SEPARATION AND LIPOPHILICITY OF SOME NEW STEROID DERIVATIVES IN NORMAL- AND REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY*

The separation ability and retention of normal and reversed phase HPLC with, respectively, three non-polar and two polar mobile phases, have been studied by measuring the retention constants of a series of newly synthesized estrone derivatives. The separation ability and retention are discussed in terms of the nature of the solute, eluent and stationary phase. Good correlation was found between the retention constants $\log k_0$ of newly synthesized estrone derivatives obtained on C-18 column and $\log P$ calculated by different methods.

Keywords: column liquid chromatography; steroids; retention behavior; correlation analysis.

The primary intention in chromatography is to achieve satisfactory resolution of the sample components. The retention, and consequently resolution, in liquid chromatography (LC) is determined by the interactions of a solute with both mobile and stationary phase of the system. Thus, the molecular structure of a solute, the polar and non-polar function of the stationary phase and the mobile phase composition influence retention in LC [1]. It is well known that the same position of different substituents in a molecule can have significant pronounced effects on retention in chromatography.

The development of steroidal compounds is often followed by research in structure-activity relationship. For purpose of initial chemical screening of the activity of newly synthesized compounds, it is recommended to determine their lipophilicity first. Lipophilicity is quantitatively characterized as $\log P$, the logarithm of the ratio of the concentrations of any analyte in a saturated 1-octanol-water system [1-4]. Many methods for estimating $\log P$, experimental as well as computational, are described in the literature [5-7]. The traditional experimental method for the determination of $P_{ow}$ is the shake flask method. Nowadays, liquid chromatography has a tendency to replace the tedious and poor interlaboratory reproducible shake flask method for measuring partition coefficients. Among liquid chromatography methods, reversed-phase liquid chromatography (RPLC) is an alternative technique that can correlate the hydrophobicity of compounds with the retention parameters [8,9].

Due to our interest in the biological activity of functionalized newly synthesized 16,17-secoestra-1,3,5(10)-triene-16-nitrile and their future derivatives, this research had two objectives:
- to investigate the separation of normal (silica gel column) and reversed phase (C-18 column) HPLC of newly synthesized steroid compounds, and
- to correlate chromatographically obtained constants in RPHPLC with $\log P$ values calculated by different methods [10].

The structures of the investigated compounds are presented in Table 1.

EXPERIMENTAL

HPLC separations were performed on an Agilent 1100 series HPLC (USA) including a degasser G1379...
A, binary G1312 pump, ALS G1313A, COLCOM G1316A and DAD G1315B. The columns used were commercially available particle size 5 μm: Spherisorb SI 250 mm×4 mm i.d. (E. Merck, Darmstadt, Germany) and Spherisorb ODS-2.5 μm, 124 mm×4 mm (Hewlett Packard, USA).

Steroid derivatives (Table 1), synthesized with original reactions or with the application of literature methods [11,12], were dissolved (0.05 mg mL⁻¹) in methanol and solutions filtered through a 0.2 μm Chromafil filter (Macherey-Nagel, Duren, Germany).

Three binary solvent systems were used as the mobile phase on the silica gel column:

(A) Benzene - ethyl acetate (0.05-0.25, increment 5%);
(B) Benzene - tetrahydrofuran (0.05-0.25, increment 5%);
(C) Benzene - acetonitrile (0.05-0.25, increment 5%).

Two binary solvent systems were used as the mobile phase on the octadecyl silica gel column:

(D) Methanol - water (0.70-0.95, increment 5%);

(E) Acetonitrile - water (0.60-0.90, increment 5%).

The eluents used to prepare mobile phases were of an analytical grade. The flow rate was 1 mL min⁻¹ at the room temperature.

The retention factor, k, was calculated from:

\[ k = \frac{t_r - t_0}{t_0} \]

where \( t_r \) is the retention time of the solute and \( t_0 \) is the column void time of methanol. Each \( t_r \) value was measured three times and then the average value was calculated.

