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Note

High-pressure liquid chromatographic separation of pharmaceutical compounds using a mobile phase containing silver nitrate

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Use of impregnated supports for the separation of closely related compounds has been reported for thin-layer chromatographic (TLC) systems[1]. Application of some of these TLC techniques has been extended to high-pressure liquid chromatography (HPLC). An argentated silica gel stationary phase has been used for the separation of *o*-, *m*- and *p*-picoline[2]. Janák *et al.*[3] have reported the use of a porous polyhydrocarbon stationary phase (Porapak Q) and a mobile phase containing silver nitrate. Very recently, Schomburg and Zegarski[4] have reported the use of an argentated mobile phase with a reversed-phase partition system for the separation of geometrical isomers of 2-alkenes and 1,5,9-cyclododecatriene, as well as for the separation of oleic and elaidic acid methyl esters.

Application of this technique for the differentiation of compounds of pharmaceutical importance was realized in this laboratory. Very similar compounds were found to be affected differentially by the presence of silver nitrate in the mobile phase. Vitamins D_2 and D_3, as well as various estrogenic compounds, were examined. Some compounds that were either only partially or totally unresolved from chromatographically similar species, were completely separated when using a mobile phase containing silver nitrate.

EXPERIMENTAL

All mobile phases were prepared using certified A.C.S. grade methanol (Fisher Scientific, Fair Lawn, N.J., U.S.A.) and demineralized water. Reagent A.C.S. grade silver nitrate was purchased from Allied Chemical (Morristown, N.J., U.S.A.). All mobile phases containing silver nitrate were shielded from light using low-actinic glassware.

A Milton Roy Mini-Pump Model 396/2396 (Laboratory Data Control, Riviera Beach, Fla., U.S.A.) was used in conjunction with a 30 cm × 4 mm I.D. μBondapak/C_{18} chromatographic column (Waters Assoc., Milford, Mass., U.S.A.). Sample introduction into a DuPont Instruments Model 830 liquid chromatograph injection port was made using a 5-μl Hamilton HP305 syringe. A DuPont Model 835 filter photometer (DuPont Instruments, Wilmington, Del., U.S.A.) and a Linear Instruments Model 252A recorder (Irvine, Calif., U.S.A.) were used. Because of the high absorbance of mobile phases containing silver nitrate, the recorder pen was electronically offset using the photometer balance and recorder zero adjustments.

Reference standard vitamins D_2 and D_3 were purchased from the United States Pharmacopoeia (Rockville, Md., U.S.A.). Estrogen samples were obtained from a variety of suppliers. All sample solutions were prepared to be approximately 1 mg/ml in methanol.

RESULTS AND DISCUSSION

In the past, chromatographers have shown the partial separation of vitamin D_2 from vitamin D_3 (ref. 5), as well as the separation of various estrogenic compounds[6–8] from each other using HPLC. Structures for some of these compounds are shown in Fig. 1. In many cases, these separations are found to be difficult to reproduce. The use of an argentated reversed-phase partition system is recommended as an alternative method for the separation of these groups of compounds.

Chromatograms showing a vitamin D_2–vitamin D_3 separation using a mobile phase both with and without silver nitrate addition are shown in Fig. 2. An essentially complete separation of vitamin D_2 from vitamin D_3 was accomplished in less than 35 min (Fig. 3). Table I relates the effect of silver nitrate concentration to the retention

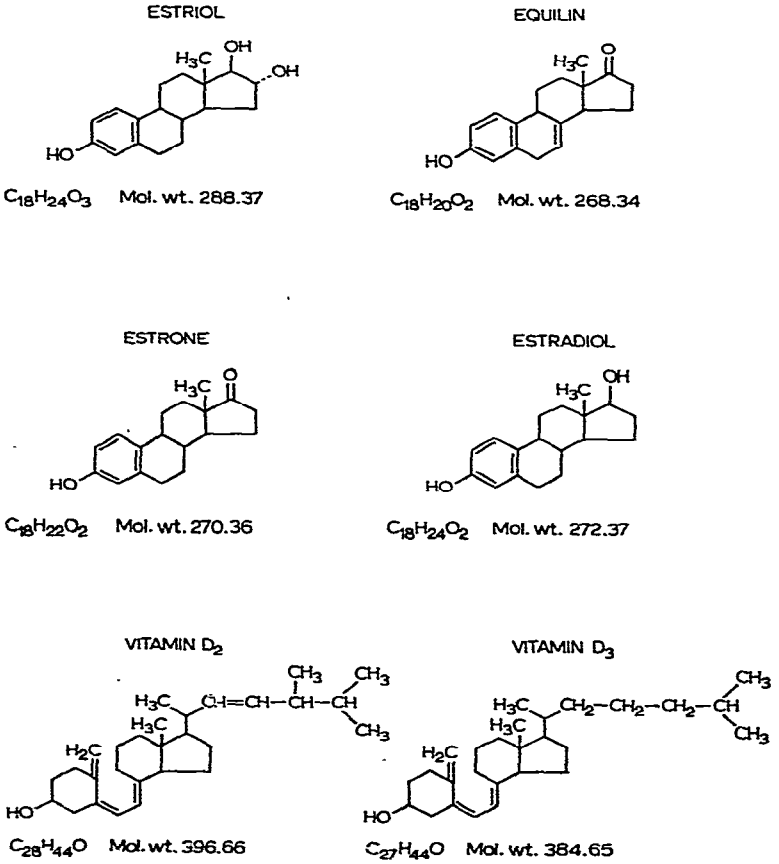

Fig. 1. Structures of various vitamin D and estrogenic compounds.

