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IRREGULAR RETENTION PROPERTIES OF 21-AMINOSTEROID ANTIOXIDANTS IN OCTYLSILANE BONDED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The retention characteristics of two novel 21-aminosteroid antioxidants, 21-[4-(2,6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl]-16α-methylpregna-1,4,9(11)-triene-3,20-dione dimethanesulfonate (I) and 21-[4-(2,5-di-N-diethylamine-2-pyridinyl)-1-piperazinyl]-16α-methylpregna-1,4,9(11)-triene-3,20-dione hydrochloride (II), in octylsilane bonded-phase high-performance liquid chromatography were examined in detail. Both I and II exhibited irregular retention behaviour which could be explained by the dual retention mechanism model proposed by A. Nahum and Cs. Horváth [J. Chromatogr., 203 (1981) 53]. Unless an amine modifier was added to competitively inhibit association with exposed silanol binding sites, the retention of both compounds in a 70% acetonitrile mobile phase was primarily due to silanophilic interactions. Addition of amine modifiers, lowering the pH, and increasing the sodium ion concentration of the mobile phase all decreased retention times, and modifiers capable of hydrogen bonding diminished peak tailing.

INTRODUCTION

A novel class of 21-aminosteroids which inhibits lipid peroxidation [1] was found to be therapeutically active in experimental models of head and spinal cord injury and hemorrhagic shock [2–4]. Most of these compounds are derivatives of 16-methylpregna-1,4,9(11)-triene-3,20-dione with a substituted piperazine ring at the C-21 position. Two representative 21-aminosteroids are compounds I and II (Fig. 1).

Analytical methodology was needed to determine plasma levels of these compounds in experimental animals. Because the 21-aminosteroids are amphiphilic and contain several amine groups, they were expected to have similar high-performance liquid chromatography (HPLC) retention properties on bonded-phase silica columns operated in the reversed-phase mode as the tricyclic antidepres-

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Fig. 1. Chemical structures of 21-[4-(2,6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl]-16α-methylpregna-1,4,9(11)-triene-3,20-dione dimethanesulfonate (I) and 21-[4-(2,5-di-N-diethylamine-2-pyridinyl)-1-piperazinyl]-16α-methylpregna-1,4,9(11)-triene-3,20-dione hydrochloride (II).

sants. The reversed-phase HPLC retention times and peak-tailing of the latter compounds are known to be influenced by mobile phase amine modifiers, pH, and ionic strength [5–7]. The present report describes the reversed-phase HPLC retention characteristics of I and II on octylsilane bonded-phase columns as a first step towards the development of an HPLC plasma assay.

EXPERIMENTAL

Compounds I and II (Fig. 1) were supplied by Dr. John McCall, Pharmaceutical Research and Development Division, The Upjohn Company (Kalamazoo, MI, U.S.A.)*. Trifluoroacetic acid and triethylamine (TEA) were purchased from Aldrich (Milwaukee, WI, U.S.A.). *tert.*-Butylammonium hydroxide (TBAH, 0.4 *M* in water) was obtained from Eastman Kodak (Rochester, NY, U.S.A.). Glacial acetic acid and sodium hydroxide pellets were purchased from Mallinckrodt (Paris, KY, U.S.A.). High-purity acetonitrile and water for HPLC were obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

HPLC was conducted with a Beckman Model 114 M pump (Beckman Instruments, Berkeley, CA, U.S.A.), an Altex 210 manual injector equipped with a 20-μl loop (Beckman), and an LDC UV III detector equipped with an Hg lamp for monitoring absorbance at 254 nm (Laboratory Data Control, Riviera Beach, CA, U.S.A.). The HPLC column was a Supelcosil LC-8, 5 μm particle size, 250 mm × 4.6 mm I.D. (Supelco, Bellefonte, PA, U.S.A.). The mobile phase composition is specified in the figure legends. The pH of the mobile phase was controlled with sodium acetate buffer unless amine modifiers were present; when using amine modifiers, the pH was adjusted to the desired level with acetic acid or trifluoroacetic acid. The column was equilibrated with 90 ml of eluent before making sample injections, which was sufficient to eliminate memory effects even when making large changes in the mobile phase composition. Approximately 200 ng of analyte dissolved in acetonitrile–water (7:3, v/v) was injected on-column. The mobile phase flow-rate was 1 ml/min.

The mobile phase volume of the column (V_m) was determined by injecting

*The Upjohn Company identification number for I is U-74006E and for II is U-74500A.

