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HIGH-EFFICIENCY RESOLUTION OF ISOMERIC ROTENONE COMPOUNDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The scope of high-performance liquid chromatography (HPLC) resolution of epimers of rotenone, 6′,7′-dihydrorotenone, 6′-ketorotenone, 6′-dinitrophenylhydrazonorotenone, and the 12a-hydroxy analogues (rotenolones) has been examined. Under optimal HPLC conditions these compounds gave elution patterns in accord with hydrophobicity and polarity predictions. In reversed-phase HPLC on spherical hydrocarbonaceous silica with a mobile phase of water (1% acetic acid) and acetonitrile, all but epimers of 6′-ketorotenone and the corresponding *cis*- and *trans*-6a,12a-rotenolones were sufficiently resolved. On the other hand, adequate separation of all the investigated epimers in the eight series was achieved by normal-phase HPLC on spherical silica with a mobile phase consisting of hexane, tetrahydrofuran, chloroform and 2-propanol. For the separation of rotenolone epimers by the process of solvophobic interactions, differences in the capacity factor between *cis*- and *trans*-6a,12a-compounds were distinctly larger than those between diastereoisomers derived from the same B/C ring junction stereochemistry. Such a conformational effect appeared to be less profound in separations where the adsorption mechanism predominated. Structural modifications on the 5′-sidechain of rotenone had a dramatic influence on resolution of epimeric components. For practical application, emphasis is placed on the potential utility of the HPLC techniques in preparative separation and in trace analysis of environmental samples.

INTRODUCTION

Rotenone is an important nonpersistent insecticide and piscicide, in part because of its ready isomerization and oxygenation in the environment to form a diversity of relatively polar but less toxic material. The major constituents of this material are isomers of rotenone (1A and 1B) and rotenolone (2A–D) shown in Fig. 1. The pesticidal rotenone (1A) is a naturally occurring substance in which structurally the B/C ring fusion adopts the *cis*-6aβ,12aβ-conformation. While 1A is known to racemize in alkaline medium giving an equilibrium mixture of 1A and 1B (6aα,12aα-epimer), the method for estimating the epimeric composition of this mixture has been solely based on the measurement of optical activity. Furthermore,

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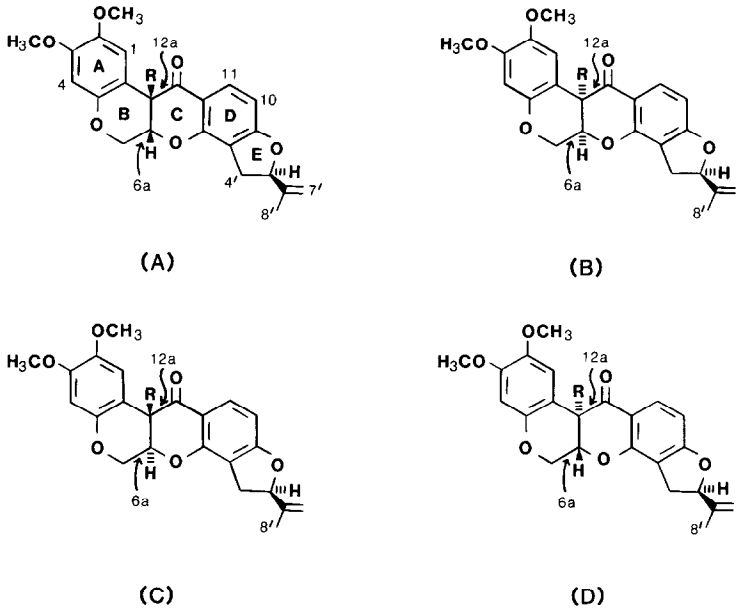

(1). R=H
(2). R=OH
(3). R=H, 6'=CH, 7'=CH_3
(4). R=OH, 6'=CH, 7'=CH_3
(5). R=H, 7'=O
(6). R=OH, 7'=O
(7). R=H, 7'=NNH $C_6H_3(NO_2)_2$
(8). R=OH, 7'=NNH $C_6H_3(NO_2)_2$

Fig. 1. Structures of 24 rotenone-related compounds.

racemization of 1A is believed to occur along with diminishing toxicity as the concentration of the lesser toxic antipode, 1B, increases. In this context, Unai[1] recently established the structure-activity relations, for a series of rotenone compounds with various isomeric structures and demonstrated that the low potency in biological activity is associated with the basic rotenone ring structure having B/C ring junction stereochemistry of *cis*-6aα,12aα-relationship, as in 1B. Knowledge of the precise composition of given epimeric mixtures is therefore essential to permit reliable evaluation and prediction of the biological properties of such mixtures.

Preparative separation of epimers of rotenone and related compounds has been difficult, and hitherto only arduous methods of repetitive fractional recrystallization and adsorption column chromatography for compounds in series 1 and 2 (Fig. 1) have been available[2]. Although thin-layer chromatography for qualitative identification of epimeric rotenone compounds has been described[3], high-resolution chromatographic techniques for efficient separation and quantitative analysis of this class of epimeric components have received little attention.

In connection with another study on the environmental fate as well as degradation kinetics of the natural rotenone 1A, we came across definitive analytical problems, apparently associated with the inefficiency of the existing high-performance liquid chromatographic (HPLC) methods[4,5] for effecting simultaneous separation

and quantification of epimeric components 1A and 1B within the kinetic time scale. A new HPLC method was obviously needed to circumvent the analytical difficulties with the dynamic systems. This method would be environmentally significant and would be of particularly practical potential in view of our recent detection of the rotenone epimer 1B and the rotenolone epimers 2A, 2B, 2C and 2D in samples isolated from the degradation of 1A under laboratory conditions simulating the natural environment[6].

The objectives of the present investigation were threefold: (i) to establish the utility of high-resolution HPLC in studying degradation kinetics and in facilitating high-efficiency separation of component epimers for analytical and preparative application, (ii) to determine chromatographic and structural factors influencing the resolution of epimeric components, and (iii) to compare resolution capabilities between normal- and reversed-phase HPLC to probe the scope and limitations of selected column systems for chromatographic characterization of isomeric rotenone compounds.

EXPERIMENTAL

Materials

All HPLC solvents (J. T. Baker, Philipsburg, NJ, U.S.A.) were of chromatography grade and were used directly. Reagent grade chemicals and solvents (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) were used in the preparation of isomeric rotenone compounds for this study. Rotenone was obtained either from Aldrich (Milwaukee, WI, U.S.A.) or Sigma (St. Louis, MO, U.S.A.) and was recrystallized twice from methanol before use. Ultrapure samples of rotenone 1A obtained in this manner were employed for the synthesis of all other compounds, following the published procedures[2]. Epimeric mixtures 1A–1B, 3A–3B, 5A–5B and 7A–7B (Fig. 1) were prepared in the medium of absolute ethanol (U.S. Industrial Chemicals, New York, NY, U.S.A.) in the presence of anhydrous sodium acetate (Alpha Products, Danvers, MA, U.S.A.). Preparation of epimeric mixtures of the four series 2, 4, 6, and 8 (2A–2B–2C–2D, 4A–4B–4C–4D, 6A–6B–6C–6D, and 8A–8B–8C–8D) was accomplished by bubbling a stream of high-purity air (Linde Specialty Gases, New York, NY, U.S.A.) through solutions of the corresponding rotenone-analogues in ethanol. Other inorganic chemicals required for the above syntheses were supplied by Alpha Products. Preliminary purification of each reaction mixture (mixture of epimers) by flash chromatography[7] with 40–60 μm silica gel 60 (E. Merck, Darmstadt, F.R.G.) afforded fractions partially enriched with one of the epimers. To obtain pure individual epimers, the enriched fractions were further purified by preparative HPLC as described later in this section.

