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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF AVENANTHRAMIDES, N-AROYLANTHRANILIC ACID ALKALOIDS FROM OATS*

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SUMMARY

The high-performance liquid chromatographic determination of N-aroylanthranilic acid alkaloids is described. The compounds were separated on a C_{18} column using methanol–aqueous buffer gradients at pH 3.5, 4.0 and 4.5. A relationship was developed between the observed retention time and the presence of the different functional groups of the molecule.

INTRODUCTION

Avenanthramides are substituted N-cinnamoylanthranilic acid alkaloids occurring in the hulls and bran of oats[1]. Over 40 distinct avenanthramides have been detected in anionic fractions from 80% aqueous methanol extracts of oat grains[2], and to date, the complete structures of ten have been elucidated[3]. Recent interest in hydroxy- and/or methoxy-substituted N-cinnamoylanthranilates has been generated by reports of their potent biochemical and pharmacological activities. N-(3′,4′-Dimethoxycinnamoyl)anthranilic acid, although not detected in oats, is an inhibitor of hyaluronidase[4] and prolyl hydroxylase[5] activities. Under the generic names Tranilast, N-5′ and Rizaben, it has been developed and marketed as an antiallergic, antiasthmatic, anti-inflammatory drug[6]. Three compounds closely related to Tranilast have subsequently been found naturally-occurring in oats[3,7]. One of these compounds N-(4′-hydroxy-3′-methoxycinnamoyl) anthranilic acid] has recently been patented as a lipoxygenase inhibitor[8].

Purification, structural elucidation and quantitative estimation of the naturally-occurring oat avenanthramides have however been hampered by several factors. First, the mixture consists of closely-related members, so far differing only in the number and position of hydroxy and methoxy substituents. Secondly, due to the presence of a conjugated styryl function, photo-isomerization generates isomeric pairs of avenanthramides even when starting with one isomerically pure compound.

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Finally, some of the avenanthramides encountered have proven extremely susceptible to auto-oxidation leading to complex di- and polymeric artefacts. To address these problems, a suitable high-performance liquid chromatographic (HPLC) method was developed which would allow rapid separation of the mixture. As an aid to ongoing structural identification, an attempt was made to correlate chromatographic behavior with geometric isomer configuration (*Z* or *E*) and with substitution pattern.

EXPERIMENTAL

Avenanthramides

Individual avenanthramides were synthesized and purified as described by Collins[3] and dissolved in 80% acetone. Avenanthramide mixtures from oat groat flour were prepared by direct ion-exchange treatment of the 80% aqueous methanol extract using Sephadex QAE A-25 anion-exchange columns (Pharmacia Canada; Pte. Claire, Canada). The columns were converted to the formate form and pre-equilibrated in 80% methanol prior to use. Recovery of the anionic components (including the avenanthramides) was effected using the solvent acetone–water–formic acid (60:35:5, v/v/v) and non-phenolic anions were removed by chromatography on Sephadex LH-20 (Pharmacia) according to previous procedures[3]. The concentrated avenanthramide mixture was taken up in 80% acetone and stored at −40°C until required.

Apparatus

Separations were carried out on a Perkin-Elmer Series 4 HPLC system. The detector was a Perkin-Elmer LC 85B UV–VIS detector fitted with an autocontroller which allowed column effluent monitoring as well as scanning. The recorder was a Perkin-Elmer LC I-100 computing integrator. A cartridge type, 10 μm C_{18} column 250 × 4.6 mm I.D., fitted with a 20 × 4.6 mm I.D. precolumn filled with the same packing, was used for all separations.

Chromatographic procedures

Mobile phases consisted of methanol and an aqueous sodium acetate or formate buffer. Buffers at pH 5.5, 4.5 and 4.0 were prepared using 0.1 *M* sodium hydroxide and adjusted to the appropriate pH with 0.1 *M* acetic acid. For the pH 3.5 buffer 0.01 *M* formic acid was used adjusted with 0.1 *M* sodium hydroxide. All solutions were prepared from double distilled water, and LC-grade methanol, sodium hydroxide, acetic and formic acids. Prior to use, the mobile phases were filtered through 0.45-μm filters and purged with helium. Solvent A: methanol–aqueous buffer (3:97, v/v). Solvent B: methanol–aqueous buffer (62:38, v/v).

