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Note

Use of organic modifiers in the mobile phase for the reversed-phase high-performance liquid chromatographic separation of steroidal hormones

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There have been several reports of the use of small percentages of various organic compounds in the mobile phases to modify chromatographic selectivity for different polar functional groups in reversed-phase high-performance liquid chromatography (HPLC)[1–7]. In the course of studies on the separation of various steroidal hormones we encountered several mixtures which we were unable to resolve completely by means of binary mixtures of acetonitrile and water or methanol and water on an octylsilane-bonded phase column. A systematic examination of several organic modifiers was initiated to improve the separations. As a result of these studies, several ethers were found to improve the separations significantly and an explanation for these effects was attempted.

EXPERIMENTAL

Reagents and materials

Estradiol-17α, estradiol-17β and estrone were purchased from Steraloids (Wilton, NH, U.S.A.). Testosterone, progesterone, estriol, mestranol, ethinylestradiol and norethindrone were products of Syntex (Palo Alto, CA, U.S.A.). Methanol, acetonitrile (UV grade), tetrahydrofuran (UV grade) and diethyl ether (distilled-in-glass grade), were obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). 1,4-Dioxane, 1,2-dimethoxyethane, diisopropyl ether and cyclohexene oxide were purchased from Fisher (Pittsburgh, PA, U.S.A.), MCB (Cincinnati, OH, U.S.A.), Mallinckrodt (St. Louis, MO, U.S.A.) and Aldrich (Milwaukee, WI, U.S.A.), respectively.

Apparatus

All HPLC separations were performed with Waters Assoc. (Milford, MA, U.S.A.) equipment. The solvent delivery system consisted of a Model 6000A and two Model 45 pumping systems. Either a Model U6K universal injector or a Model 710B sample processor was used for the injection of samples. The detector was a Model 450 variable-wavelength UV monitor. The flow-rate and solvent composition were controlled by a Model 720 system controller. Peak heights, peak areas and retention times were measured by means of a Model 730 data module. The columns used were

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prepacked with 10-μm or 5-μm LiChrosorb RP-8, containing covalently bonded octylsilane functions (Rheodyne, Cotati, CA, U.S.A.).

High-performance liquid chromatography

Binary solvent mixtures of water–acetonitrile and water–methanol and ternary mixtures of these systems with ethers as modifiers were used as the mobile phases. Mobile phases were degassed in an ultrasonic bath for 30 min. The flow-rates used were 2 ml/min for the 10-μm RP-8 column and 1 ml/min for the 5-μm RP-8 column. Further details are given in the legends to the figures.

RESULTS AND DISCUSSION

Separation of a mixture of estrone, estradiol-17α and estradiol-17β by use of an acetonitrile–water system with or without diethyl ether as a modifier

The conditions employed for this comparison and the results obtained are summarized in Fig. 1. Addition of ether shortened the retention times, improved the resolution, and sharpened the peaks, thus increasing the sensitivity. Without ether, the separation of the epimeric estradiols was incomplete.

Effect of diethyl ether concentration on the elution volumes for estrone, estradiol-17α, estradiol-17β, testosterone and progesterone

The results of this study are summarized in Fig. 2. Elution volumes, expressed as a capacity ratio (k'), are plotted as a function of the volume percent of diethyl ether