High-performance liquid chromatography

For all HPLC studies, a Varian Model 5000 liquid chromatograph interfaced with a variable-wavelength (200–800 nm) detector (Varian Varichrom) and a Varian Model 9176 strip chart recorder was used. In a normal instrumental operation the detector was set at 295 nm for monitoring the analytes emerging from a column. In some experiments involving samples of analytes at trace levels, appropriate detector settings were chosen at the absorption maximum, λ_{max}, of individual rotenone deriv-

atives so that the sample detectability during each analysis was fully utilized. Retention data and peak area measurements were automatically performed on a Varian Model CDS-111-L data system. Typical mobile phases for reversed- and normal-phase HPLC were aqueous 1% acetic acid–acetonitrile and hexane–tetrahydrofuran (THF)–chloroform–2-propanol (ISP), respectively. Specific mobile phase compositions and other solvent systems employed in various experiments are detailed in the Results and discussion section. Three reversed-phase columns were used in the present work: (i) Varian MicroPak MCH-10, irregular octadecylsilica packing, 30 cm × 4.6 mm I.D., (ii) Altex Ultrasphere ODS, spherical octadecylsilica packing, 25 cm × 4.6 mm I.D., and (iii) Altex Ultrasphere OS, spherical octylsilica packing, 25 cm × 4.6 mm I.D. For normal-phase HPLC, two columns were evaluated: (i) Altex Ultrasphere Si, spherical silica packing, 25 cm × 4.6 mm I.D., and (ii) Altex Ultrasphere Cyano, spherical cyanosilica packing, 25 cm × 4.6 mm I.D. For sample injections, each column was connected to a Valco CV-6-UHPa-N60 injection valve and a 10-μl loop (Valco, Houston, TX, U.S.A). Unless otherwise indicated, the eluent was passed through the column at a flow-rate of 2 ml/min for reversed-phase HPLC and 3 ml/min for normal-phase HPLC. Ambient temperature was maintained throughout the analyses. Retention times were computed on the basis of the average values of three replicate determinations by injecting 30–60 ppm (μg/ml) aliquots of analytes in acetonitrile and methylene chloride in reversed- and normal-phase HPLC experiments, respectively.

Isolation of pure epimers by preparative HPLC

A mixture of the enriched epimers obtained from flash chromatography was separated by preparative normal-phase HPLC on the Altex Ultrasphere preparative Si column (25 cm × 10 mm I.D.). In general, aliquots containing about 10 mg of sample in methylene chloride were injected into the column via a 100-μl loop and the Valco injection valve attachment (see above). The following mobile phases were found suitable for the separation of different sets of epimeric mixtures: (i) 1A–1B and 3A–3B, 9:1 hexane–(19:1 CH_2Cl_2–ISP); (ii) 5A–5B, 9:1 hexane–(1:1:0.2 THF–CH_2Cl_2–ISP); (iii) 7A–7B and 8A–8B–8C–8D, 83:17 hexane–(1:1:0.2 THF–CH_2Cl_2–ISP); (iv) 2A–2B–2C–2D and 4A–4B–4C–4D, 9:1 hexane–(1:1:0.2 THF–$CHCl_3$–ISP); (v) 6A–6B–6C–6D, 4:1 hexane–(1:1:0.2 THF–$CHCl_3$–ISP). The column was eluted isocratically with the mobile phase specified at a flow-rate of 0.5 ml/min. Collection of column effluents with an ISCO Retriever III linear automatic fraction collector yielded 0.5-ml fractions. Upon analysis of each fraction by analytical HPLC–UV, fractions that were epimerically homogeneous were combined and evaporated to dryness under reduced pressure. Structures of the pure individual epimers isolated in this fashion were unequivocally characterized by ^{13}C nuclear magnetic resonance spectroscopy[8] and by comparison of physical constants with those reported in the literature[2,3].

Epimerization of rotenone (rate measurement)

A general procedure for the kinetic measurement of epimerization of 6aβ,12aβ-compounds (1A, 3A, 5A and 7A in Fig. 1) is described hereby using the natural rotenone 1A as an example. In a typical kinetic experiment, an accurately measured amount of absolute ethanol (4 ml) was placed in a graduated vial, which

had been immersed in a constant temperature bath (60°C in the example in Table I). Thirty minutes later when the vial had come to the bath temperature, rotenone 1A (1 mg) and anhydrous sodium acetate (20–40 mg) were added. The PTFE-lined screw cap septum was put on, and the reaction mixture was stirred uniformly at constant temperature. The reaction was quenched at 15-min intervals initially and then at 30-min intervals by withdrawing aliquots (200 μl) with the aid of a syringe through the septum, followed by treatment of each aliquot with 50% aqueous acetic acid (1 ml) at 0°C and extraction of the resulting solution twice with 1-ml portions of methylene chloride. The combined extract was dried over anhydrous sodium sulfate, filtered, and concentrated to exactly 1 ml under a stream of nitrogen. A volumetric flask made of low-actinic glass was used for storing samples prior to analysis by either reversed- or normal-phase HPLC.

TABLE I

KINETIC MEASUREMENTS OF EPIMERIZATION OF ROTENONE (1A–1B) AT 60°C

Time (min)	*Chromatographic peak area (cm^2)**					
	*Experiment I***		*Experiment II***		*Experiment III***	
	1A	*1B*	*1A*	*1B*	*1A*	*1B*
0	12.4	0.00	12.4	0.00	12.4	0.00
15	10.8	0.67	10.1	0.96	9.63	1.79
30	9.07	1.53	8.47	2.56	8.05	3.57
60	7.23	3.68	6.55	4.26	6.30	5.10
90	6.54	4.15	6.20	4.44	6.00	5.39
120	6.25	4.21	6.15	4.52	5.84	5.48

* Reversed-phase HPLC on spherical octadecylsilica (ODS) was used to determine the concentration of epimers. Mobile phase: 1% aqueous acetic acid–acetonitrile (1:1). Peak area measurements are based on the mean values of three replicate determinations, coefficient of variation: 3.6–6.7%.

** The amount of anhydrous sodium acetate used in experiments I, II and III was 20, 30 and 40 mg, respectively (see Experimental for details).

Oxygenation of rotenone (rate measurement)

The procedure for measuring the rate of oxygenation of rotenone 1A was nearly the same as that for epimerization (see above), except that the reaction mixture was composed of rotenone 1A (2 mg), butanol (10 ml), and sodium methoxide (0.1 ppm) and was stirred in the presence of air. This procedure was used for studying the oxygenation kinetics of the analogous compounds 3A, 5A and 7A. Mobile phases for both reversed- and normal-phase HPLC were carefully optimized so that the minimum resolution (R) value for all adjacent components was 1.00 to ensure reliable quantification of peak areas.

RESULTS AND DISCUSSION

Although most commercial normal- and reversed-phase HPLC columns of moderate efficiency were found satisfactory for the separation a mixture of roten-

oids[4,5], in the early stage of this work we were unsuccessful in finding a suitable column for obtaining kinetic data due to overlapping peaks of rotenone epimers 1A–1B and of rotenolone epimers 2A–2B–2C–2D as illustrated by the left and right chromatograms (B) respectively in Fig. 2. The inability to resolve these epimeric