The solvent program for separation consisted of 60% B in A for 5 min followed by a linear gradient over 20 min to 80% B in A, then maintained isocratically for a further 20 min followed by a linear gradient return over 3 min to initial conditions and a 5-min equilibration period. The flow-rate was maintained at 2.0 ml/min. Although the avenanthramides exhibit different UV maxima between 320 and 355 nm this principle absorption band is broad[3]. Compounds used in this study all show substantial absorption at 340 nm, this wavelength was used for monitoring column eluates. To confirm peak identity, individual peaks were routinely analyzed using the stop-flow scanning mode of the LC 85B UV–VIS detector.

RESULTS AND DISCUSSION

Using single standards, several different programs and solvent combinations were tried. Initially, methanol–water or acetonitrile–water gradients were used with no buffer. Under these conditions the majority of avenanthramides eluted over a very narrow range, even with a very gradual gradient. Similar behaviour has previously been observed and attributed to intramolecular hydrogen bonding (*i.e.* disturbing solute interaction with the mobile phase) for *ortho* substituted phenolic acids, aldehydes and ketones which bear a substituent capable of hydrogen-bonding with carbonyl oxygen[9,10]. Increasing the ionic strength by buffering the aqueous solvent component[9] greatly improved resolution, and the most efficient system was a methanol–sodium acetate buffer gradient as noted in the Experimental section. Buffered acetonitrile systems generated much less acceptable resolution of mixed standards and were abandoned.

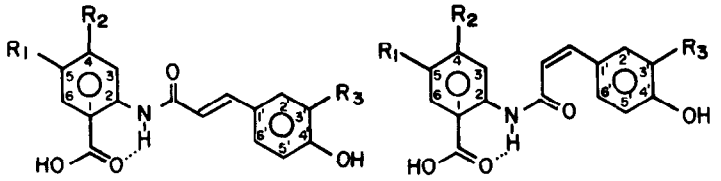
Table I summarizes the structures and retention times for the avenanthramides used in this study which were obtained using a pH 4.5 sodium acetate buffered solvent system. Standards, freshly prepared from recrystallized *E* isomers, gave single peaks but on exposure to daylight or UV light, a second peak (usually abut 5–10% of the major peak when allowed to reach equilibrium) was clearly discernable. The identity of this secondary peak as the corresponding *Z* isomer was confirmed by collecting the eluate from the minor peak using several chromatographic separations and reducing the volume *in vacuo*. Re-injection of the concentrate produced the same two peak pattern as previously obtained. Stop-flow UV absorption spectra obtained for both elution peaks showed them to be identical. Similarly, rechromatography of the eluate from the major peak also produced the same pattern of two spectrally identical but chromatographically distinct components. Such photo-mediated *Z,E*-isomerization has been observed with free and conjugated hydroxycinnamic acids[11–14], but has often been overlooked. For example, in a recent paper on the HPLC determination of Tranilast (*i.e.* avenanthramide E 4′-methyl ether) in body fluids[15], no mention was made of possible *E* or *Z* isomers in either the starting materials or metabolites recovered in the plasma and urine (including avenanthramide E, a reported Tranilast metabolite). However from a pharmacological standpoint, the *Z* isomer is known to possess in fact over ten times the antiallergic activity of the *E* isomer[4,16]. Additional examples of gross difference in chemical and biological properties between *E* and *Z* geometric isomers involving cinnamates abound in the literature and further illustrate the necessity of care in the preparation, quantitative estimation and evaluation of biological functionalities of cinnamic acid derivatives.

As shown in Table I, the retention time for the *Z* form of each isomer pair was approximately half that of the corresponding *E* form, despite the relatively minor difference in structure. Consideration of the comparative aqueous solubilities and three-dimensional structures (as observed in space-filling models) suggest that the higher aqueous solubility and more compact overal shape of the *Z* isomer, with a less exposed hydrophobic vinylic function, weaken stationary phase–solute interaction and therefore reduce elution time.

To facilitate a more detailed discussion of the relationship between substitution pattern and retention time, the avenanthramides listed in Table I can be considered as analogously-substituted cinnamoyl derivatives of anthranilic (D, E and F), 4-hy-

TABLE I

STRUCTURES AND RETENTION TIMES FOR OAT AVENANTHRAMIDE ALKALOIDS



E form *Z* form

Aqueous component, 0.1 *M* sodium acetate buffer (pH 4.5); flow-rate, 2.0 ml/min.