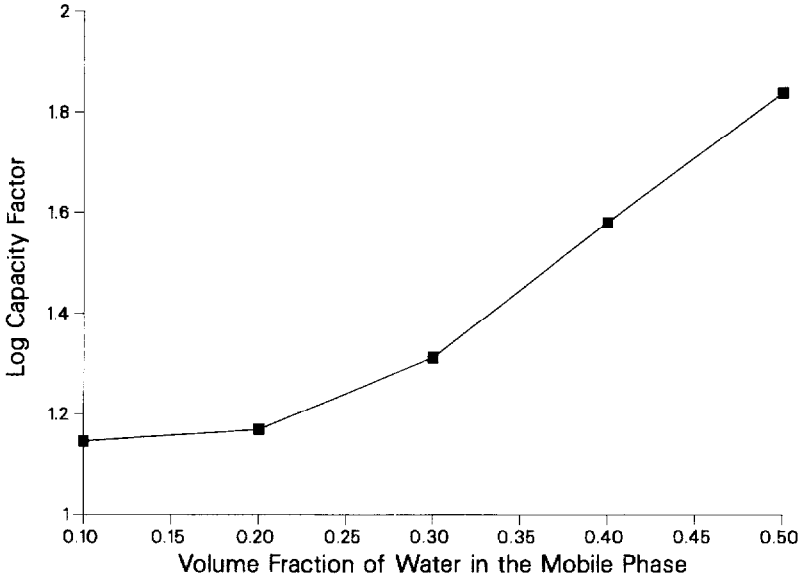

Fig. 2. Dependence of the logarithm of the capacity factor of I on the volume fraction of water in the mobile phase. The mobile phase contained 10 m*M* sodium acetate buffer, pH 6.1.

water on-column and marking the first deflection from the UV detector baseline. The capacity factor (k') was calculated as $(V_R - V_m)/V_m$, where V_R is the peak retention volume measured from the time of injection. Peak asymmetry factors were calculated as CB/AC, where CB is the distance from the peak tailing edge at 10% peak height to a perpendicular drawn from the peak apex, and AC is the distance from the peak leading edge at 10% peak height to the perpendicular [8]. Values greater than 1.0 indicate peak tailing.

RESULTS AND DISCUSSION

Using an acetonitrile–water mobile phase buffered to pH 6.1 with 10 m*M* sodium acetate and an octylsilane reversed-phase column, a plot of the logarithmic capacity factor of I versus the volume fraction of water was non-linear (Fig. 2). This type of "irregular" retention behavior has been explained by Nahum and Horváth [9] and Bij et al. [10] by a dual retention mechanism model in which the analyte interacts with both the column alkyl chains and silanol groups (solvophobic and silanophilic interactions, respectively). The dual retention model is particularly relevant to the reversed-phase chromatography of amphiphilic amines such as the tricyclic antidepressants because amine groups are capable of both hydrogen bonding and ion pairing with surface silanols [5,6]. Kiel et al. [6] reported that the silanol binding was stronger with tertiary compared to secondary or primary amino tricyclic antidepressants. Both I and II have four tertiary amine groups per molecule and were a priori good candidates for irregular retention behavior.

According to model theory, the relative importance of the solvophobic and sil-

anophilic retention mechanisms can be assessed graphically from data collected in the presence of varying concentrations of amine modifier in the mobile phase [10]. Thus

$$k' = k'_1 + k'_2 \quad (1)$$

$$k'_0 = k'_1 + k'_{2\max} \quad (2)$$

$$A/(k'_0 - k') = 1/(k'_{2\max} K_a) + A/k'_{2\max} \quad (3)$$

where k' = observed capacity factor, k'_1 = capacity factor due to solvophobic interactions, k'_2 = capacity factor due to silanophilic interactions, $k'_0 = k'$ in the absence of amine modifier, $k'_{2\max} = k'_2$ in the absence of amine modifier, K_a = association constant for the silanol amine complex, and A = amine concentration in the mobile phase. Plots of $A/(k'_0 - k')$ versus A should therefore yield a straight line with the reciprocal of the maximal silanophilic factor as the slope. The solvophobic capacity factor can then be obtained by difference according to eqn. 2.

The retention behavior of I and II was evaluated according to this model using both TEA and TBAH as amine modifiers and an acetonitrile concentration in the mobile phase of 70% (v/v). The pH was held constant at 6.1 by adding acetic acid. Both amines decreased the capacity factors of the aminosteroids to apparent asymptotic values when varied from 2 to 65 m*M* (Fig. 3). Sodium acetate alone caused a reduction in the capacity factor of I when varied over this range, but it was much less potent than either TEA or TBAH (Fig. 4). Using k'_0 values of 110 for I and 98 for II, plots of $A/(k'_0 - k')$ versus A were linear with correlation coefficients exceeding 0.990. Parameters derived from the plots are presented in Table I.