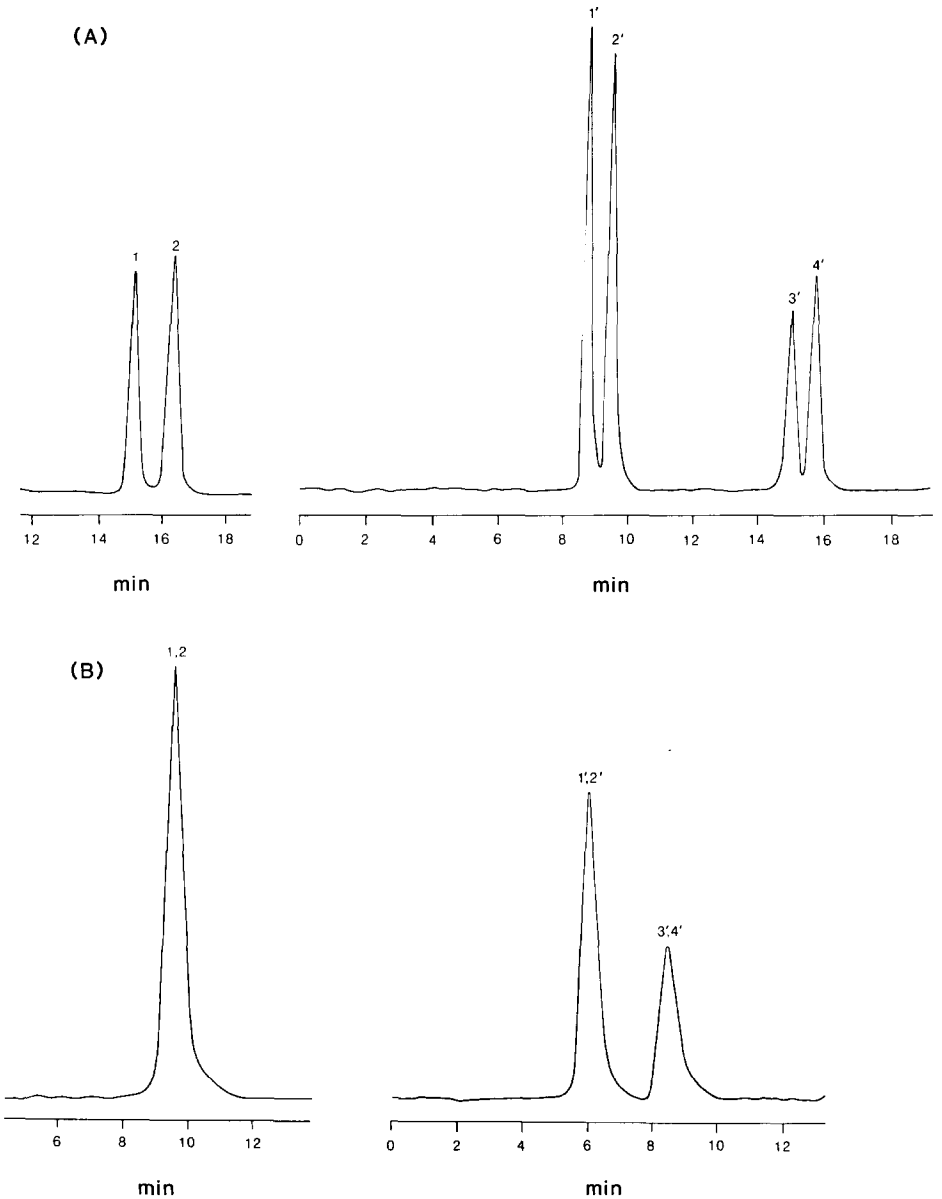

Fig. 2. Reversed-phase HPLC separation of epimers of rotenone and rotenolone. Peak identities: 1 = 1B; 2 = 1A; 1′ = 2B; 2′ = 2A; 3′ = 2D; 4′ = 2C. Column: (A) spherical ODS; (B) irregular ODS. Mobile phase: (A) 1% aqueous acetic acid–acetonitrile (11:9); (B) 1% aqueous acetic acid–acetonitrile (1:1). Flow-rate: 2 ml/min.

mixtures led us to explore a few commercial high-efficiency columns. Initial use of an Altex spherical octadecylsilica (ODS) column with a mobile phase consisting of 1% aqueous acetic acid–acetonitrile (11:9) led to excellent resolution of the seemingly inseparable sets of epimers 1A–1B and 2A–2B–2C–2D into their respective components (chromatograms A in Fig. 2). Encouraged by the results, we applied this technique to the isolation of pure epimers on the preparative scale and to rate measurements for the epimerization and oxygenation of rotenone and related compounds. An example of kinetic measurements of the epimerization of rotenone is given in Table I. It is deemed worthwhile at this point to clarify the precursor–product relationship among the eight series of epimers investigated. With reference to Fig. 1, epimerization of 1A, 3A, 5A and 7A produced corresponding epimeric mixtures 1A–1B, 3A–3B, 5A–5B and 7A–7B, of which all the B-epimers had the *cis*-6aα,12aα-conformation. On the other hand, oxygenation of 1A, 3A, 5A and 7A yielded epimeric mixtures 2A–2B–2C–2D, 4A–4B–4C–4D, 6A–6B–6C–6D and 8A–8B–8C–8D, respectively. In each set of these rotenolone epimers, the *trans*-6aα,12aβ-epimer (C) and the *trans*-6aβ,12aα-epimer (D) were found to be the minor components of the oxygenation products.

Reasoning that a majority of degradation products derived from rotenone are oxygen-containing polar substances and yet their insufficient solubility in water precludes the use of a high proportion of water in the reversed-phase mobile phase, we considered it appropriate to incorporate normal-phase HPLC in this study to provide a versatile approach for the chromatographic resolution of isomeric rotenone compounds. We chose eight series of compounds (Fig. 1) for study, since they were accessible by structural variation on the 5′-side chain of rotenone and should be ideally suited for a comparison of normal- and reversed-phase HPLC. These compounds include epimers of rotenone (1), rotenolone (2), 6′,7′-dihydrorotenone (3), 6′,7′-dihydrorotenolone (4), 6′-ketorotenone (5), 6′-ketorotenolone (6), 6′-dinitrophenylhydrazonorotenone (7), and 6′-dinitrophenylhydrazonorotenolone (8). Results of normal- and reversed-phase HPLC studies are discussed in separate paragraphs.

Reversed-phase HPLC on spherical octadecylsilica with a mobile phase of 1% aqueous acetic acid–acetonitrile (1:1) effected adequate separation of most epimeric components of epimerization and oxygenation products (Tables II and III). The chromatographic profiles were comparable with those shown in Fig. 2A. However, epimers of 6′-ketorotenone (5A and 5B) and the corresponding *cis*- and *trans*-6a,12a-rotenolones (R = 0 for 6A and 6B, R = 0 for 6C and 6D, Table III) were not separable under any of the reversed-phase conditions tried. The chromatogram on the left in Fig. 3 displays the unresolved component peaks. The fact that a high degree of resolution is shown by the dinitrophenylhydrazones (7A, 7B, 8A, 8B, 8C and 8D) in contrast to the underivatized compounds (5A, 5B, 6A, 6B, 6C and 6D) clearly indicates that the dinitrophenylhydrazono group increases the differential hydrophobic interactions. Thus, subjecting a sample of 7A to epimerizing conditions gave the two-component peaks, 7A and 7B, quantifiable for kinetic studies (Fig. 3), but it was impossible to obtain similar results for 5A. By virtue of their highly sensitive detectability for both UV and electrochemical detection, the dinitrophenylhydrazone derivatives described have been useful in the trace analysis of rotenone residues in environmental samples[9].

Tables II and III summarize the results of reversed-phase HPLC for the epi-

TABLE II

REVERSED-PHASE HPLC OF EPIMERIZATION PRODUCTS OF ROTENONE AND RELATED COMPOUNDS

*Mobile phase**	*Chromatographic characteristics***											
	Epimeric pair											
	1B		*1A*	*3B*		*3A*	*5B*		*5A*	*7B*		*7A*
	k'	*R*	*k'*	*k'*	*R*	*k'*	*k'*	*R*	*k'*	*k'*	*R*	*k'*
Spherical ODS												
I	2.73	1.20	2.92	4.10	1.10	4.40	0.92	0.00	0.92	5.61	2.00	6.02
II	7.91	0.00	7.91	11.8	0.00	11.8	1.20	0.00	1.20	15.6	1.52	17.7
III	9.52	1.36	10.0	13.0	1.20	13.8	2.64	0.00	2.64	30.3	4.25	26.6
IV	10.1	1.32	10.6	13.9	1.17	14.6	2.83	0.00	2.83	31.2	4.06	28.1
Spherical OS												
I	2.20	1.00	2.41	3.03	0.60	3.10	1.11	0.00	1.11	3.40	1.11	3.60
II	7.23	0.00	7.23	10.1	0.00	10.1	1.20	0.00	1.20	12.5	0.82	14.7
III	6.30	1.38	6.81	8.40	1.25	9.00	2.51	0.00	2.51	15.5	2.00	17.5
IV	7.03	1.33	7.43	9.14	1.22	9.65	2.72	0.00	2.72	17.3	1.89	19.1
Irregular ODS												
I	3.62	0.00	3.62	4.00	0.00	4.00	1.53	0.00	1.53	6.22	0.00	6.22
II	8.04	0.00	8.04	11.3	0.00	11.3	1.72	0.00	1.72	32.5	0.00	32.5
III	8.61	0.00	8.61	11.5	0.00	11.5	2.41	0.00	2.41	20.6	1.05	23.4
IV	9.11	0.00	9.11	12.1	0.00	12.1	2.50	0.00	2.50	21.8	1.00	24.0

* Mobile phase conditions: I, acetonitrile–1% aqueous acetic acid (65:35); II, methanol–1% acetic acid (65:35); III, acetonitrile–1% aqueous acetic acid (50:50); IV, acetonitrile–water (50:50). For column specifications see Experimental; ODS = octadecylsilica column; OS = octylsilica column.