Avenanthramide pair				*Structure*	*Retention time (min)*		
Desig-nation	R_1	R_2	R_3		*E Form*	*Z Form*	*Ratio Z/E*
A	OH	H	H	N-(4′-Hydroxycinnamoyl)-5-hydroxyanthranilic	5.72	2.74	0.48
B	OH	H	OCH_3	N-(4′-Hydroxy-3′-methoxycinnamoyl)-5-hydroxyanthranilic acid	6.43	3.28	0.51
C	OH	H	OH	N-(3′,4′-Dihydroxycinnamoyl)-5-hydroxyanthranilic acid	4.31	2.17	0.50
D	H	H	H	N-(4′-Hydroxycinnamoyl)-anthranilic acid	10.90	5.50	0.50
E	H	H	OCH_3	N-(4′-Hydroxy-3′-methoxycinnamoyl)-anthranilic acid	12.41	5.89	0.47
F	H	H	OH	N-(3′,4′-Dihydroxycinnamoyl)-anthranilic acid	7.80	3.90	0.50
G	H	OH	H	N-(4′-Hydroxycinnamoyl)-4-hydroxyanthranilic acid	8.60	4.70	0.55
H	H	OH	OCH_3	N-(4′-Hydroxy-3′-methoxycinnamoyl)-4-hydroxyanthranilic acid	10.10	5.00	0.50
K	H	OH	OH	N-(3′,4′-Dihydroxycinnamoyl)-4-hydroxyanthranilic acid	6.31	3.58	0.57

droxyanthranilic (G, H and K) and 5-hydroxyanthranilic (A, B and C) acids. The data shown in Table I indicates that, within each of these three series, the order of increasing retention time followed the sequence: 3′.4′-dihydroxycinnamoyl < 4′-hydroxycnnamoyl < 4′-hydroxy-3′-methoxycinnamoyl.

This order of elution has been previously observed for free cinnamic acids[9,10,17,18]. The same order of elution applies to both the *E* forms and the *Z* forms of these compounds when considered separately. Between the three series, those compounds with identical cinnamoyl moieties (*e.g.* D, G and A) were increasingly retained according to the order: amido-5-hydroxyanthranilic < amido-4-hydroxyanthranilic < amidoanthranilic. From the above two sequences, it is evident that in general, addition of a hydroxyl group results in a decrease in retention time while methoxylation produces the opposite response. Both effects can be attributed to characteristic changes in hydrophobicity of aromatic compounds arising from methoxyl and hydroxyl substitution[19]. Addition of a methoxyl group decreases polarity (*i.e.* increases affinity for the hydrophobic stationary phase) while the polarity is increased (*i.e.* lower stationary phase affinity) with increasing hydroxylation. Both the 4- and 5-hydroxylated types have shorter retention times than the correspondig unsubstituted derivatives. From a comparison of the hydrophobicity of mono-hydroxylated benzoic acid analogues as determined by their octanol–water partition coefficients, the more polar 4-hydroxy derivatives might be expected to elute before the 5-hydroxyisomers (partition coefficinets: *para*-hydroxybenzoic acid = 31.6; *meta*-hydroxybenzoic acid = 38.1)[19]. However, since the opposite elution order was observed it suggests that either, the amido substitution changes the effects of the 4- or 5-hydroxy polarity or, polarity differences between these two isomers cannot account for the relatively large differences in resolution time.