RESULTS AND DISCUSSION

Two types of compounds were studied: steroid derivatives hydroxylated at 3-position (odd numbered compounds) and their 3-benzyloxy counterparts (even numbered compounds), Table 1. All derivatives were examined by the normal- and reversed-phase HPLC on silica gel and C-18 bonded silica gel columns.
Normal-phase chromatography

The retention sequence of investigated compounds in chromatography in normal-phases is basically the consequence of the polarity of the compounds. The odd numbered compounds were generally more retained compared to their 3-benzyloxy counterparts, because the hydroxy group is much more polar in comparison to the benzyloxy group (Table 1). The change of retention of the steroid compounds with the change of the volume rate of the more polar component in the mobile phase (Figure 1) is in accordance with the well known equation Eq. (1):

\[ \log k = \log k_0 - n \log \phi \]  

(1)

The coefficients \( \log k_0, n, \hat{r} \pm SD \) as well as the degree of freedom are presented in Table 2.

The sequence of separation on the silica gel column with eluents A-C of all compounds is as follows: \( 3 > 4 \geq 5 = 7 > 1 > 6 = 8 > 2 \).

Figure 1. Correlation lines of Eq. (1) for eluents A-C. Designation of solutes is presented in Table 1.
The most retained compound was compound 3. The least retained compound was compound 2. Compounds 5 and 7 as well as 6 and 8 were not resolved because the number of non-polar methylene groups in alkyl chain in position 17 did not affect the retention. In this research, as in the previous one [13], we also showed that silica gel did not always indicate real polarity of the compound, however, it can be stated that the retention behavior of new steroid derivatives on silica gel was in accordance with general principles in the normal-phase liquid chromatography.

**Reversed-phase chromatography**

The change in compounds retention of steroid derivatives with an increasing volume fraction of the modifier in aqueous mobile phases was in accordance with the well known equation, generally accepted in the partition chromatography, Eq. (2):

\[
\log k = \log k_0 - s \phi
\]

(2)

where \( \phi \) is the volume fraction of the organic component of the binary aqueous mobile phase, \( \log k_0 \) is the value of \( \log k \) extrapolated to \( \phi = 0 \), and \( s \) is constant. The relationship between the retention factor, \( \log k \), of the examined compounds and volume fraction, \( \phi \), of the modifier in the aqueous mixture was linear (Figure 2).

The compounds were more mobile in eluent with acetonitrile as the eluent modifier than with methanol due to the lower polarity of acetonitrile. It is empirically known that a change from methanol to acetonitrile generally decreases selectivity.

Numerical values of the absolute value constants \( s \), \( \log k_0 \), \( \pm SD \) as well as the degree of freedom for each examined compound and mobile phases containing water and methanol or acetonitrile as a modifier are presented in Table 3. Retention data obtained on C-18 bonded silica gel column are generally typical of the reversed phase chromatographic behaviour: less polar solutes are more strongly retained. The retention order of the compounds with both aqueous mobile phases was very similar and increases in the following order: \( 8 > 6 > 2 > 4 > 7 > 5 > 1 > 3 \).

Compounds 5 and 7 as well as 6 and 8 which were not resolved on silica gel column were clearly resolved on C-18 bonded silica gel (Figure 2), because retention of compounds was determined with hydrophobicity of compounds. Compounds 2 and 4 were poorly resolved because in reversed-phase the retention was determined by the benzyloxy function only, *i.e.*, the polar keto- and hydroxy-groups at position 17 did not affect the retention [14].

It is apparent from the data presented in Table 3 that on C-18 bonded silica gel column with both mobile phases the constant \( \log k_0 \) and absolute value of the constant \( s \) increase with the increase in the compound retention. There are, therefore, linear relationships between these two constants, with high correlation coefficients, Figure 3 (\( N = 8, p < 0.0001 \)).

This linear dependence with no physical meaning is just a consequence of the coupled parameters of the fit. The same has been observed earlier with tosylated xylitol derivatives [15], 1,2,4-triazole derivatives [16], benzimidazole derivatives [17] and estradiol and estrone derivatives [9,18].