** For structural identification see Fig. 1. Resolution (R) values were determined on the basis of the peak characteristics of epimeric pairs.

merization and oxygenation products of rotenone and related compounds. The retention (k') and resolution (R) data were obtained with three different columns (spherical ODS, spherical OS, and irregular ODS) under four different mobile phase conditions (see table footnotes for details). Comparisons of the chromatographic characteristics suggest that the spherical reversed-phase columns had resolution capabilities superior to the irregular particle columns and that resolution of epimeric components was better achieved with water–acetonitrile (mobile phases I, III, and IV) than with water–methanol (mobile phase II). While the spherical OS column tended to be less retentive (lower k' values) than the spherical ODS, as expected, fairly similar R values were observed with these two columns. When we compared the resolution data for the different structural types, we were able to arrange the compounds in the order of increasing R values as follows: $5 < 3 < 1 < 7$.

The general trends in the effect of the acetonitrile content in the mobile phase of reversed-phase HPLC on the component retentivity are shown in Figs. 4–6. In relation to the spherical OS column (A) the greater tendency for the spherical ODS column (B) to retain analyte solutes throughout a wide range of solvent compositions is evident from the plots in all three figures. The potential advantage of using a

TABLE III

REVERSED-PHASE HPLC OF OXYGENATION PRODUCTS OF ROTENONE AND RELATED COMPOUNDS

*Epimeric** component		*Chromatographic characteristics*								
		*Mobile phase***								
		I	*II*	*III*	*I*	*II*	*III*	*I*	*II*	*III*
		Spherical ODS			*Spherical OS*			*Irregular ODS*		
		k'	*k'*	*k'*	*k'*	*k'*	*k'*	*k'*	*k'*	*k'*
2B		1.49	5.61	5.33	1.33	4.93	4.48	2.47	5.18	4.86
	(*R*)	(0.70)	(0.00)	(1.14)	(0.41)	(0.00)	(0.81)	(0.00)	(0.00)	(0.00)
2A		1.73	5.61	5.70	1.41	4.93	4.92	2.47	5.18	4.86
	(*R*)	(3.06)	(5.21)	(5.78)	(1.48)	(3.78)	(4.25)	(1.25)	(3.08)	(3.33)
2D		2.27	11.4	8.82	1.64	9.11	6.60	3.23	9.11	7.43
	(*R*)	(0.80)	(0.58)	(1.10)	(0.67)	(0.55)	(1.00)	(0.00)	(0.38)	(0.00)
2C		2.52	12.1	9.40	1.84	9.63	6.93	3.23	9.90	7.43
4B		2.38	8.20	7.50	1.74	6.00	5.34	3.22	7.34	6.46
	(*R*)	(0.40)	(0.00)	(0.83)	(0.00)	(0.00)	(0.72)	(0.00)	(0.00)	(0.00)
4A		2.60	8.23	8.04	1.74	6.00	5.80	3.22	7.34	6.46
	(*R*)	(5.31)	(7.27)	(8.36)	(1.50)	(4.10)	(5.75)	(1.22)	(3.33)	(2.89)
4D		3.5	16.4	12.6	2.44	8.78	7.62	4.24	13.4	10.3
	(*R*)	(0.52)	(0.28)	(1.06)	(0.43)	(0.25)	(1.00)	(0.00)	(0.00)	(0.00)
4C		3.82	16.8	13.1	2.63	8.81	8.08	4.24	13.4	10.3
6B		0.45	1.23	1.51	0.83	1.13	1.50	0.80	0.93	1.02
	(*R*)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)
6A		0.45	1.23	1.51	0.83	1.13	1.50	0.80	0.93	1.02
	(*R*)	(1.01)	(0.00)	(1.19)	(0.85)	(0.00)	(0.95)	(0.00)	(0.00)	(0.80)
6D		0.93	1.23	1.90	0.92	1.13	1.77	0.80	0.93	1.14
	(*R*)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)
6C		0.93	1.23	1.90	0.92	1.13	1.77	0.80	0.93	1.14
8B		3.25	16.6	14.1	2.48	11.7	9.50	4.17	18.7	10.0
	(*R*)	(1.10)	(1.23)	(1.36)	(0.87)	(1.20)	(1.20)	(0.00)	(0.00)	(1.36)
8A		3.60	18.8	16.7	2.75	13.5	10.4	4.17	18.7	11.9
	(*R*)	(3.50)	(10.1)	(4.69)	(2.95)	(7.90)	(3.44)	(1.15)	(5.00)	(3.41)
8D		4.78	44.3	25.3	3.41	25.6	15.3	5.33	35.2	17.1
	(*R*)	(1.59)	(2.00)	(2.10)	(1.36)	(1.86)	(1.79)	(0.00)	(1.00)	(0.72)
8C		5.33	48.7	28.7	3.79	29.3	18.7	5.33	38.2	18.2

* For structural identification see Fig. 1. Resolution (*R*) values were determined on the basis of the peak characteristics of adjacent components.

** Mobile phase conditions I, II and III were the same as in Table II. ODS = octadecylsilica column. OS = octylsilica column.

spherical OS column in the reversed-phase HPLC analysis of isomeric rotenone compounds was the significant reduction in the analysis time without impairment of resolution. This may find utility in environmental sample analysis. Inspection of the retention curves revealed that, as expected for reversed-phase HPLC, the separation

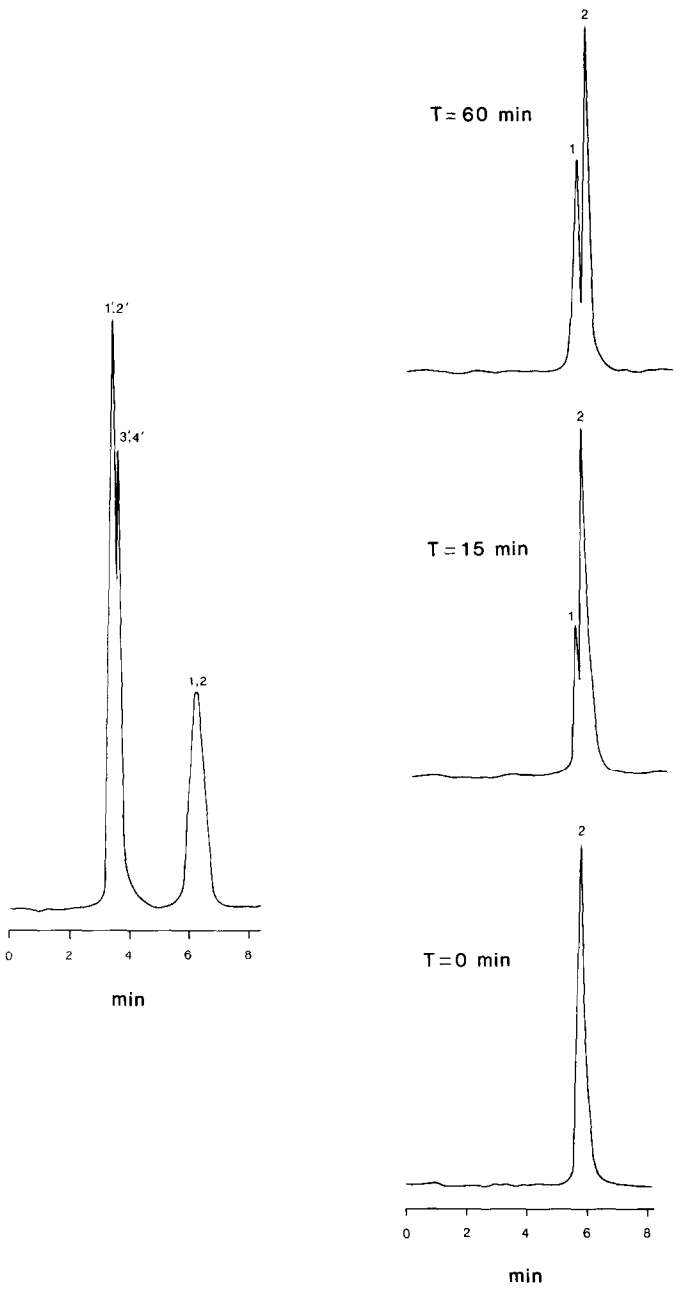

Fig. 3. Reversed-phase HPLC separation of epimers of 6′-keto-compounds (left) and 6′-dinitrophenylhydrazonorotenone (right). Peak identities: (left) 1 = 5B; 2 = 5A; 1′ = 6B; 2′ = 6A; 3′ = 6D; 4′ = 6C; (right) 1 = 7B; 2 = 7A. Column: spherical ODS. Mobile phase: (left) 1% aqueous acetic acid–acetonitrile (3:2); (right) 1% aqueous acetic acid–acetonitrile (7:13). Flow-rate: 2 ml/min.