Since the avenanthramides are weak carboxylic acids, the effects of suppressing acid dissociation on retention times was studied by decreasing the pH of the solvent buffer. Although the precise pK_a values for the compounds in the aqueous methanol gradient system employed were not known, it was felt that some effects due to differences in dissociation would be observed over the pH range 4.5 to 3.5 (*e.g.* pK_a for N-acetylanthranilic acid in water = 3.63; pK_a increases with increasing organic solvent content[20,21]). Indeed, as shown in Fig. 1 for the *E* isomers, lowering the pH resulted in longer retention times, with most of the increase occurring between pH 4.0 and 3.5. Throughout the range studied, the order of elution did not change and the *Z* isomers followed precisely the same pattern. Significantly, retention times for avenanthramides containing the amido-5-hydroxyanthranilic acid moiety (*i.e.* A, B and C) did not increase between pH 4.5 and 4.0, while those of the corresponding amido-4-hydroxyanthranilate isomers exhibited moderate increases (11% for H to 17% for G). The general effect of increasing retention time with decreasing pH (*i.e.* increasing H^+ concentration) is undoubtedly associated with a decrese in polarity of the acids when in the undissociated form and a concommitant increase in affinity for the hydrophobic stationary phase[22]. The rapid increase in retention time of all the aventhramides between pH 4.0 and 3.5 simply reflects the increasing mole fraction of the acids in the undissociated form as the pH is lowered through the pK_a ranges for these acids. The preferential increase in retention time exhibited by the 4-hydroxylated amidoanthranilates may in fact result from a lower degree of dissociation over the pH range 4.5 to 4.0. Indirect support for this explanation can be found by

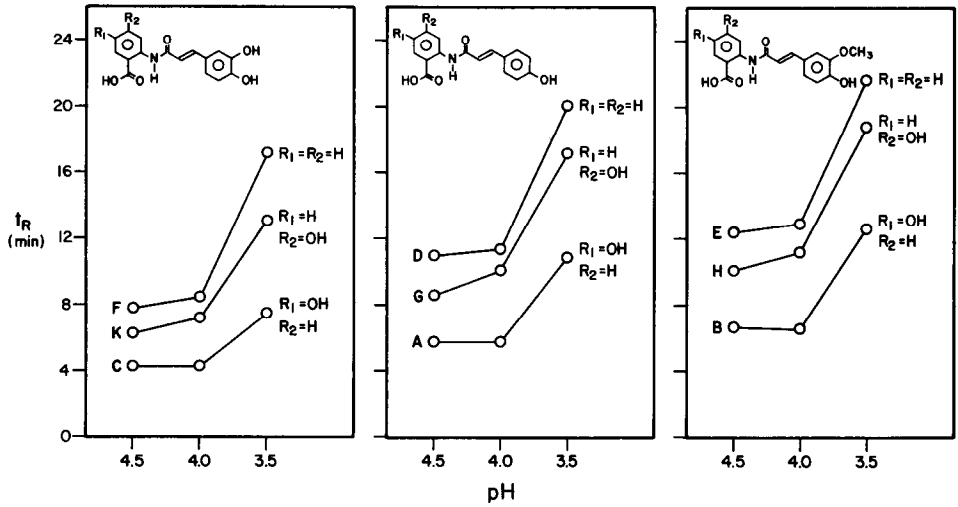


Fig. 1. Effect of pH on retention times of *E* isomers of avenanthramides.

comparing the pK_a values for *para verus meta* hydroxylated benzoic acids in aqueous media[20]. Introduction of a hydroxyl group in benzoic acid (pK_a = 4.20) in a position *para* to thecarbocxyl function suppresses dissociation (pK_a = 4.07). Thus, at pH values near the pK_a values for the isomers, the weaker 4-hydroxylated aventhramides might be expected to exhibit a somewhat lower degree of dissociation and therefore display a greater affinity for the stationary phase. The advantage of chromatographing phenolic acids (and bases) over a pH range close to their pK_a values may be of broader practical use for optimizing resolution of otherwise troublesome isomeric mixtures[23].

To measure the relative effects of the various hydroxyl and/or methoxyl substitution patterns on the chromatographic properties, substitute effect factors, were calculated[24]. These factors describe the proportional effect of particular functional groups when attached at specific positions on retention times (t_R) of individual members of an homologous series according to the relationship:

$$\alpha_i = \frac{t_R \text{ of homologue with substituent } i \text{ at position X}}{t_R \text{ of homologue without substituent } i}$$

Since the factor is a proportionality, the deviation from unity (either greater than 1.00 if retention time is increased by substituent, or less than 1.00 if retention time is increased) can be used to measure the effect of the substituent. For the study compounds, four such factors are identifiable: α_1 (3′-OCH_3), α_2 (3′-OH), α_3 (5-OH) and ;a_4 (4-OH). The results are summarized for the *E* series at three different pH values in Table II. Several conclusions emerge from an evaluation of the data. First, for each pair of homologues the four individual factors were not greatly affected over the ph range studied. For example, based on avenanthramides B and A varied less than 2% between pH 4.5 and 3.5. Secondly the value of each factor was not signifi-