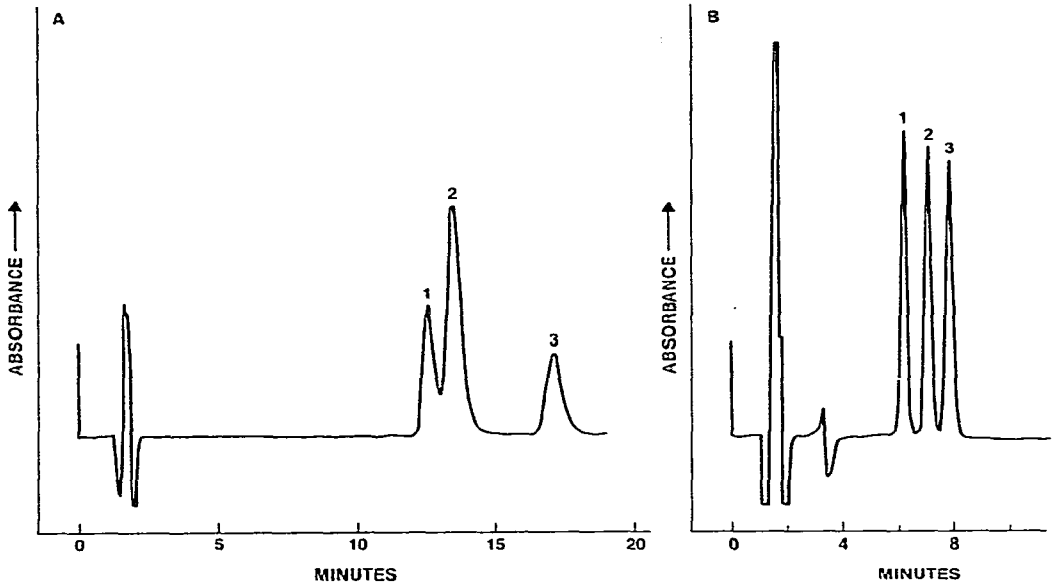

Fig. 1. HPLC of three estrogens with binary and ternary solvent systems. (A) Mobile phase: acetonitrile–water (35:65); (B) Mobile phase:(acetonitrile–water, 35:65)–diethyl ether (90:10, v/v). Column: LiChrosorb RP-8, 10 μm, 25 cm × 4.6 mm I.D.; flow-rate: 2.0 ml/min, pressure: 700 p.s.i.; UV detection at 280 nm, 0.04 a.u.f.s. Peaks: 1 = estradiol-17β; 2 = estradiol-17α; 3 = estrone.

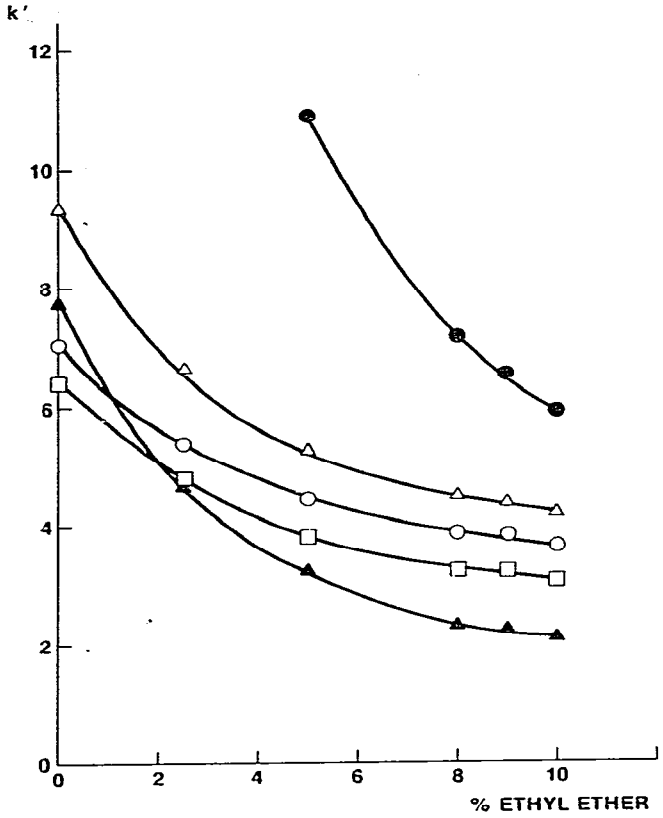

Fig. 2. k' Values of estrogens, testosterone and progesterone as a function of diethyl ether content of the mobile phase. Curves: ▲ = testosterone; □ = estradiol-17β; ○ = estradiol-17α; △ = estrone; ● = progesterone. Column: LiChrosorb RP-8, 10 μm, 25 cm × 4.6 mm I.D.; Solvent system: acetonitrile–water (35:65, v/v) plus diethyl ether.

in the mobile phase. In each case, lower values of k' were obtained when the concentration of diethyl ether was increased. However, the magnitude of the effect was different for different functional groups[1,2]. The retention times of α,β-unsaturated ketonic steroids were more drastically shortened.