of epimeric components was invariably favored by increasing the water content of the mobile phase. We were aware of the inevitable peak broadening caused by a high percentage of water in the mobile phase. For analytical application, the upper limit of the amount of water in the mobile phase was determined by drawing a line from the k' axis at $k' = 10$–15 across the curves (Figs. 4–6) and reading the abscissa at the

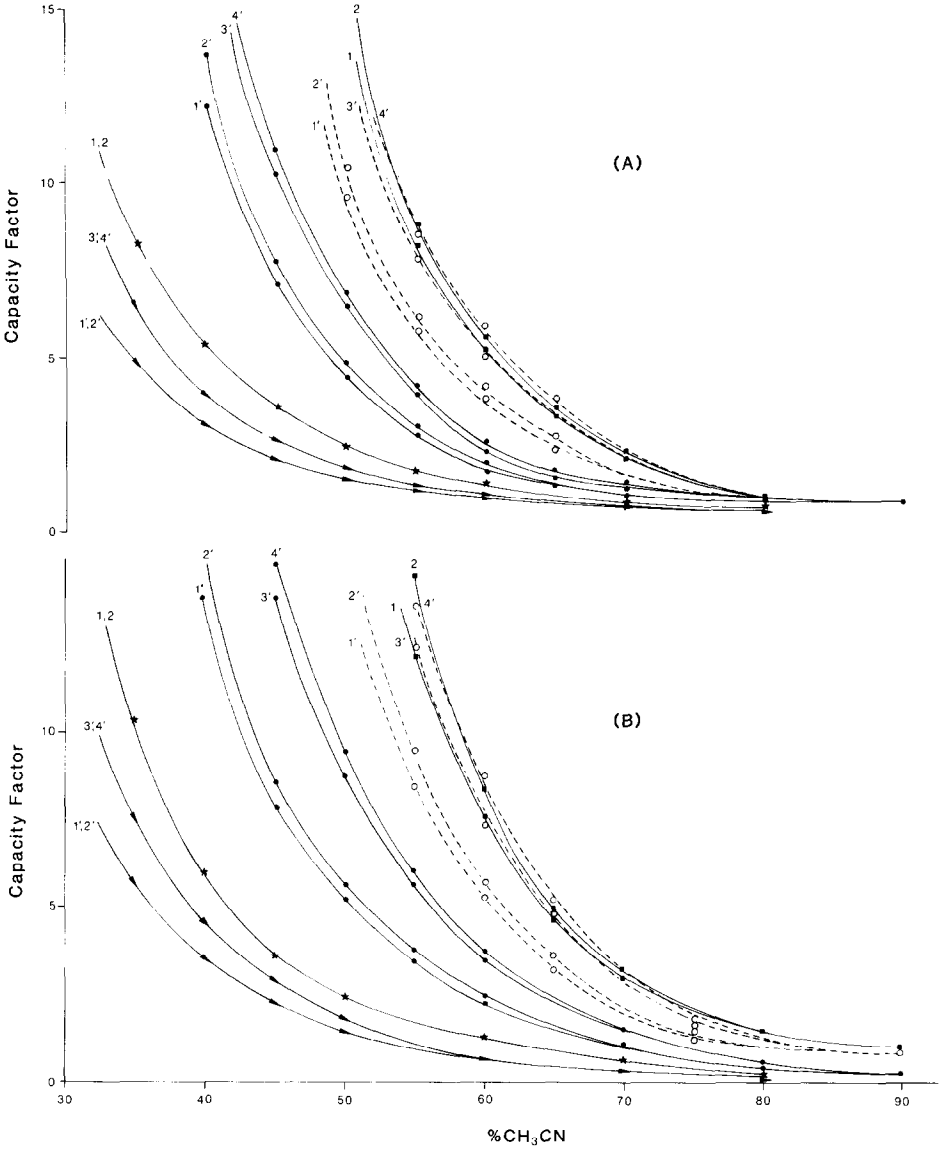

Fig. 4. Dependence of k' on the acetonitrile content in the mobile phase (1% aqueous acetic acid–acetonitrile). Column: (A) spherical OS; (B) spherical ODS. Symbols for structural series: square = 7; circle = 8; dot = 2; star = 5; arrow = 6. Components of rotenone-type structures: 1 = B (6aα,12aα-epimer); 2 = A (6aβ,12aβ-epimer). Components of rotenolone-type structures: 1′ = B (6aα,12aα-epimer); 2′ = A (6aβ,12aβ-epimer); 3′ = D (6aα,12aβ-epimer); 4′ = C (6aβ,12aα-epimer).

intercept with each curve. It is of interest to note that in each set of curves representing a given set of epimers there was at least one mobile phase composition at which the epimeric components coalesced (R = 0). Evidently, that solvent composition varied with the different structural types of epimers. Knowledge of this solvent composition could be of analytical importance.

In order to gain insight into the stereochemical effect on the structure retentivity relationship in reversed-phase HPLC, we conducted comparative studies on the retention behavior of epimeric compounds with different stereochemistry at the B/C

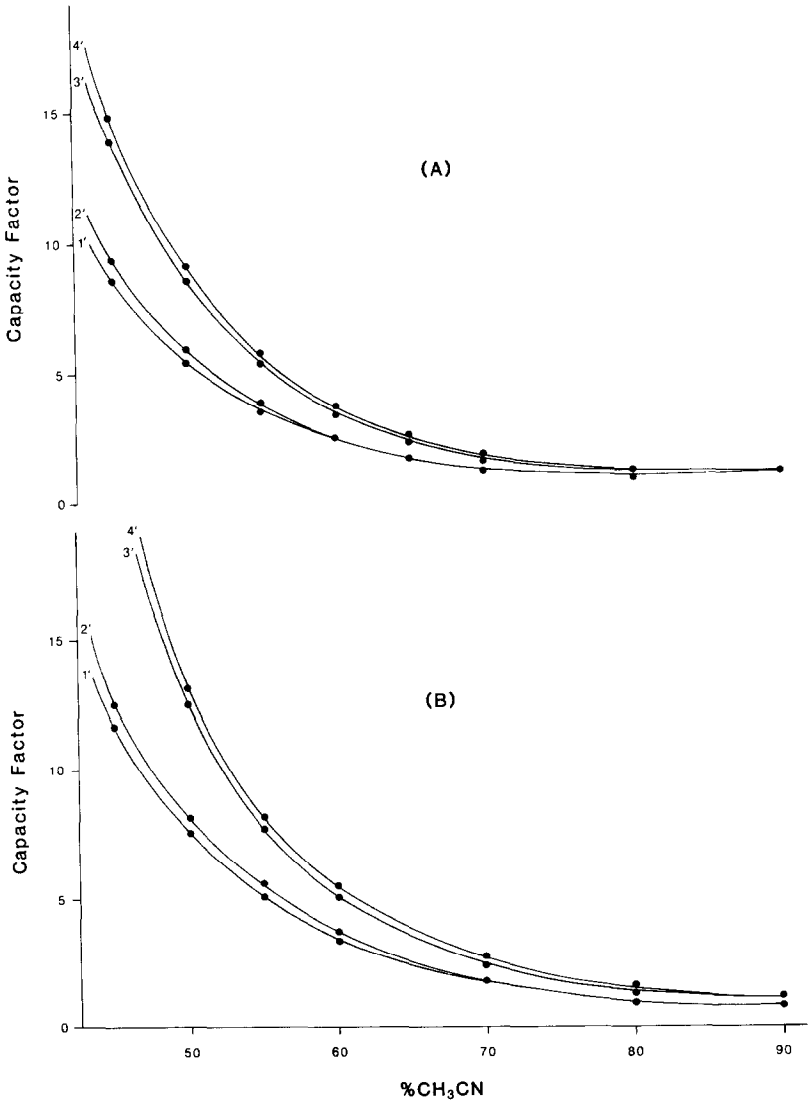

Fig. 5. Dependence of k' on the acetonitrile content in the mobile phase (1% aqueous acetic acid–acetonitrile). Column: (A) spherical OS; (B) spherical ODS. Components: 1′ = 4B; 2′ = 4A; 3′ = 4D; 4′ = 4C.

