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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF 15-METHYL $PGF_{2\alpha}$ METHYL ESTER ISOMERS

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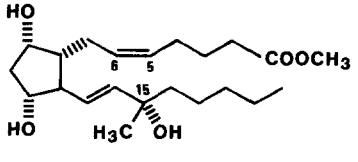
SUMMARY

A normal-phase high-performance liquid chromatography system was developed to separate the prostaglandin, 15(*S*)-15-methyl $PGF_{2\alpha}$ methyl ester, from its 5,6-*trans* isomer and 15(*R*)-epimer. Several other related prostaglandins are resolved from their 5,6-*trans* isomers on silica columns after esterification of the free acid with a large chromophore. Similar derivatization is not possible with the methyl esters. Detection at 214 nm was therefore utilized, although it severely restricted solvent choices for the mobile phase. Resolution was achieved on a Zorbax-CN column with a hexane–dioxane–water mobile phase. Water plays an important role in the mobile phase. The concentration of water, soluble to 0.2%, has a unique effect on the retention behavior of these prostaglandins. A plot of water concentration *versus* capacity factor (k') increases approximately exponentially as the water concentration approaches saturation. At the same time, the number of theoretical plates also greatly increases. The resolution of the isomers was concluded to depend on the formation of an effective stationary phase formed from the interaction of the mobile phase with the bonded cyanopropyl group. Highly adsorptive sites on the stationary phase were masked, resulting in a large increase in efficiency.

INTRODUCTION

Several high-performance liquid chromatography (HPLC) systems have been developed to separate isomers of $F_{2\alpha}$ type prostaglandins. Epimers involving the



METHYL CARBOPROST
15(S)-15-METHYL $PGF_{2\alpha}$ METHYL ESTER

Fig. 1. Structure of methyl carboprost.

polar hydroxyls are easily separated. Isomers in the orientation of the 5,6-double bond have proven to be more difficult to separate (Fig. 1). Several assays have been developed which separate the 5,6-*trans* isomer from the natural *cis*-isomer of several related $PGF_{2\alpha}$ type prostaglandins[1–3]. In all of these assays, the prostaglandins existed as the free acids and were esterified with a bulky chromophore to enable detection at 254 nm. Morozowich and Douglas[1] developed two systems to resolve the isomers of $PGF_{2\alpha}$. They used two silica columns in series to separate the 5,6-*trans* isomer from $PGF_{2\alpha}$ *p*-nitrophenacyl ester. Merritt and Bronson[2] used a silver-loaded ion-exchange column also to separate the isomers of $PGF_{2\alpha}$ *p*-nitrophenacyl ester. Brown and Carpenter[3] separated the 5,6-*trans* isomer and the 15(*R*)-epimer from carboprost [15(*S*)-15-methyl $PGF_{2\alpha}$] as the naphthacyl esters.

Since methyl carboprost, 15(*S*)-15-methyl $PGF_{2\alpha}$ methyl ester, is already esterified, derivatization and separation by the previous methods was not feasible. A different method for achieving the desired selectivity was therefore required. The goal of this work was to develop a method which would separate the underivatized compounds. The use of a hexane–dioxane–water mobile phase with a Zorbax-CN column was found to produce the desired selectivity within the constraints of a mobile phase of low UV absorbance. The water concentration was found to have a critical effect on the desired resolution.

Mobile phase components are known to extract into and become part of the bonded stationary phase[4–6] as originally proposed by Knox and Pryde[7]. The bonded stationary phase, including the extracted mobile phase components, is referred to as the effective stationary phase. Quantitative measurements of the concentrations of the common organic modifiers (methanol, acetonitrile, and tetrahydrofuran) in alkyl bonded phases have been reported by a number of investigators[8–14]. Water has also been determined in alkyl and alkylamine bonded phases[10,13–15].

