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CHROMATOGRAPHIC BEHAVIOUR OF A SERIES OF NITRO AND AZA STEROIDS

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SUMMARY

The chromatographic properties and mass spectra of several synthetic nitro and aza steroids have been examined. Reversed-phase chromatography was performed with methanol, acetonitrile, or mixtures of these with aqueous phosphate buffer as mobile phases. The relationship between calculated lipophilicity and chromatographic capacity factors of the solutes was evaluated. Mass spectra were studied using soft ionization techniques. Calculated lipophilicity was a linear function of retention when methanol and methanol–phosphate buffer were used as mobile phases but not when acetonitrile and acetonitrile–phosphate buffer were used. It did not seem possible to detect intramolecular hydrogen-bond formation by use of chromatographic retention factors. Intramolecular hydrogen-bond formation affected the mass spectra, suggesting it affected the stability of the molecular ions.

INTRODUCTION

The properties of chemical compounds depend on their structure. Observed physicochemical and/or biological properties are, in fact, net effects of intermolecular interactions between the molecules of an individual chemical entity and the molecules forming its environment [1]. The same fundamental intermolecular interactions determine the behaviour of chemical compounds in both biological and chemical environments [2]. Lipophilicity, assumed to be one of the most important physicochemical properties, is regarded as a “driving force” for liquid–liquid partitioning, passive transport through biological membranes, and drug–receptor binding [3]. Thus pharmacokinetic processes of drug absorption into the systemic

circulation, distribution among different body compartments, and excretion, all of which involving penetration of lipid membranes and aqueous extracellular and intracellular fluids, must be affected by drug lipophilicity.

It is well known that steroids are involved in crucial biochemical pathways in mammals; compounds such as sexual hormones, corticosteroids, and bile acids are members of this group. Aza steroids may also be biologically important, because finasteride (**1**; Fig. 1), a 5α-reductase inhibitor used clinically for treatment of benign prostatic hyperplasia [4], is a member of this group of compounds. Structure–retention relationships have

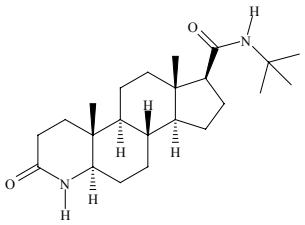


Fig. 1

The structure of finasteride

already been discussed for some groups of steroids. Georgakopoulos and Kiburis used theoretically derived structural data for correlation of gas chromatographic relative retention times of anabolic steroids [5]. Salo et al. calculated the topological indices of a series of steroid hormones and found they could be used to predict the retention and/or migration of the hormones in reversed-phase liquid chromatography or electrokinetic capillary chromatography when the solutes form a congeneric series and when stereochemical properties do not affect the separation process [6]. This was in agreement with the general observation that valid predictions of even the most simple properties, for example boiling point temperature, chromatographic retention, or anaesthetic potency, are only possible within series of homologues or otherwise closely congeneric compounds [1].

In the work discussed in this paper we examined the chromatographic properties and mass spectra of several synthetic nitro and aza steroids. The compounds were obtained by use of reported procedures (compounds **2**–**9**, Ref. [7]; compound **10**, Ref. [8]; compound **11**, Ref. [9]; compound **12**, Ref. [10]). Reversed-phase chromatography was performed with methanol–water and acetonitrile–water mobile phases. The relationship between calculated lipophilicity of the solutes and chromatographic capacity

factors was evaluated. Mass spectra were studied using soft ionization techniques, because of recently stressed advantages of electrospray ionization spectra in steroid analysis [11,12].

EXPERIMENTAL

Chemicals and Reagents

Methanol and acetonitrile of HPLC grade were purchased from Merck. Double-distilled water was used. Other reagents were p.a. grade and were purchased from Witko, Łódź, Poland.

HPLC

Chromatography was performed with a LabAlliance liquid chromatograph equipped with two Series III solvent pumps, a 525 dual-wavelength detector, a Rheodyne 7725i manual injection valve with 20-μL loop, and a Data Ally system controller. Compounds were separated on a 250 mm × 4.6 mm i.d., 5-μm particle, RP-18 Luna column (Phenomenex). The mobile phases used were methanol, methanol–phosphate buffer, pH 7.38, 85:15 (*v*/*v*), acetonitrile, and acetonitrile–phosphate buffer, pH 7.38, 9:1 (*v*/*v*). The mobile phase flow rate was 1 mL min^{-1} and detection was performed at 220 nm. Dead volumes were determined by injection of 10^{-3} M aqueous KNO_3.