The results of this analysis clearly show that, with 70% acetonitrile in the mobile phase, the column retains both compounds by predominately silanophilic interactions. Only 1% of the retention of I and 2% of the retention of II can be attributed to interaction with the hydrocarbonaceous phase of the packing material. Amine modifiers can compete for silanol binding sites and thereby diminish the importance of this interaction. TBAH has a two-fold higher association constant for silanol groups under the conditions of this experiment than TEA (Table I), making it a more potent modifier of capacity factors. Eqn. 3 can be used to predict the concentration of amine modifer required to occupy a given percentage of silanol binding sites, since for a given association constant the value of k'_2 is dependent on the concentration of free silanol groups. Masking of 90% of the silanol groups at pH 6.1 and with 70% acetonitrile mobile phase requires approximately 1.8 m*M* TEA and 0.9 m*M* TBAH. Masking of 99% of silanol groups requires a ten-fold higher concentration of either modifier.

At pH 6.1, interaction of these 21-amino steroids with silanol groups probably consists of both charge pairing and hydrogen bonding. The pK_a of I is approximately 8.1 in water but is suppressed by the organic modifier, probably to between 6 and 7. Compound I therefore exists in both ionic and non-ionic forms in the HPLC mobile phase. As a tertiary ammonium salt, it can ion pair with anions and act as a hydrogen bond donor. As the amine base, and even as the ammon-

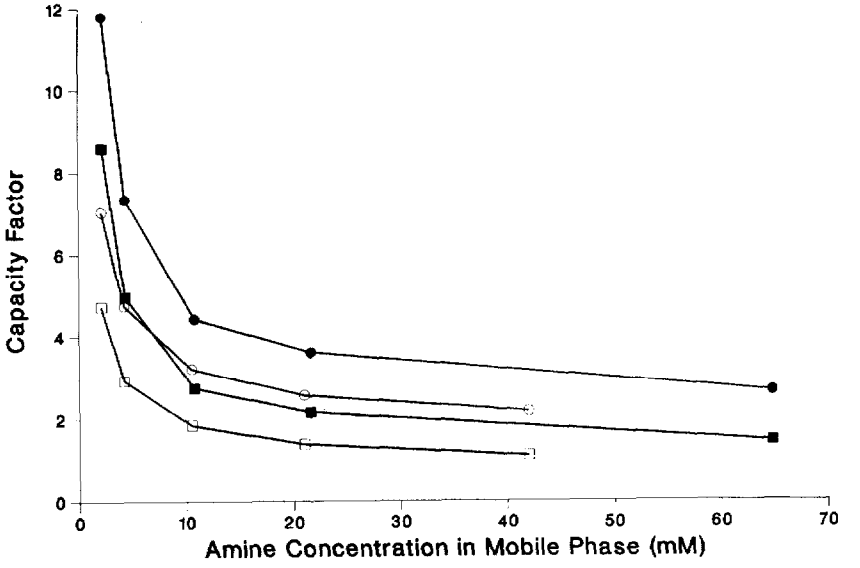

Fig. 3. Dependence of the capacity factors of I (closed symbols) and II (open symbols) on the TEA concentration (squares) and TBAH concentration (circles) in the mobile phase. The mobile phase consisted of acetonitrile–water (7:3, v/v) adjusted to pH 6.1 with acetic acid. At [TEA] or [TBAH] = 0, k'(I) = 110 and k'(II) = 98.

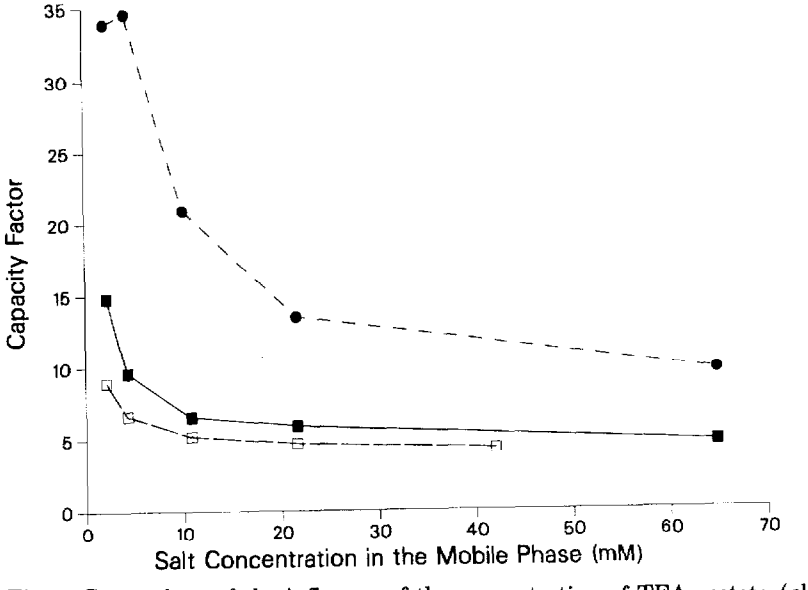

Fig. 4. Comparison of the influence of the concentration of TEA acetate (closed squares), TBA acetate (open squares), and sodium acetate (closed circles) in the mobile phase on the capacity factor of I. See Fig. 3 for the mobile phase composition.