TABLE II

SUBSTITUENT EFFECT FACTORS FOR *E* ISOMERS OF OAT AVENANTHRAMIDES

Substituent effect factor	*Homologues used*	*pH*			*Mean value (± S.D.)*
		4.5	*4.0*	*3.5*	
α_1	B/A	1.12	1.14	1.16	1.14 ± 0.02
(3'-OCH_3)	E/D	1.14	1.13	1.10	1.12 ± 0.02
	H/G	1.17	1.11	1.09	1.12 ± 0.02
	Mean value (± S.D.)	1.14 ± 0.03	1.13 ± 0.02	1.12 ± 0.04	1.13 ± 0.03
α_2	F/D	0.72	0.75	0.77	0.75 ± 0.03
	K/G	0.73	0.71	0.76	0.73 ± 0.03
(3'-OH)	C/A	0.75	0.74	0.69	0.69 ± 0.04
	Mean value (± S.D.)	0.73 ± 0.02	0.73 ± 0.02	0.74 ± 0.04	0.74 ± 0.03
α_3	A/D	0.52	0.51	0.55	0.53 ± 0.02
	C/F	0.55	0.51	0.49	0.52 ± 0.03
(5-OH)	B/E	0.52	0.51	0.58	0.54 ± 0.04
	Mean value (± S.D.)	0.53 ± 0.02	0.51 ± 0.00	0.54 ± 0.04	0.53 ± 0.03
α_4	G/D	0.79	0.89	0.87	0.85 ± 0.05
	K/F	0.81	0.85	0.86	0.84 ± 0.03
(4-OH)	H/E	0.81	0.87	0.87	0.85 ± 0.03
	Mean value (± S.D.)	0.80 ± 0.01	0.87 ± 0.02	0.87 ± 0.01	0.85 ± 0.04

cantly altered by other substituents already present in the molecule. Thus the α_1 factor calculated using pairs A and B, D and E, and H and G differed by less than 4%. The factors therefore appear to be independent of both pH and the presence of other functional groups. Thirdly, in comparing the mean values obtained for the individual factors, the greatest effect on retention time resulted from introduction of an hydroxyl group at the 5 position ($|1.00-\alpha_3|=0.47$), producing a decrease in retention time by almost 50%. Hydroxylation at either the 3' position ($|1.00-\alpha_2|=0.26$) or the 4 position ($|1.00-\alpha_4|=0.15$) had less effect. Methoxylation at the 3' position also had a small ($|1.00-\alpha_1|=0.13$) but opposite effect on retention time.

The independent influence of each substituent effect factor would appear to be a valuable tool in predicting retention time and optimizing separation conditions. For example, the retention time of avenanthramide K, containing 3',4' and 4-OH, could be predicted at any of the pH values studied by multiplying the observed retention time of a lesser substituted homologue by appropriate factor(s) *i.e.*:

$$t_R K = t_R f(\alpha_4) = t_R D(\alpha_2)(\alpha_4)$$

which leads to the general equation:

$$t_{R_i} = t_R(\alpha_1)(\alpha_2) \ldots (\alpha_i)$$

where t_{R_i} is the retention time of any aventhramide with i substituents, t_R = retention time of avenanthramide lacking all substituents in t_R, and α_1, α_2 ... α_i are the group substituent effect factors.

The avenanthramides have very high molar extinction coefficients, typically 10^4–10^3 which makes UV detection ideal. Although some compromise was necessary in the selection of 340 nm as the optimum wavelength for monitoring, the limit of detection was estimated to be in the picomol range. For example, a pure sample of avenanthramide F was dissolved in methanol–acetone to give a concentration of $4.85 \cdot 10^{-5}$ mol/l. A 5-μl injection of this standard gave a well-defined peak on the chromatogram and it was estimated that a further ten-fold reduction in concentration would still allow satisfactory quantitation. Am more thorough study of the quantitative aspects is being considered to determine the feasability of using microcolumns and to check detection limits and also the linearity of response for each compound.

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