Mass Spectrometry

Methanolic solutions of the steroids were studied by mass spectrometry with soft ionization techniques, which are suitable when compounds are introduced into the mass spectrometer in the HPLC mobile phase. EI mass spectra of some nitro and aza steroids have been published elsewhere [7]. Analysis was performed with a Finnigan MAT SSQ 7000 single-stage quadrupole mass spectrometer (ThermoFinnigan, USA) equipped with electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) interfaces. APCI source settings were corona current 5 μA and capillary temperature 150°C. ESI source settings were electrospray needle potential 4.5 kV and capillary temperature 200°C. For both techniques the sheath gas was nitrogen at ca. 45 psi, the tube lens offset potential was ±100 V, the octapole potential was ±3.1 V (± depending on polarity), and the accelerating potential 5 kV. Samples were delivered into the mass spectrometer source by means of a Rheodyne (USA) injector with 5-μL

sample loop, into a stream of mobile phase introduced at a flow-rate of 10–20 μL min^{-1}, by means of a syringe infusion pump (Harvard Apparatus; Model 22). Full-scan positive and negative-ion mass spectra were acquired (2 s each).

Theoretical Calculations

Theoretical calculations were performed with the aid of Hyperchem 6.0.

RESULTS AND DISCUSSION

The compounds examined, **2**–**12** in Table I, seemed quite hydrophobic; their calculated lipophilicity was in the range 3.04–7.12, preventing direct measurement by the shake-flask method [13]. Retention values from reversed phase chromatography can be related to classical shake-flask log *P* values by eq. (1) [14]

$$\log P = a\log k + b \quad (1)$$

where *k* is the chromatographic capacity factor and *a* and *b* are constants, although often the correlation is moderate only.

Chromatographic retention factors, *k* (as log *k*) or values extrapolated to 100% water, k_w (as log k_w) are often used as measures of lipophilicity. For the very hydrophobic compounds **2**–**12** calculation of k_w would be impossible and log *k* values (Table I) were used directly as indicators of lipophilicity. It was found that calculated lipophilicity was a linear function of retention factors obtained with methanol (eq. 2) or methanol–phosphate buffer pH 7.38, 85:15 (*v*/*v*) (eq. 3). Compounds **4**, **9**, and **12** were excluded from the latter correlation when methanol–phosphate buffer mobile phases were used, because their retention times were too long to be determined, but not when acetonitrile or acetonitrile–phosphate buffer were used. This result was in agreement with the observation of Bączek et al., who found that dependence of retention on analyte hydrophobicity (log *P*) was greater for methanolic mobile phases than for acetonitrile-modified mobile phases [15].