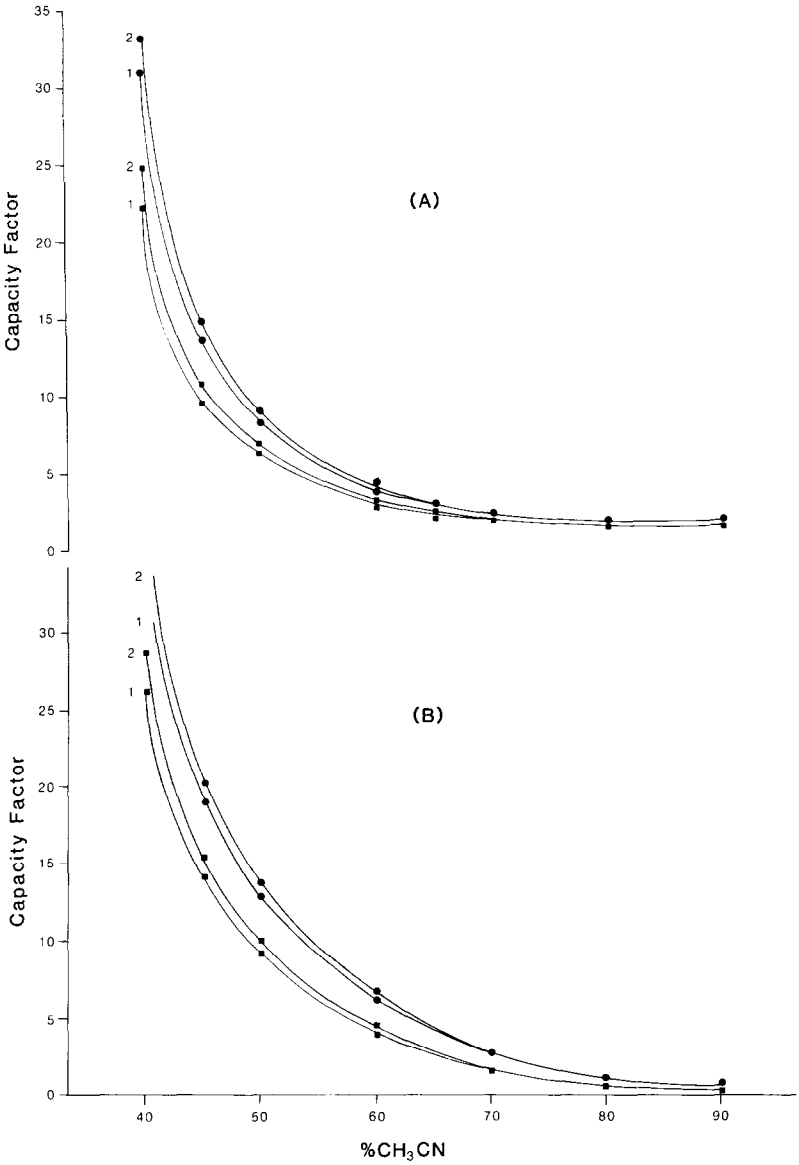

Fig. 6. Dependence of k' on the acetonitrile content in the mobile phase (1% aqueous acetic acid–acetonitrile). Column: (A) spherical OS; (B) spherical ODS. Symbols for structural series: dot = 3; square = 1. Component designations are as in Fig. 4.

ring junction and of different structural types. With the exception of the unresolved pair 5A–5B, the 6aβ,12aβ-epimers (Tables II and III, and Figs. 4–6) and the *trans*-6a,12a-epimers (Table III and Figs. 4 and 5) were found to be more retained than the corresponding 6aα,12aα-epimers and *cis*-6a,12a-epimers, respectively. For the separation of epimeric rotenolones, the differences in k' (capacity factor) values be-

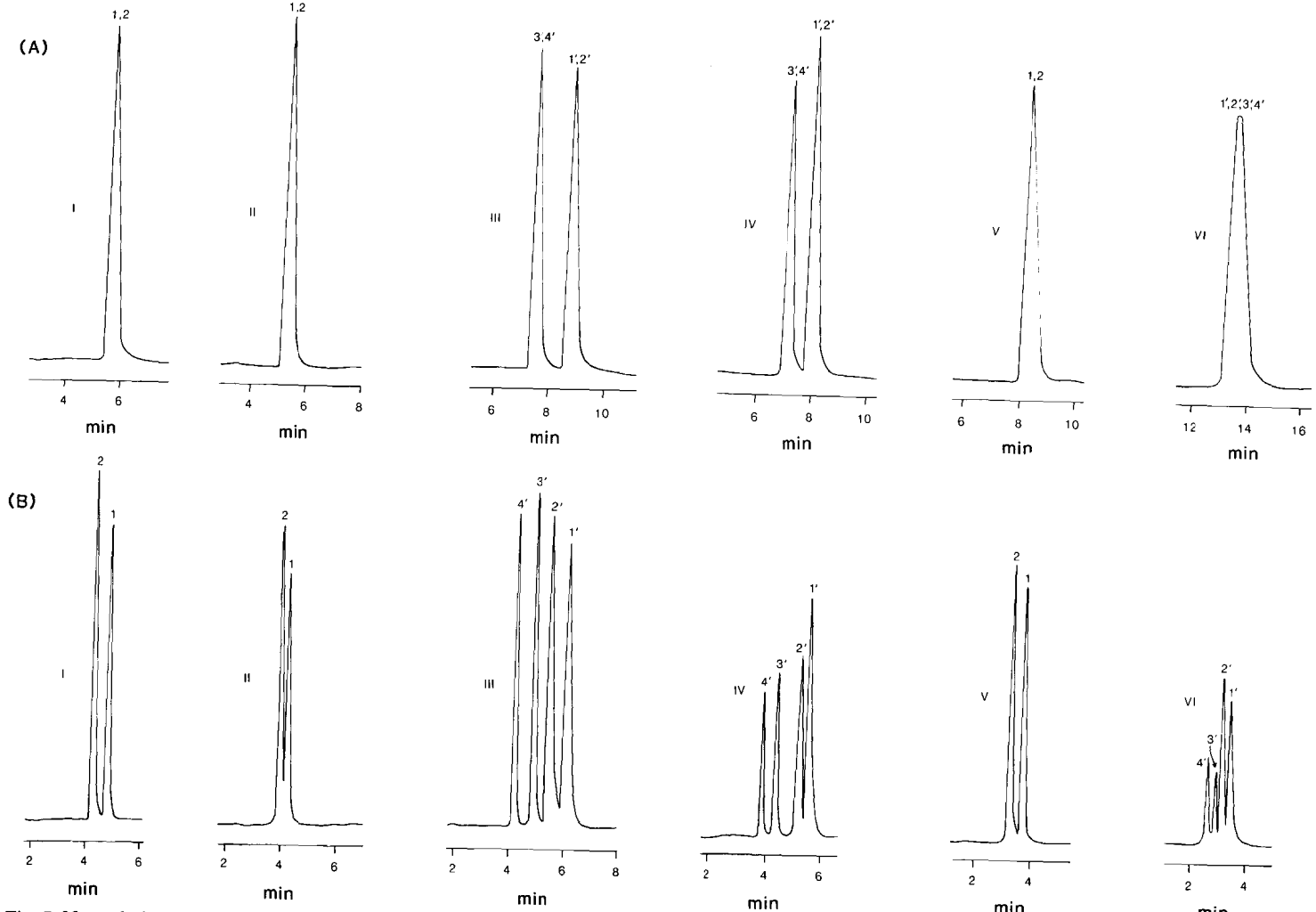

Fig. 7. Normal-phase HPLC separation of epimers of six series of rotenone-related compounds. Structural series: I = 1; II = 3; III = 2; IV = 4; V = 5; VI = 6. Component designations are as in Fig. 4. Column: (A) spherical cyano-silica; (B) spherical silica. Mobile phase: (A) 9:1 hexane–(1:1:0.2 THF–$CHCl_3$–ISP) for I, II, III and IV; 4:1 hexane–(1:1:0.2 THF–CH_2Cl_2–ISP) for V and VI; (B) 19:1 hexane–(1:1 THF–CH_2Cl_2) for all structural series. Flow-rate: 3 ml/min.