**Correlation between retention constant \( \log k_0 \) of steroid derivatives obtained on C-18 column and ACDlog \( P \)**

For purpose of avoiding practical difficulties that often arise in the direct determination of the partition coefficient, extrapolated retention parameters of both eluents were used for measuring hydrophobicity. The intercept \( \log k_0 \) corresponds to the retention in water.
as the mobile phase, and represents the commonly employed chromatographic hydrophobicity parameter 
[1,6,7].

Several different log $P$ values were calculated for investigated steroid derivatives, Table 4 [10]. Statistical analysis is constantly evolving [19]. The dendrogram of Pearson’s $r$ correlation [20] of nine different methods for the calculation of log $P$ values (correlation coefficient is on the Y-axis) show that the similarity in different methods (listed in Table 4) exists (Figure 4).

Table 3. Constants $s$, log $k_0$, $r^2$±SD and the degree of freedom of Eq. (2) for the linear relationship between retention and eluents D and E composition. Designation of solutes is presented in Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Eluents</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D</td>
</tr>
<tr>
<td></td>
<td>log $k_0$</td>
</tr>
<tr>
<td>1</td>
<td>1.9446</td>
</tr>
<tr>
<td>2</td>
<td>5.1350</td>
</tr>
<tr>
<td>3</td>
<td>1.5892</td>
</tr>
<tr>
<td>4</td>
<td>5.0585</td>
</tr>
<tr>
<td>5</td>
<td>2.2953</td>
</tr>
<tr>
<td>6</td>
<td>5.7175</td>
</tr>
<tr>
<td>7</td>
<td>2.6428</td>
</tr>
<tr>
<td>8</td>
<td>6.1510</td>
</tr>
</tbody>
</table>
Correlations between the retention constant $\log k_0$ of the 16,17-secoestrone derivatives with average $\log P$ (last column of Table 4) are very high for both eluents (Figure 5).

**CONCLUSION**

Newly synthesized estrone derivatives have been investigated by normal- and reversed-phase HPLC. Compounds which were not resolved in normal phase were resolved in reversed phases and vice versa, because the spatial orientation of newly synthesized
estrone derivatives between the mobile and stationary phases determined retention. The correlation between the retention constants log k0 determined by RP-HPLC on C-18 column and log P was found.

Acknowledgements

The authors gratefully acknowledge the financial support from the Ministry of Education and Science of the Republic of Serbia (Project III 46005).

REFERENCES

MARIJANA M. AČANSKI1
ĐURA N. VUJIĆ1
SUZANA JOVANOVIĆ-ŠANTA2

1Katedra za primenjene i inženjerske hemije, Tehnološki fakultet, Univerzitet u Novom Sadu, Novi Sad, Srbija
2Prirodno-matematički fakultet, Departman za hemiju, biohemiju i zaštitu životne sredine, Univerzitet u Novom Sadu, Novi Sad, Srbija

NAUČNI RAD

RAZDVAJANJE I LIPOFILNOST NEKIH NOVIH DERIVATA STERIODA U NORMALNO- I REVERSNO-FAZNOJ TEČNOJ HROMATOGRAFIJI POD VISOKIM PRITISKOM

U radu je ispitano razdvajanje i retencija novosintetizovanih derivata steroida u normalno- i reversno-faznoj tečnoj hromatografiji pod visokim pritiskom, primenom tri nepolarne i dve polarne pokretne faze. Razdvajanje i retencija ispitivanih jedinjenja je diskutovana sa aspekta prirode rastvorka, pokretne i nepokretne faze. Ustanovljena je dobra korelacija između retencionih konstanti log k i lipofilnosti log P različitih derivata steroida dobijenih primenom C-18 kolone i vrednosti log P računatih na različite načine.

Ključne reči: kolonska tečna hromatografija; steroidi; retencija; korelaciona analiza.