$$\log P = 3.49 + 3.59 \times \log k \quad (2)$$

$$r = 0.9312,\ s = 0.5016,\ P < 0.001,\ n = 11$$

$$\log P = 2.78 + 1.43 \times \log k \quad (3)$$

$$r = 0.9632,\ s = 0.2564,\ P < 0.001,\ n = 8$$

Table I

Retention factors and calculated lipophilicity for compounds **2**–**8**

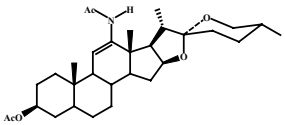
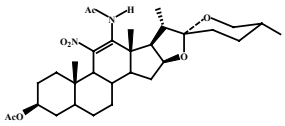
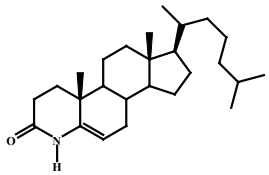
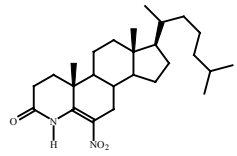
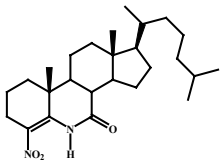
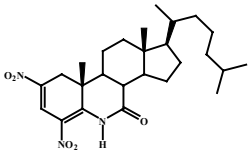
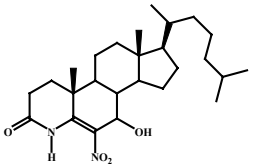
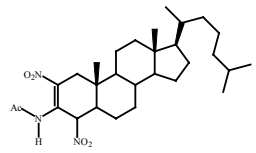
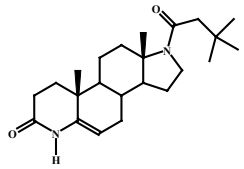
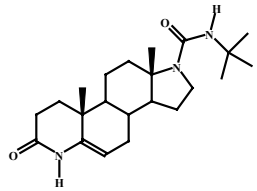
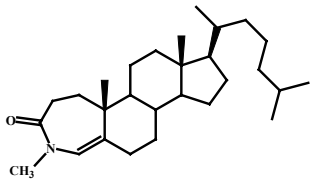
Compound	log *k*				log *P*
	MeOH	MeOH–H_2O, 85:15	CH_3CN	CH_3CN–H_2O, 90:10	
 **2**	0.19	0.97	0.30	0.62	4.21
 **3**	0.03	0.71	−0.05	0.33	3.32
 **4**	0.37	–	0.45	1.15	6.10
 **5**	0.55	1.57	0.67	1.32	5.14
 **6**	0.58	1.66	0.69	1.39	5.37
 **7**	0.37	1.12	0.35	1.08	4.30
 **8**	0.27	1.24	0.40	0.92	4.44

Table I (continued)

Retention factors and calculated lipophilicity for compounds **9**–**12**

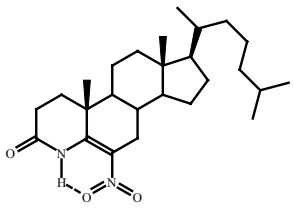
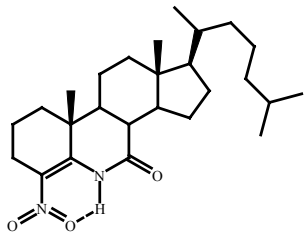
Compound	log *k*				log *P*
	MeOH	MeOH–H_2O, 85:15	CH_3CN	CH_3CN–H_2O, 90:10	
 **9**	0.55	–	0.72	1.47	5.75
 **10**	−0.08	0.16	−1.02	0.09	3.04
 **11**	−0.10	0.06	−1.22	−0.01	3.16
 **12**	1.05	–	1.31	1.88	7.12

Interestingly, by use of this method it was possible to detect the presence of an intramolecular hydrogen bond. For compounds **4** and **5** (**5** was a nitro derivative of **4**) calculated lipophilicity was 6.10 and 5.14, respectively, and introduction of the nitro group reduced lipophilicity. The retention factors obtained were, however, higher for compound **5** than for compound **4**, suggesting an increase in lipophilicity. The experimental lipophilicity of compound **5** was similar to that of **6**, a structural analogue of **5** (Table I). For other compounds, for example **2** and **3** or **6** and **7**, introduction of a nitro group resulted in a decrease of theoretical and experimental lipophilicity. This result could be explained by assuming hydrogen-bond formation between the N–H and nitro groups in compounds

5 and **6** [2] because of the coplanar conformation of the nitro group and the adjacent carbon–carbon double bond (Table II). Calculated C(5)–C(6)–N–O and H(N_4)–N(4)–C(5)–C(6) dihedral angles in AM1 optimised compound **5** were found to be −14.31° and 10.18°, respectively, thus placing both hydrogen and oxygen atoms almost in the plane of the molecule (with β-face preference) with the H(N4)–O(N) distance being 2.05 Å. In compound **3** the coplanar conformation for hydrogen bonding is not possible for steric reasons. Thus theoretical and experimental lipophilicity changes for compounds **2** and **3** were consistent.