tween the epimeric *cis*- and *trans*-6a,12a-compounds were distinctly larger than those of diastereoisomers derived from the same B/C ring junction. This is better understood by an illustration with rotenolone type 2 compounds: $\Delta k'$ (2D–2B), $\Delta k'$ (2C–2B), $\Delta k'$ (2D–2A), $\Delta k'$ (2C–2A) > $\Delta k'$ (2A–2B), $\Delta k'$ (2C–2D) (Table III). We presume that in a reversed-phase separation the *trans*-6a,12a-conformation, which could confer hydrophobic properties upon the molecular surface would make for a higher degree of solvophobic interaction than the *cis*-conformation. Notwithstanding the slight structural dissimilarity between structures 1 and 3, the marked differences in their k' values (Table II) reflect the significant differential effect of the 6'-methylene- (in 1A and 1B) and 6'-methyl-group (in 3A and 3B) on the hydrophobicity and polarity of isomeric rotenone structures.

In normal-phase HPLC, we compared the effectiveness of a spherical silica column and a spherical cyano-silica column in terms of resolution. Representative

TABLE IV

NORMAL-PHASE HPLC OF EPIMERIZATION PRODUCTS OF ROTENONE AND RELATED COMPOUNDS

Mobile phase*	Chromatographic characteristics**											
	Epimeric pair											
	1B		1A	3B		3A	5B		5A	7B		7A
	k'	R	k'	k'	R	k'	k'	R	k'	k'	R	k'
Spherical silica												
a	13.3	1.94	11.7	12.1	0.67	11.7	ND	–	ND	ND	–	ND
b	8.91	2.62	7.63	8.56	1.16	7.60	ND	–	ND	ND	–	ND
c	4.40	1.37	3.81	3.82	0.80	3.61	ND	–	ND	ND	–	ND
d	2.89	1.00	2.56	2.40	0.50	2.49	ND	–	ND	ND	–	ND
e	4.80	2.12	4.20	4.43	0.89	4.24	ND	–	ND	ND	–	ND
f	5.00	1.75	4.31	4.55	1.09	4.36	ND	–	ND	ND	–	ND
g	3.21	1.92	3.82	3.32	1.14	3.05	12.2	3.08	9.80	21.3	5.28	16.9
h	3.38	1.69	2.90	2.92	0.91	2.74	11.1	2.84	9.23	20.2	4.73	16.1
i	2.42	1.56	2.19	2.20	0.88	2.00	10.0	2.73	8.51	19.4	3.89	15.3
j	3.53	1.37	3.02	3.13	1.01	2.81	9.22	2.43	7.49	15.6	5.25	11.9
k	2.70	1.65	2.30	2.17	0.95	1.80	8.53	2.52	6.44	14.5	5.30	10.6
l	3.07	1.73	2.55	2.93	1.12	2.62	9.03	2.59	6.08	15.0	5.37	11.3
Spherical cyano-silica												
m	4.72	0.00	4.72	4.33	0.00	4.33	7.36	0.00	7.36	30.5	4.40	23.7
n	9.40	0.18	9.20	9.11	0.33	8.81	ND	–	ND	ND	–	ND
o	9.40	0.12	9.20	9.20	0.27	9.01	ND	–	ND	ND	–	ND
p	ND	–	ND	ND	–	ND	2.20	0.00	2.20	6.42	2.33	5.33

* For column specifications, see Experimental. Mobile phases: (a) 9:1 hexane–(19:1 CH_2Cl_2–ISP); (b) 9:1 hexane–(19:1 $CHCl_3$–ISP); (c) 9:1 hexane–(19:1 THF–ISP); (d) 9:1 cyclohexane–(19:1 THF–ISP); (e) 9:1 hexane–(1:1:0.1 THF–CH_2Cl_2–ISP); (f) 9:1 hexane–(1:1:0.1 THF–$CHCl_3$–ISP); (g) 9:1 hexane–(1:1:0.2 THF–$CHCl_3$–ISP); (h) 9:1 hexane–(1:1:0.3 THF–$CHCl_3$–ISP); (i) 9:1 hexane–(1:1:0.4 THF–$CHCl_3$–ISP); (j) 9:1 hexane–(1:1:0.2 THF–CH_2Cl_2–ISP); (k) 9:1 isooctane–(1:1:0.2 THF–CH_2Cl_2–ISP); (l) 9:1 isooctane–(1:1:0.2 THF–$CHCl_3$–ISP); (m) 19:1 hexane–(1:1 THF–CH_2Cl_2); (n) 49:1 hexane–(1:1 THF–CH_2Cl_2); (o) 49:1 hexane–THF; (p) 17:3 hexane–(1:1 THF–CH_2CL_2).

** For R determinations, see footnote to Table II. ND = None detected.

chromatograms obtained with these columns are shown in Fig. 7. In contrast to the poorly resolved (or unresolved) peaks obtained with the cyano-silica stationary phase (chromatograms B), the well-resolved peaks (chromatograms A) attributable to the various epimers strongly indicate the high selectivity of the silica stationary phase for isomeric rotenone compounds. Table IV compiles the results on normal-phase HPLC of the epimerization products of rotenone (1A) and related compounds (3A, 5A and 7A). The normal-phase HPLC data for oxygenation products of rotenone (1A) and 6',7'-dihydrorotenone (3A) are summarized in Table V, and those for 6'-ketorotenone (5A) and the dinitrophenylhydrazone derivative (7A) are in Table VI. Two features of these results are of immediate interest. One is that epimeric components of 6'-keto-compounds (5A, 5B, 6A, 6B, 6C and 6D) were adequately resolved on spherical silica with most of the mobile phases employed. In contrast, in reversed-phase HPLC the epimers remained either unresolved (5A–5B) or partially resolved (6A–6B and 6C–6D) in all cases studied. Separation of a mixture of epimeric 6'-ketorotenolones 6A, 6B, 6C and 6D into well-separated four components by normal-phase HPLC on spherical silica proved to be unique, because it was difficult to resolve such a mixture by other chromatographic means. Also, in comparison to the data from reversed-phase HPLC normal-phase HPLC gave rather small differences in the k' values between epimeric *cis*- and *trans*-6a,12a-rotenolone compounds relative to those of diastereoisomers derived from the same B/C ring junction.