TABLE I

GRAPHICAL ESTIMATES OF DUAL RETENTION MECHANISMS MODEL PARAMETERS WITH TEA AND TBAH MOBILE PHASE MODIFIERS

Mobile phase; acetonitrile–water (7:3, v/v), adjusted to pH 6.1 with glacial acetic acid.

Amine modifier	I			II		
	k'_2	k'_{2max}	K_a	k'_2	k'_{2max}	K_a
TEA	1.2	109	6.1	2.3	96	4.0
TBAH	0.9	109	11.9	1.9	96	7.5

ium salt it has several non-ionic amine groups, it is a hydrogen bond acceptor. No information is available regarding the pK_a of II. Column silanol groups have a pK_a of approximately 6 and are therefore capable of ion pairing with cations as well as hydrogen bond formation. Sodium, TBAH and TEA cations added as mobile phase modifiers can compete with the ionized drugs for pairing with the anionic silanoxide groups. TEA is also a hydrogen bond donor.

Both I and II exhibited peak tailing in the absence of amine modifiers. The peak asymmetry factors of I and II were 19 and 6, respectively. TEA was more effective than TBAH at improving peak symmetry for both aminosteroids (Fig. 5), despite its lower apparent affinity for silanol groups. Sodium acetate, which also decreased the capacity factor of I, was completely ineffective at improving peak symmetry. Of the three modifiers, only TEA can interact with the silanols by hydrogen bonding, and the relative efficiency of TEA at preventing tailing suggests that hydrogen bonding is an important determinant of peak shape. This conclusion is supported by the observation that peak symmetry for both compounds decreased as the pH dropped below 6 (Fig. 6), coinciding with the protonation of silanoxide anions to a form capable of hydrogen bond donation to the remaining unionized steroid amines. In addition, tailing was more severe with I than II under all conditions, as expected if it is due to hydrogen bonding with the tertiary amines. The pyrrolinidyl amines of I are less sterically hindered than the diethylamines of II.

Under conditions where both electrostatic and hydrogen bonding silanophilic interactions were inhibited with 11 m*M* TEA, lowering the pH of the mobile phase decreased the capacity factors of both aminosteroids (Fig. 7). The sharpest decrease, especially for II, was centered near pH 6, which is near the pK_a of both the silanol groups and the I amine. At pH 3.5, the capacity factors of both compounds were below the level due to solvophobic retention alone at pH 6 (Table I).

The retention characteristics of I were evaluated on two Supelcosil LC-8 columns and a Brownlee RP-8 cartridge column (Brownlee Labs., Santa Clara, CA, U.S.A.). There were differences between the columns in the capacity factor of compound I, as expected when a high percentage of the retentivity depends on the end-capping efficiency, but the factors affecting retention were unchanged.

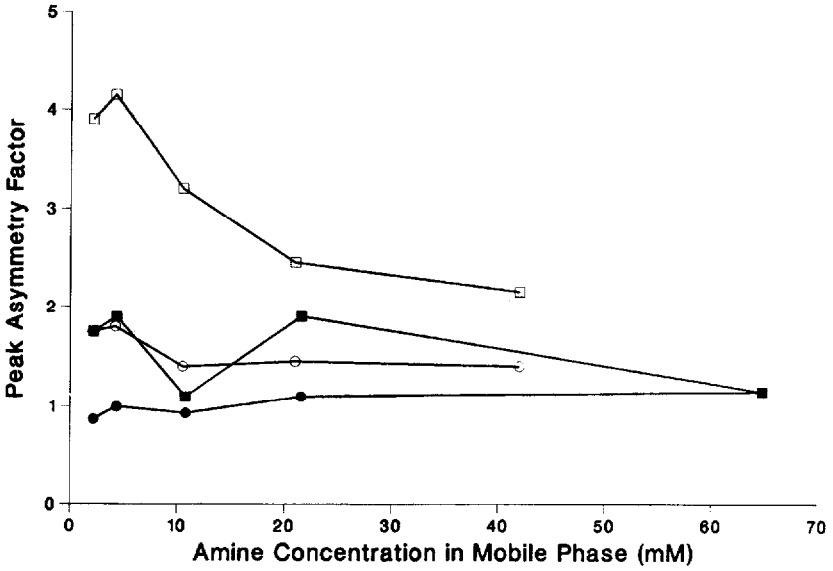

Fig. 5. Dependence of the peak asymmetry factors of I (closed symbols) and II (open symbols) on the mobile phase TEA concentration (squares) and TBAH concentration (circles). Mobile phase composition as in Fig. 3.