Use of diethyl ether and tetrahydrofuran in the separation of ketonic from non-ketonic steroids

In Fig. 3 are plotted the k' values for 17α-ethinylestradiol, mestranol, norethindrone and progesterone as a function of the concentration of acetonitrile in water. Based on the closeness of the k' values for norethindrone and 17α-ethinylestradiol at all concentrations of acetonitrile, it is apparent that this binary system is unsuitable for the separation of these steroids. However, the addition of 10% (v/v) diethyl ether to the eluent containing 40% acetonitrile in water resulted in a lowering of the k' value for norethindrone in comparison with that obtained for 17α-ethinylestradiol and an improvement of the separation. Similarly, the addition of 10% (v/v)

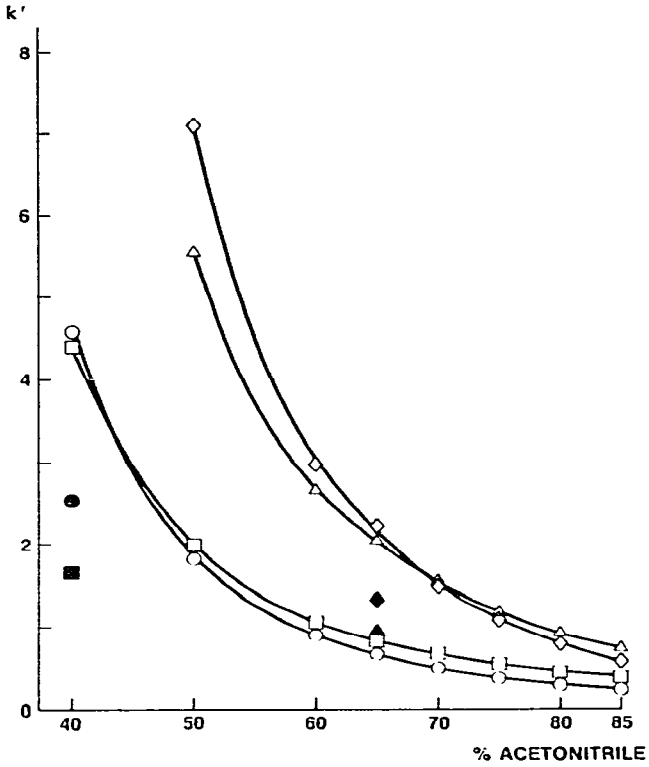

Fig. 3. k' Values of four steroids in binary and ternary solvent systems. Ethinylestradiol (○), norethindrone (□), mestranol (◇), and progesterone (△) were eluted with binary solvent systems of acetonitrile in water, as shown on the horizontal scale. Ethinylestradiol (●) and norethindrone (■) were eluted with a ternary solvent system of (acetonitrile–water, 40:60)-diethyl ether (90:10, v/v). Mestranol (◆) and progesterone (▲) were eluted with a ternary solvent system of (acetonitrile–water, 65:35)–tetrahydrofuran (90:10, v/v). Column: LiChrosorb RP-8, 5 μm, 25 cm × 4.6 mm I.D.

tetrahydrofuran to the eluent containing 65% acetonitrile provided a differential lowering of the k' values for mestranol and progesterone and allowed them to be separated. Although progesterone and mestranol could be separated from one another with a binary system at a lower concentration of acetonitrile, the presence of tetrahydrofuran reduced the elution volumes by at least a factor of 2 and shortened the time required for separation accordingly.

Selectivity effects for steroids of several organic modifiers with ether linkages

Since diethyl ether and tetrahydrofuran had significant effects on the retention behavior of various steroids and these modifiers both contain an ether linkage in their structures, the selectivity effects of other organic modifiers with ether linkages were investigated.