Table II

Calculated (log *P*) and experimental (log *k*) lipophilicity of compounds **5** and **6**

Compound	MeOH	85% MeOH	MeCN	90% MeCN	log *P*
 **5**	0.55	1.57	0.67	1.32	5.14
 **6**	0.58	1.66	0.69	1.39	5.37

Mass Spectrometry

In ESI MS in positive-ion mode the nitro and aza steroids readily formed protonated $[M + H]^+$ and sodiated $[M + Na]^+$ molecular ions and bimolecular/dimeric ions $[2M + Na]^+$. In the APCI source protonated molecular ions and some fragments ions were recorded in positive-ion mode. Negative ions for this class of compound were not observed for either ionization technique.

Below we discus the most characteristic mass spectral features of the compounds. Compounds **5** and **6** in the ESI source formed mainly pseu-

domolecular ions at *m/z* 453 $[M + Na]^+$ and *m/z* 431 $[M + H]^+$, dimeric ions $[2M + Na]^+$ at *m/z* 883, and did not tend to form fragment ions, giving only ions at *m/z* [M − 17] corresponding to elimination of water from the protonated molecules, after collision-induced dissociation. The same results were obtained by using positive ionization in the APCI source, which gave ions at *m/z* 431 corresponding to the protonated molecular ion $[M + H]^+$ and *m/z* 413 $[M + H - H_2O]^+$ for both compounds, which are structural analogues. Typical fragmentation of nitro compounds, e.g. [M − 16], [M − 30], or [M − 46], corresponding to elimination of oxygen, NO, and NO_2, respectively, was not observed (Fig. 2).

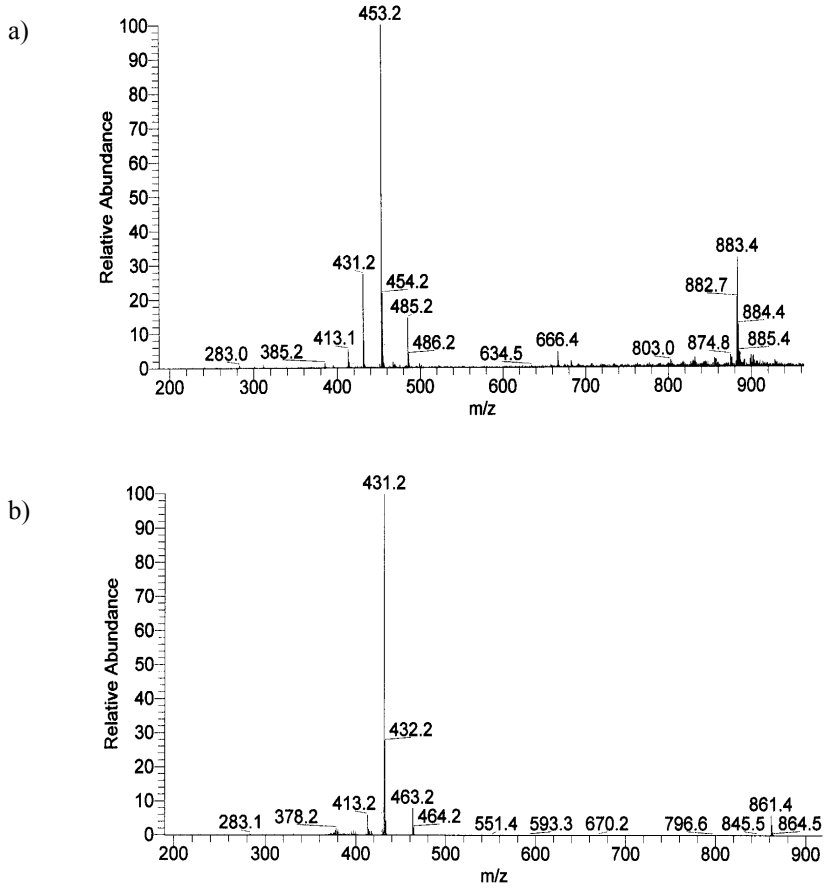


Fig. 2

ESI (a) and APCI (b) mass spectra of compound **5**

Fragmentation of steroid **8** ($M = 446$), a hydroxy derivative of **5**, occurs with elimination of molecules of water. The sodiated pseudomolecular ion at *m*/*z* 469 is observed in ESI; no protonated molecular ion is observed in APCI, but only fragment ions originating from elimination of a molecule of water to give *m*/*z* 429 then loss of either a nitro group to give *m*/*z* 383 or loss of another molecule of water to give *m*/*z* 411 (Fig. 3).