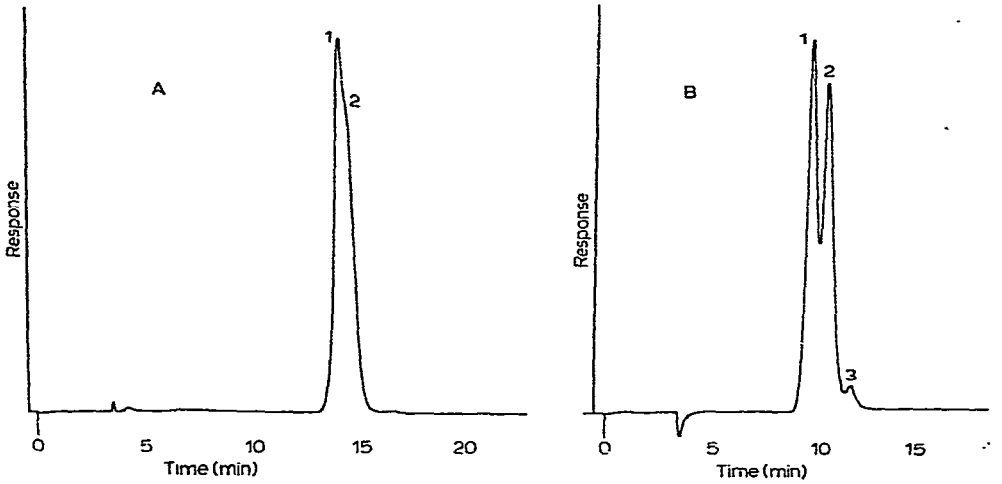

Fig. 2. HPLC separation of vitamin D compounds using a μBondapak/C_{18} column (30 cm × 4 mm I.D.). (A) Mobile phase, 95 ml methanol + 5 ml water; flow-rate, 0.8 ml/min. (B) Mobile phase, 95 ml methanol + 5 ml water + 2 g silver nitrate; flow-rate, 0.8 ml/min. 1 = Vitamin D_2; 2 = vitamin D_3; 3 = impurity.

time of each component. From a study of this set of data, it is evident that the role of the silver nitrate is to allow π-complexation with available sites on these molecules. Both the retention times of vitamin D_2 and vitamin D_3 are noticeably decreased on the addition of silver nitrate to the mobile phase because each of these compounds

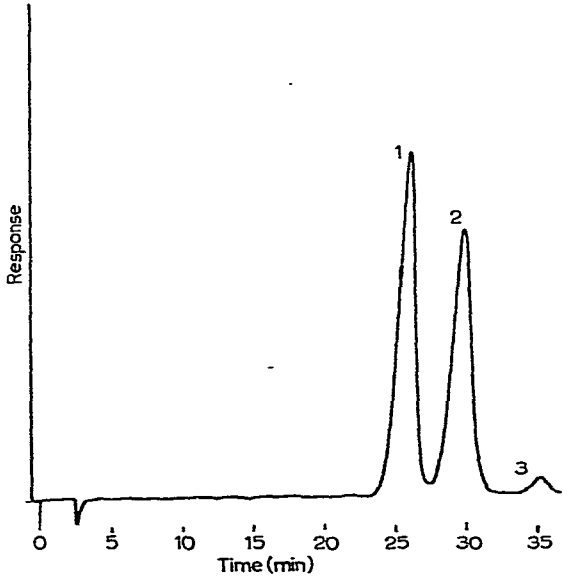

Fig. 3. HPLC separation of vitamin D_2 from vitamin D_3. Column, μBondapak/C_{18}, 30 cm × 4 mm I.D.; mobile phase, 86.5 ml methanol + 13.5 ml water + 2.4 g silver nitrate; flow-rate, 1.1 ml/min. 1 = Vitamin D_2; 2 = vitamin D_3; 3 = impurity.

TABLE I

RETENTION TIMES FOR VITAMIN D_2 AND VITAMIN D_3 IN THE PRESENCE OF VARYING AMOUNTS OF SILVER NITRATE

All runs were made using the following conditions: column, μ-Bondapak/C_{18}; mobile phase, methanol–water (95:5) + varying amounts of silver nitrate; detector, UV at 254 nm; flow-rate, 0.8 ml/min.

Grams of $AgNO_3$/500 ml of mobile phase	*Retention time (min)*	
	Vitamin D_2	*Vitamin D_3*
0	14.3	14.5
2.53	12.5	13.0
5.00	11.6	12.3
7.50	10.3	11.1
10.01	10.0	10.6

has the ability to π-complex with the silver atom. Vitamin D_2, however, has an additional complexation site on the side chain, which provides the main basis for the separation of vitamin D_2 from vitamin D_3.