It is evident from earlier reports[8,9] and from our own results that the order of elution of the steroids used in this study depends on whether aqueous acetonitrile or methanol is used as the eluent in reversed-phase HPLC. For example, estradiol-17α

TABLE I

CAPACITY FACTORS (k') AND SELECTIVITY FACTORS (α) OF STEROIDS FOR DIFFERENT ORGANIC MODIFIERS WITH ETHER LINKAGES

	Steroids	*Water–methanol (45:55, v/v)*	*Ternary mixture of water–methanol (40.5:49.5, v/v) with 10 parts of*					*Water–methanol–cyclohexene oxide (44.1:53.9:2, v/v/v)*
			Diethyl ether	*Tetra-hydrofuran*	*Diisopropyl ether*	*1,2-Dimethoxy-ethane*	*1,4-Dioxane*	
Part A: k' values	Estrone	7.88	1.94	2.32	1.79	3.49	3.53	5.27
	Estradiol-17α	9.27	2.19	2.82	1.87	3.91	3.91	6.35
	Estradiol-17β	9.35	2.06	2.65	1.59	3.79	3.77	6.06
	Testosterone	11.3	1.59	2.09	0.56	4.12	4.53	5.47
	Progesterone	23.9	2.97	4.0	1.42	7.82	9.0	10.88
	Norethindrone (NET)	8.65	1.47	1.92	0.52	3.32	3.71	4.59
	17α-Ethinylestradiol (EE)	9.29	2.18	2.82	1.95	3.88	3.82	6.5
Part B: α values	α for NET and EE	1.07	1.48	1.47	3.75	1.17	1.03	1.42

and estradiol-17β are eluted before estrone by the eluent containing acetonitrile and after estrone by the eluent containing methanol. In addition, the order of elution of the two epimeric estradiols is reversed in these systems. However, when various modifiers containing ether linkages are added to both binary systems, the selectivity effect for polar functional groups is similar.

Table I shows the changes in elution volumes resulting from the addition of different ethers to aqueous methanol. Significant changes in elution order are observed. The elution volumes of all steroids are decreased by the use of an ether as a modifier. However, in systems containing diethyl ether, tetrahydrofuran, diisopropyl ether or cyclohexene oxide, the magnitude of the decreases in the elution volumes for steroids with a phenolic structure in the A ring (estrone, estradiol-17α, estradiol-17β and 17α-ethinylestradiol) is smaller than that for steroids containing an α,β-unsaturated ketone in the A ring (testosterone, progesterone and norethindrone). Similar results were obtained (Figs. 2 and 3) with the system containing acetonitrile. An example of polar functional group selectivity is shown in Part B of Table I. The values of the selectivity factor (α) for 17α-ethinylestradiol to norethindrone are significantly larger in the systems containing these monoether modifiers. These two steroids differ only in the A ring which is phenolic in the former and contains an unsaturated ketone in the latter. The role of organic modifiers on the retention mechanism in reversed-phase HPLC has previously been investigated by McCormick and Karger[10,11]. They indicated that polar group selectivities of solutes could in large part be rationalized on the basis of specific solute–modifier interactions in the stationary phase. The greater retardation of phenolic solutes may result from hydrogen bonding to the ethers which are partially extracted by the stationary phase. On a mole-percent basis in the mobile phase, diisopropyl ether, which is more hydrophobic than diethyl ether or tetrahydrofuran, provides the largest selectivity effect of the ethers. A large selectivity effect was also obtained for the relatively hydrophobic epoxide, cyclohexene oxide, which was tested at a concentration of only 2% (v/v) in the mobile phase. The modifiers containing two ether linkages, such as 1,2-dimethoxy ethane and 1,4-dioxane, do not show the differential effect for phenolic and ketonic steroids (Table I). The reduced hydrophobicity in these modifiers compared to monoethers could be responsible for the decreased effect, since it is reasonable that the concentration of these extracted modifiers in the non-polar stationary phase would be lower[10]. If this interpretation is correct, then a good modifier for mobile phases in reversed-phase HPLC must be a compound with a strong hydrophobic region to insure that it is adequately sorbed by the hydrophobic bonded phase, but it must also contain a polar region (such as an ether linkage), which can interact selectively (probably via hydrogen bonding) with certain compounds in the mixture to be separated.