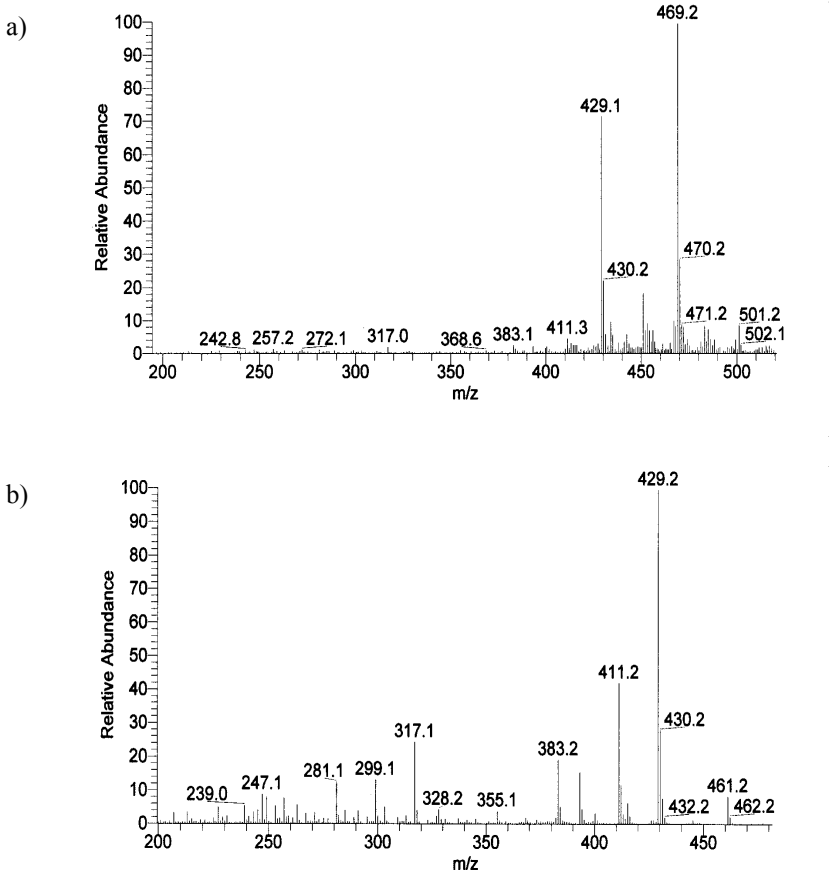


Fig. 3

ESI (a) and APCI (b) mass spectra of compound **8**

In contrast, the APCI positive-ion mass spectrum of dinitrosteroid **7** ($M = 473$) contained evidence of many fragment ions originating from the protonated molecular ion *m*/*z* 474 $[M + H]^+$. Loss of a hydroxyl group

gave an ion at *m*/*z* 457 $[M + H - OH]^+$ and this was followed by loss of a nitro group to give *m*/*z* 411 $[M + H - OH - NO_2]^+$ (Fig. 4).

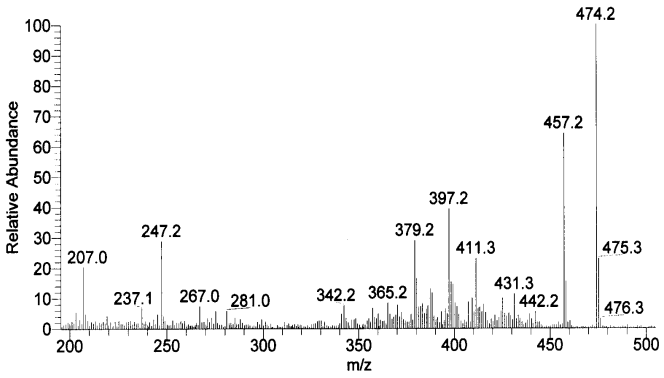


Fig. 4

APCI mass spectrum of compound **7**

For the acetyl, dinitro-substituted derivative **9** (*M* = 517) in the ESI source, loss of a nitro group from sodiated molecular ion (*m*/*z* 540) to give *m*/*z* 494 and fragmentation of an acetyl group were observed; in the APCI source loss of acetyl and nitro groups from the weak protonated molecular ion $[M + H]^+$ at *m*/*z* 518, gave ions with high abundance at *m*/*z* 476 $[MH - CH_2CO]^+$ and *m*/*z* 471 $[MH - HNO_2]^+$ (Fig. 5).