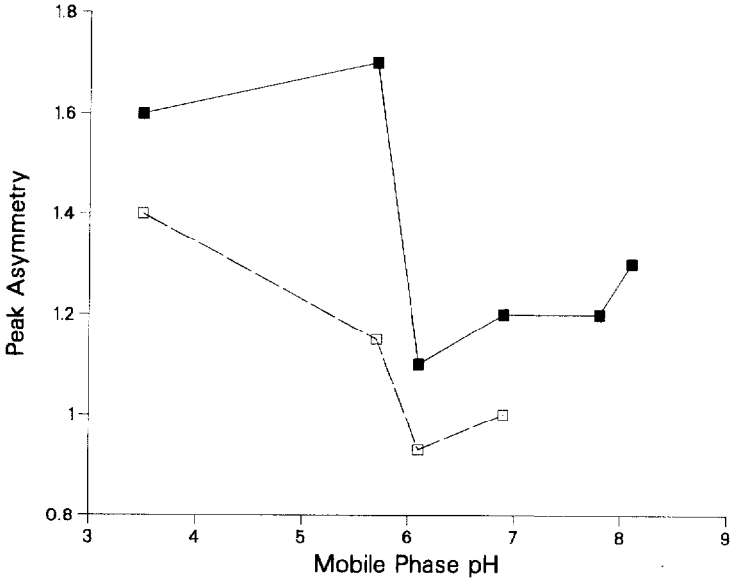

Fig. 6. Dependence of the I (closed squares) and II (open squares) peak asymmetry factors on the mobile phase pH. The mobile phase consisted of acetonitrile–water (7:3, v/v) with 11 m*M* TEA. The pH was adjusted with trifluoroacetic acid.

CONCLUSIONS

Both I and II exhibited irregular retention behavior on a silica support octyl-

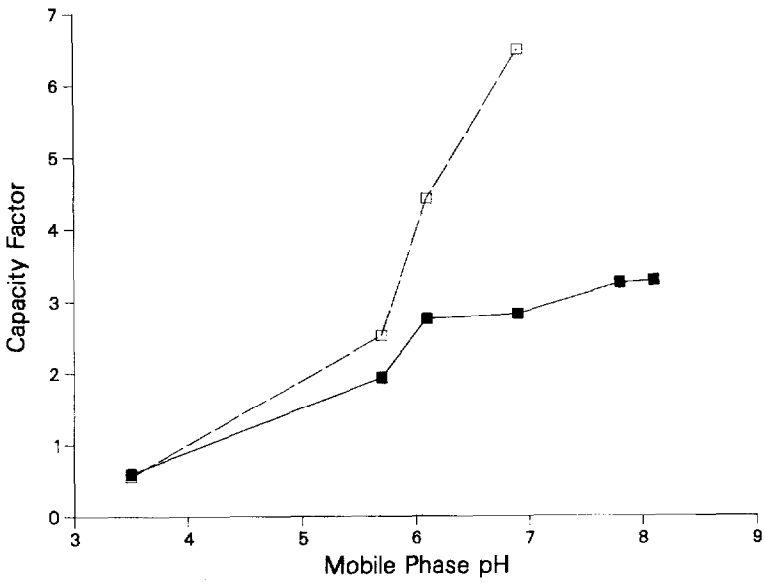

Fig. 7. Dependence of the capacity factor I (closed squares) and II (open squares) on the mobile phase pH. See Fig. 6 for the mobile phase composition.

silane bonded-phase column operated in the reversed-phase mode. Factors found to influence retention were the organic modifier concentration in the mobile phase, the identity and concentration of amine modifier, pH, and the sodium ion concentration. The retention characteristics can be rationalized according to a model which includes both solvophobic and silanophilic interactions. The latter can be made to predominate by deleting amine modifier from the mobile phase and operating above pH 5. These findings are particularly relevant to the development of a plasma HPLC assay for these compounds. By modifying the HPLC mobile phase to emphasize or deemphasize the silanophilic contribution, it should be possible to achieve a high degree of selectivity for complex mixture analyses.

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