 14.38 | 7–9–11 |
| 12 | 40.5:8.6:50.9 | 15.52 | 9–11–12 |
| 13 | 54.5:8.15:37.35 | 12.67 | 9–11–13 |

In the second step the composition of chosen mobile phase was optimized first by "simplex" method and then by "prisma" method.

The "simplex" method consists into a geometric figure in the variable space of criteria function, figure whose number of vertices is bigger by one than the number of variable. The objective function, $F_{obj}$ (Eq. (4)) is evaluated in each vertex of the figure, the most unfavorable vertex corresponding to the worst response is rejected and then a new favorable vertex is established by searching the direction that is experienced by this unfavorable vertex and the centroid of the other vertices [14]. The new simplex is thus determined and the algorithm is repeated until the optimum response is obtained.

Successive mobile phase compositions were used for the optimization of androstane isomers separation (Table 3). The

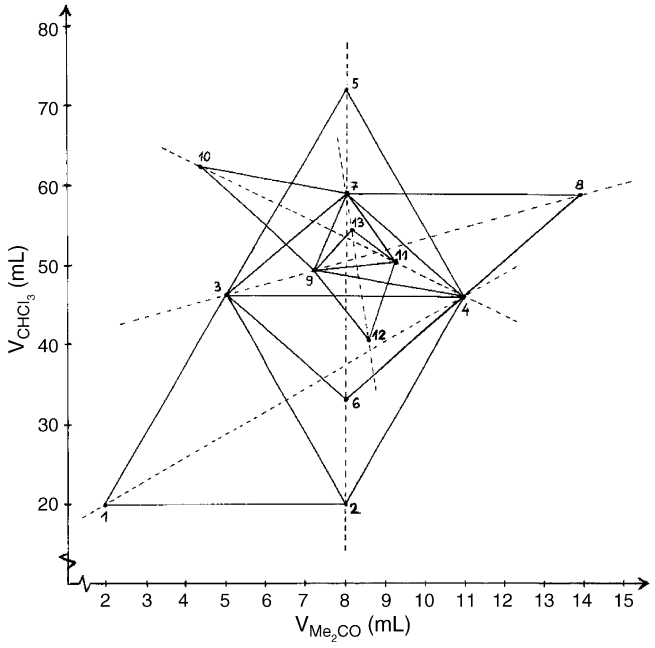


Fig. 3. The generation of simplex.

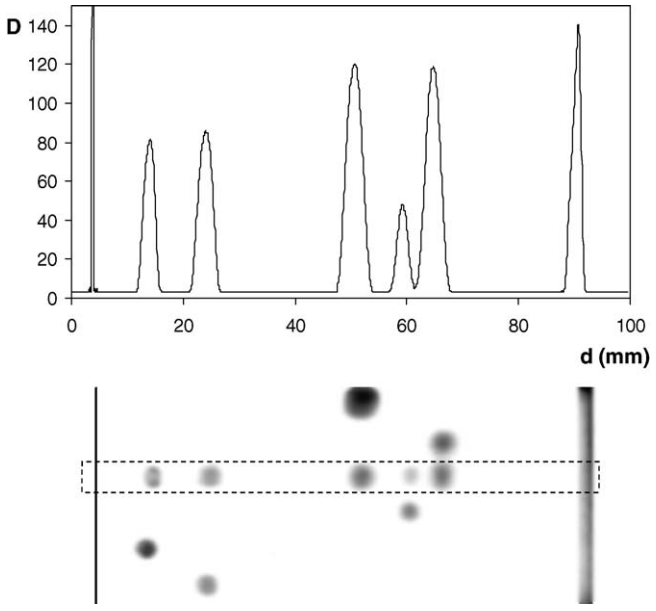


Fig. 4. The chromatographic separation obtained by elution with optimum mobile phase determined by "simplex" method.

optimum composition of mobile phase was obtained graphically from a bidimensional diagram (Fig. 3) after evaluation of chromatographic separations by means of $F_{obj}$ (Table 3).

The optimum separation of androstane derivatives was obtained with composition 13 of mobile phase as it can be seen from Fig. 4.

When "prisma" method was applied the optimization commenced with the compositions of mobile phase (chloroform–acetone–petroleum ether) corresponding to the center of the triangle top face of the regular part of prism, $P_s = 333$ and three other selectivity points close to the apexes of the triangle, $P_s = 811$, 118 and 181 (Fig. 5). Because the obtained separation was insufficient other selectivity points were tested around the solvent combination that gave the best separation (chloroform–acetone–petroleum ether, 80:10:10 v/v/v) and this process was repeated until the best solvent composition was obtained.