We observed that for compounds with nitro groups involved in hydrogen-bond formation (either as nitro or as aci tautomers – Fig. 6, compounds **5**, **6**, and **8**) no fragmentation of the nitro group was observed. In contrast, such fragmentation of nitro and aza steroids did occur when formation of this hydrogen-bond on one of the two nitro groups was not possible (compounds **7** and **9**). We therefore suggest that intramolecular hydrogen-bond formation affects the stability of molecular ions during ionization in the mass spectrometer source.

CONCLUSIONS

Linear correlations were obtained between calculated lipophilicity and retention factors obtained by use of methanol and methanol–phosphate buffer mobile phases but not for those obtained with acetonitrile and ace-

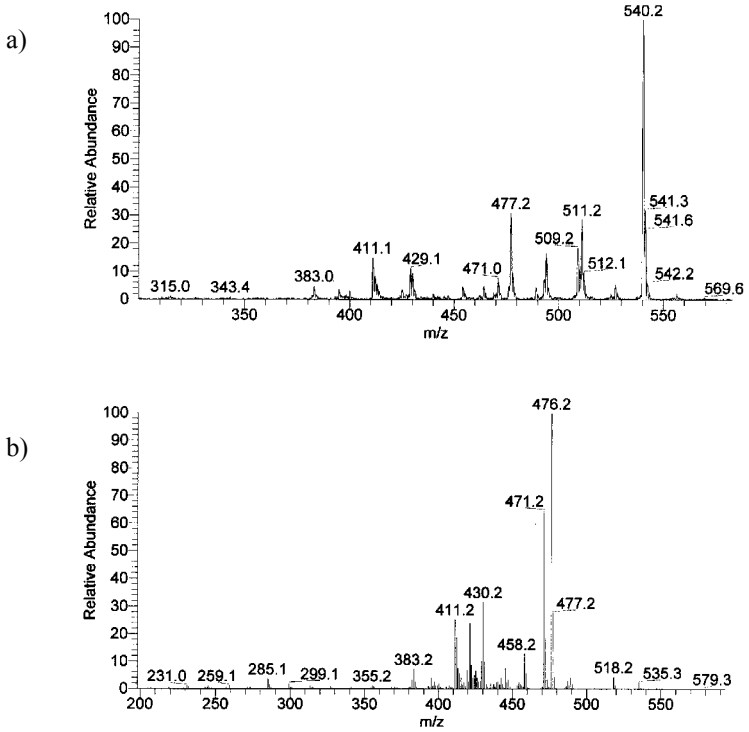


Fig. 5

ESI (a) and APCI (b) mass spectra of compound **9**

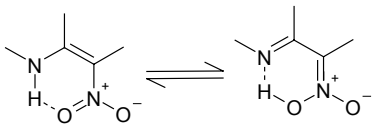


Fig. 6

Hydrogen-bond formation between nitro and aci tautomers

tonitrile–water mobile phases. Introduction of a nitro group to compound **4** led to compound **5** and resulted in an increase in apparent lipophilicity (chromatographic retention) even though, according to theoretical calculations, the lipophilicity should decrease. This could be explained by assuming hydrogen-bond formation between the N–H and nitro groups in compound **5**, because of the coplanar conformation of the nitro group and the adjacent

carbon–carbon double bond. Thus lipophilicity determined chromatographically seemed to be suitable for detection not only of structural differences but also of intramolecular hydrogen-bond formation.

Hydrogen-bond formation also affected mass spectra. We observed that for compounds in which nitro groups were involved in hydrogen-bond formation (compounds **5** and **6**) no fragmentation of the nitro group was observed. Such fragmentation did occur for nitro and aza steroids in which formation of a hydrogen-bond with one of the two nitro groups was not possible, (compounds **7** and **9**). We therefore suggest that the intramolecular hydrogen-bond formation affects the stability of molecular ions during ionization in the mass spectrometer source.

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