Complete separation of a mixture of testosterone, estriol, estradiol-17β, estradiol-17α, estrone and progesterone with an eluent containing acetonitrile, water and diethyl ether

The conditions used and the separation obtained are shown in Fig. 4. In the absence of diethyl ether, the elution volume for progesterone was excessive and the separation of testosterone from estradiol-17α was marginal. The addition of diethyl ether to the binary mobile phase of water-acetonitrile changed the elution order of these steroids in a way that permitted rapid baseline separation of this complex mixture of steroids.

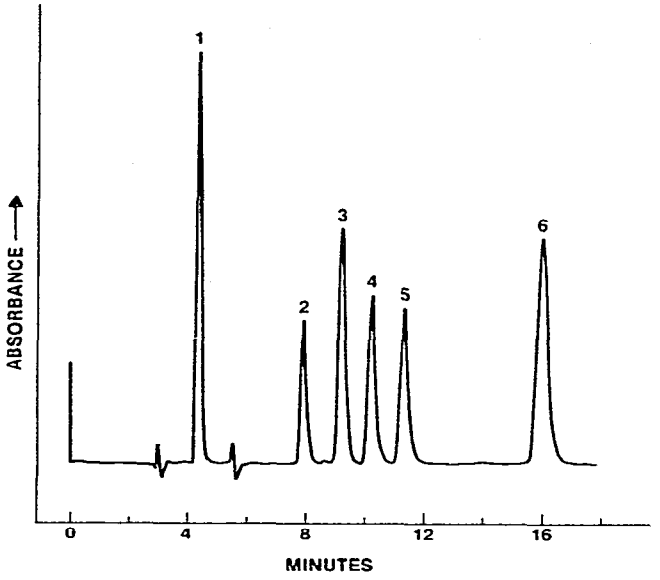

Fig. 4. HPLC separation of estrogens, testosterone and progesterone. Peaks: 1 = estriol; 2 = testosterone; 3 = estradiol-17β; 4 = estradiol-17α; 5 = estrone; 6 = progesterone. Column: LiChrosorb RP-8, 5 μm, 25 cm × 4.6 mm I.D.; Solvent system: (acetonitrile–water, 40:60)–diethyl ether (90:10, v/v); flow-rate: 1.0 ml/min; pressure: 2100 p.s.i.; UV detection at 270 nm, 0.04 a.u.f.s.

REFERENCES

1 S. R. Bakalyar, R. McIlwrick and E. Roggendorf, *J. Chromatogr.*, 142 (1977) 353.
2 N. Tanaka, H. Goodell and B. L. Karger, *J. Chromatogr.*, 158 (1978) 233.
3 Sj. van der Wal and J. F. K. Huber, *J. Chromatogr.*, 149 (1978) 431.
4 R. Spatz and E. Roggendorf, *Z. Anal. Chem.*, 299 (1979) 267.
5 F. Klink, *Amer. Lab.*, 12, No. 5 (1980) 81.
6 E. Roggendorf and R. Spatz, *Chromatography Review (Spectra-Physics)*, 6 (1980) 10.
7 C. D. Hathaway, *Amer. Lab.*, 12, No. 10 (1980) 134.
8 R. J. Tscherne and G. Capitano, *J. Chromatogr.*, 136 (1977) 337.
9 R. W. Roos, *J. Ass. Offic. Anal. Chem.*, 63 (1980) 80.
10 R. M. McCormick and B. L. Karger, *J. Chromatogr.*, 199 (1980) 259.
11 R. M. McCormick and B. L. Karger, *Anal. Chem.*, 52 (1980) 2249.