TABLE V

NORMAL-PHASE HPLC OF OXYGENATION PRODUCTS OF ROTENONE AND 6',7'-DIHYDROROTENONE

*Mobile phase**	*Chromatographic characteristics***													
	Epimeric component													
	2C		*2D*		*2A*		*2B*	*4C*		*4D*		*4A*		*4B*
	k'	R	k'	R	k'	R	k'	k'	R	k'	R	k'	R	k'
Spherical silica														
a	ND	–	ND	–	ND	–	ND	ND	–	ND	–	ND	–	ND
b	8.56	0.00	8.56	1.03	10.0	0.00	10.0	ND	–	ND	–	ND	–	ND
c	3.40	2.16	4.10	2.30	9.15	0.00	9.15	3.14	1.80	3.61	2.89	8.62	0.00	8.62
d	2.22	1.25	2.73	2.29	6.62	0.00	6.62	2.12	1.00	2.45	1.94	5.95	0.00	5.95
e	4.63	4.33	5.95	5.23	9.11	0.59	10.0	4.33	3.50	5.15	2.00	7.88	0.00	7.88
f	4.20	2.02	5.20	0.00	5.20	1.39	6.06	3.85	2.04	4.47	1.78	5.29	0.38	5.47
g	3.11	3.45	3.84	2.15	4.56	1.40	5.10	3.00	2.49	3.51	2.83	4.32	0.94	4.64
h	2.90	2.75	3.53	0.87	3.74	1.33	4.17	2.85	2.26	3.26	0.83	3.40	0.71	3.70
i	2.22	1.23	2.50	0.00	2.50	1.11	2.80	2.15	1.59	2.41	0.00	2.41	0.69	2.54
j	2.13	1.63	2.51	0.57	2.73	0.56	3.03	1.94	1.55	2.21	1.00	2.73	0.00	2.73
k	2.07	2.00	2.41	0.84	2.73	0.37	2.91	1.83	1.38	2.00	0.92	2.69	0.00	2.69
l	2.53	1.81	3.00	0.00	3.00	1.06	3.52	2.10	2.07	2.53	0.85	3.08	0.00	3.08
Spherical cyano-silica														
m	6.50	0.00	6.50	3.25	7.83	0.00	7.83	6.15	0.00	6.15	2.25	7.04	0.00	7.04

* For mobile phase conditions, see footnote to Table IV.

** For R determinations, see footnote to Table III. ND = None detected.

TABLE VI

NORMAL-PHASE HPLC OF OXYGENATION PRODUCTS OF 6′-KETOROTENONE AND THE DINITROPHENYLHYDRAZONE DERIVATIVE

*Mobile phase**	*Chromatographic characteristics***													
	Epimeric component													
	6C		*6D*		*6A*		*6B*	*8C*		*8D*		*8A*		*8B*
	k′	*R*	*k′*	*R*	*k′*	*R*	*k′*	*k′*	*R*	*k′*	*R*	*k′*	*R*	*k′*
Spherical silica														
g	9.58	1.73	10.1	0.64	10.8	1.19	11.1	9.67	1.42	11.3	1.93	13.3	1.11	14.8
h	8.37	1.69	9.00	0.39	9.52	1.08	10.0	8.41	1.36	9.71	1.26	12.0	0.81	13.4
i	7.19	1.14	8.46	0.00	8.51	1.05	9.04	7.35	1.28	8.80	1.18	11.3	1.53	12.0
j	6.45	1.36	7.23	0.64	7.67	1.19	8.16	6.93	1.37	8.47	1.88	10.5	1.05	11.7
k	6.11	1.41	6.80	0.53	7.34	1.16	7.83	6.18	1.54	7.30	1.51	9.33	1.00	10.9
l	6.37	1.50	7.12	0.75	7.53	1.41	8.00	6.36	1.68	8.12	1.73	9.78	1.22	11.3
Spherical cyano-silica														
m	12.4	0.00	12.4	0.00	12.4	0.00	12.4	25.5	1.50	29.0	2.46	31.8	1.20	34.2
n	ND	–	ND	–	ND	–	ND	ND	–	ND	–	ND	–	ND
o	ND	–	ND	–	ND	–	ND	ND	–	ND	–	ND	–	ND
p	3.68	0.00	3.68	0.00	3.68	0.00	3.68	6.23	1.30	7.31	1.00	8.20	1.23	8.94

* For mobile phase conditions, see footnote to Table IV.

** For *R* determinations, see footnote to Table III. ND = None detected.

In general, the retention (k') and resolution (R) parameters were notably responsive to the polarity of mobile phase constituents used in normal-phase HPLC. When mobile phases of insufficient elution strength were employed, often the peaks of the more polar components of an epimeric mixture tended to broaden and more frequently, none of the analyte components was detectable due to adsorption on the stationary phase. In accordance with polarity predictions, lower k' values were observed for the less polar epimers and both the k' and R values increased with a decrease in the mobile phase polarity. As noted in Table IV, the resolution (R) data for the epimeric pairs listed are illustrative of an ordering of increasing R values in the sequence of following structural types: $3 < 1 < 5 < 7$. It is instructive to compare the resolution characteristics (R) of adjacent components within each set of oxygenation products. Analysis of the results in Tables V and VI indicated that without exception the R values for the C and D epimers in each of the four epimeric sets 2, 4, 6, and 8 appeared to be higher than those for the A and B epimers.

Of the numerous mobile phases investigated (see footnote to Table IV for detailed mobile phase conditions) in the current normal-phase HPLC experiments, the system comprising 9:1 hexane–(1:1:0.2 THF–CH_2Cl_2–ISP) was found to be the most satisfactory for general application to all the compound types under consideration. In addition, the use of mixed solvents in lieu of individual solvents for mobile phases was advantageous for improving resolution and other chromatographic characteristics, such as peak symmetry and column selectivity. Fig. 8 shows typical examples of the dependence of k' on the amount of hexane in the mobile phase. A

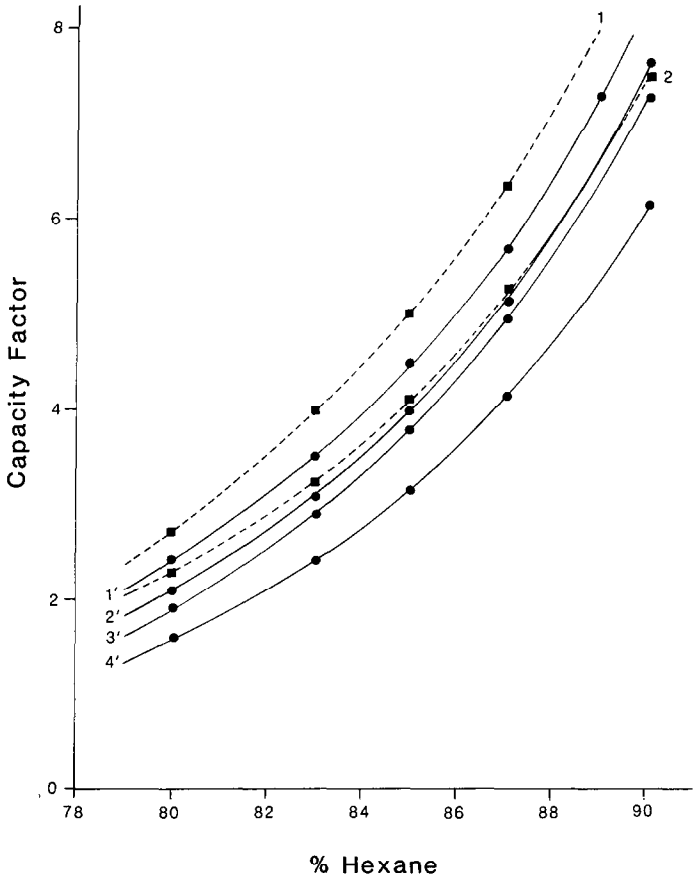

Fig. 8. Dependence of k' on the hexane content in the mobile phase [hexane–(1:1:0.2 THF–CH_2Cl_2–ISP)]. Column: spherical silica. Symbols for structural series: square = 5; dot = 6. Components: 1 = 5B; 2 = 5A; 1′ = 6B; 2′ = 6A; 3′ = 6D; 4′ = 6C.

consistent large increase in k' values of 5A, 5B, 6A, 6B, 6C and 6D was observed as a result of a small increase in the hexane content of the mobile phase. This trend of solvent effect on retention is parallel to that reported for the separation of nitrosamines[10]. An important finding in the use of the cyano-silica column needs to be mentioned. In spite of predictions of the inadequacy of the cyano-silica stationary phase in the resolution of epimers in series 1–6, application of the same normal-phase system with the spherical cyano-silica column to compounds in series 7 and 8 led to excellent resolution of epimers in mixtures 7A–7B (Table IV) and 8A–8B–8C–8D (Table VI). The mobile phases used for these separations were 19:1 hexane–(1:1 THF–CH_2Cl_2) and 17:3 hexane–(1:1 THF–CH_2Cl_2), corresponding to mobile phases m and p.

Both the reversed- and normal-phase HPLC methods developed in this study can be used to separate closely related isomeric rotenone compounds and are applicable to studies of degradation mechanisms of rotenone. For resolution of compo-

nent epimers, resolution characteristics can be favorably adjusted by chemical derivatization and by selection of high-efficiency stationary phases and suitable mobile phases.

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