Chromatograms showing the separation of estriol, equilin, estrone and estradiol both before and after argentation of the mobile phase are shown in Fig. 4. Addition of 20 g/l of silver nitrate resulted in a retention time decrease of 6 min for

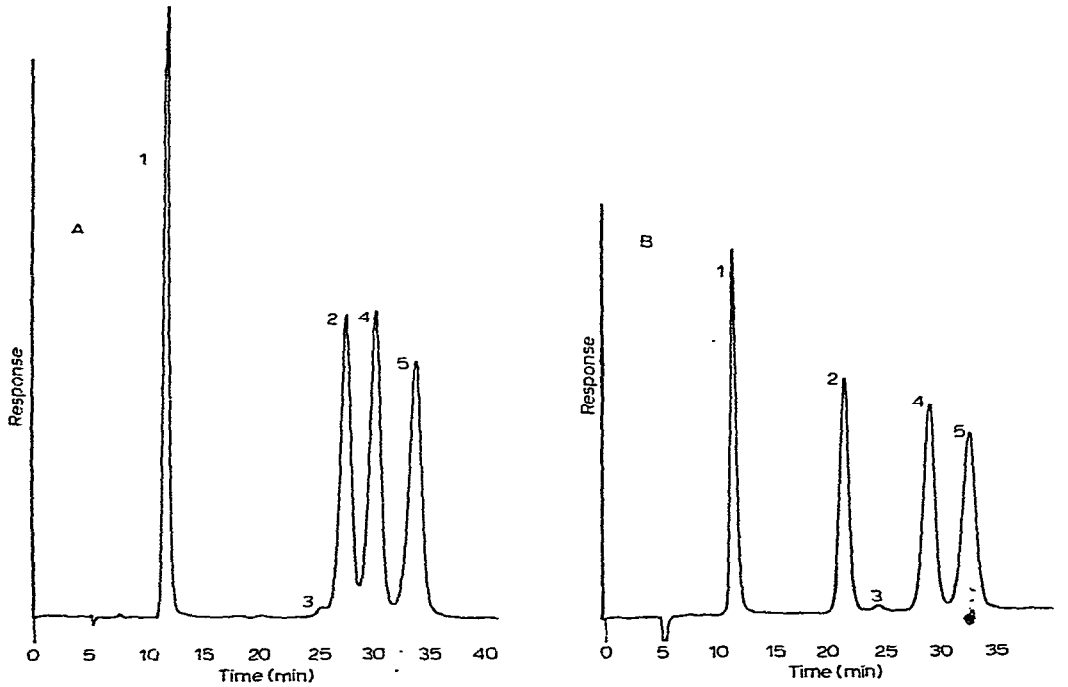

Fig. 4. HPLC separation of estrogenic compounds using a μBondapak/C_{18} column. (A) Mobile phase, 60 ml methanol + 40 ml water; flow-rate, 0.55 ml/min. (B) Mobile phase, 60 ml methanol + 40 ml water + 2 g silver nitrate; flow-rate, 0.55 ml/min. 1 = Estriol; 2 = equilin; 3 = impurity; 4 = estrone; 5 = estradiol.

the equilin compound, while the retention times for estriol, estrone, and estradiol were virtually unchanged. In this case, the double bond between No. 7 and No. 8 carbon atoms in the equilin molecule allows for π-complexation with the silver atom. Because of this phenomenon, the retention time of equilin could be decreased almost independently of the other compounds simply by silver nitrate addition to the mobile phase (Table II).

TABLE II

RETENTION TIMES FOR ESTROGENS IN THE PRESENCE OF VARYING AMOUNTS OF SILVER NITRATE

All runs were made using the following conditions: column, μ-Bondapak/C_{18}; mobile phase, methanol–water (60:40) + varying amounts of silver nitrate; detection, UV at 280 nm; flow-rate, 0.8 ml/min.

Grams of $AgNO_3$/500 ml of mobile phase	*Retention time (min)*			
	Estriol	*Equilin*	*Estrone*	*Estradiol*
0	11.8	27.8	30.7	34.1
2.48	11.7	25.7	30.0	33.7
5.46	11.6	23.9	29.6	33.2
7.60	11.7	22.7	29.3	32.9
10.12	11.7	21.7	29.7	33.3

Complete reproducibility of all systems using silver nitrate was observed. To prevent metallic silver build-up, each evening a water–methanol (50:50) mobile phase was used to thoroughly flush excess silver nitrate from the system. Even so, a small amount of metallic silver build-up was observed on the inner walls of the tubing over a period of time. Rinsing with dilute nitric acid or replacement of the tubing itself was used to remedy this situation.

By no means is the use of this argentation technique expected to be the answer to all separation problems of this type, but it is an alternative. The versatility of the system is shown in the ability to completely regenerate the μBondapak/C_{18} column back to the state in which it was prior to the use of the argentated mobile phase. Applicability of this technique of adding metal ions to the mobile phase should not stop with these two series of compounds, but should be expanded for use on a wide variety of compounds with possibly other metal ions as complexing agents, such as mercury and nickel salts.

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