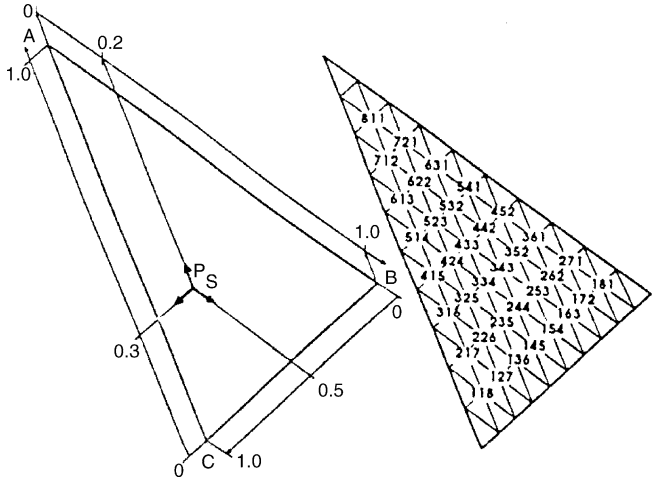


Fig. 5. The selectivity points in the "prisma" model.

Table 4
The mobile phase compositions used in "prisma" method and the values of $F_{obj}$

| Chloroform–acetone–petroleum ether (v/v/v) | $F_{obj}$ |
|---|---|
| 33:33:33 | 35.35 |
| 10:10:80 | 81.71 |
| 80:10:10 | 13.72 |
| 10:80:10 | 397.2 |
| 70:20:10 | 38.79 |
| 70:10:20 | 13.51 |
| 60:10:30 | 12.71 |
| 60:20:20 | 18.01 |
| 50:10:40 | 13.35 |
| 50:20:30 | 21.22 |
| 55:10:35 | 12.64 |

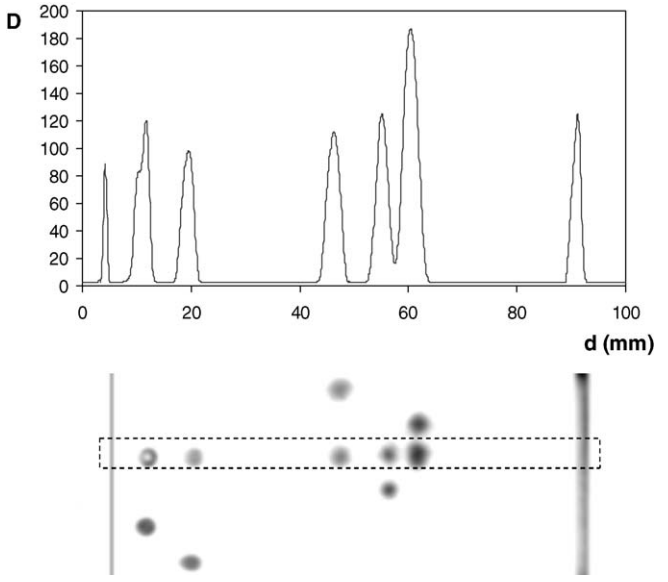


Fig. 6. The chromatographic separation obtained by elution with optimum mobile phase determined by "prisma" method.

The mobile phase compositions tested and the values of $F_{obj}$ are presented in Table 4. It can be seen from Table 4 that the optimum composition of mobile phase is the last, which give the separation of all compounds (Fig. 6).

It can be seen, from Figs. 4 and 6, that the optimum separation obtained with both optimization methods were almost identical.

## 4. Conclusions

Numerical taxonomy allows a rational and logical choice of optimum solvent system using the information quantity and objective function as selection criterion on the basis of formal classification of solvent systems.

The "simplex" and "prisma" methods for optimization are simple and rapid and many mobile phase compositions can be evaluated simultaneously. The advantage of these optimization methods is that the optimum composition of mobile phase can be easily obtained. Using the optimum mobile phases all androstane isomers can be separated from mixtures even they have similar structures.

The optimum mobile phase compositions determined by these two methods were very similar. This fact is due to the moderately polar character of the separated compounds. So, it can be concluded that in this case the modification of mobile phase compositions by small increments do not lead to major modification of components retention.

The optimum composition of mobile phase can be applied for the separation of androstane isomers from real samples such as drug formulation, biological and natural sources.

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