The extent to which the effective stationary phase influences the chromatographic process is not well-defined. The distribution of ion-pair reagents into an effective stationary phase is central to current theory of retention in ion-pair chromatography[16,17] but is difficult to account for quantitatively. Chiral reagents in the stationary phase have been shown to be responsible for the selectivity among amino acid enantiomers[18]. McCormick and Karger[11] demonstrated that small amounts of a third solvent in the mobile phase could have a great effect on the selectivity of a system for solutes of varying polarities. They attributed the magnitude of the selectivity differences to the effective stationary phase formed by the distribution of the ternary solvent into the bonded phase. Colin *et al.*[19] also have reported on the role of the stationary phase in selectivity. Schoenmakers *et al.*[20] suggested that predictions of retention in gradient elution can be improved if the modification of the stationary phase by the mobile phase is taken into account. In general, however, the role of the effective stationary phase in retention is considered to be insignificant compared to the mobile phase[5,6].

In this paper the retention characteristics of 15-methyl $PGF_{2\alpha}$ methyl ester isomers are shown to be greatly influenced by the effective stationary phase formed with water extracted from the mobile phase.

EXPERIMENTAL

Instrumentation

The component high-performance liquid chromatograph used for the separation consisted of a pump (Altex Model 110A, Beckman, Berkeley, CA, U.S.A.), a fixed loop injector (Rheodyne Model 7125, Berkeley, CA, U.S.A.), a detector fitted with a 214 nm filter and a zinc lamp (Laboratory Data Control, Riveria, FL, U.S.A) and a recorder (Sargent Welch Model XKR, Detroit, MI, U.S.A.). The flow-rate was 1.5 ml/min. The injection volume was 20 μl. The detector and recorder were set for 0.1 absorbance units full scale. Integration was completed on an in-house computer system.

Columns

Four columns were used: a Zorbax-SIL, a Zorbax-CN, a Zorbax-TMS (DuPont, Wilmington, DE, U.S.A.), and a μPorasil (Waters, Milford, MA, U.S.A.). The Zorbax columns were 250 × 4.6 mm I.D. The μPorasil column was 300 × 4.6 mm I.D. The nominal particle size for the Zorbax packings was 6 μm. The particle size of the μPorasil packing was 10 μm. The Zorbax-CN column is made from a bonding of a cyanopropyl moiety to Zorbax silica. The Zorbax-TMS is made from the bonding of trimethylsilane groups to Zorbax silica.

Reagents and samples

All solvents used were distilled-in-glass grade (Burdick & Jackson, Muskegon, MI, U.S.A.). Samples of 15(*S*)-15-methyl $PGF_{2\alpha}$ methyl ester, 15(*R*)-15-methyl $PGF_{2\alpha}$ methyl ester and the 5,6-*trans* isomer of 15(*S*)-15-methyl $PGF_{2\alpha}$ methyl ester were obtained from the research laboratories of The Upjohn Company.

Sample preparation

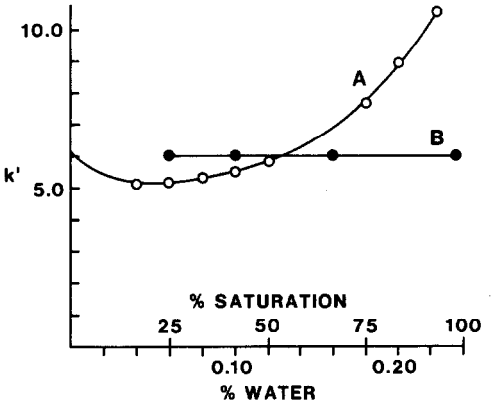
Methyl carboprost (*ca.* 6 mg) was dissolved in 5.0 ml of either internal standard solution or mobile phase. The internal standard solution was medroxyprogesterone acetate made up in mobile phase at a concentration of 0.050 mg/ml.

RESULTS AND DISCUSSION

Chromatographic system

In most prostaglandin analyses, the acid is derivatized to the naphthacyl or the *p*-nitrophenacyl esters before the separation. This allows for sensitive detection at 254 nm with equivalent responses for different prostaglandins. Methyl carboprost, already a methyl ester, cannot be directly derivatized at the carboxyl group and has only limited UV absorption below 220 nm with a maximum at 202 nm. At these wavelengths, solvent selection for the mobile phase becomes very limited. Miscibility problems further restrict the number of solvent choices.

Normal-phase chromatography was needed to distinguish the small steric differences between the isomers, especially in the carbon skeleton. These constraints led to the investigation of a mobile phase containing selections of hexane, 2-propanol, acetonitrile and water mixtures. Initial work on silica columns showed improvements in the number of theoretical plates as polar solvents such as acetonitrile were added.



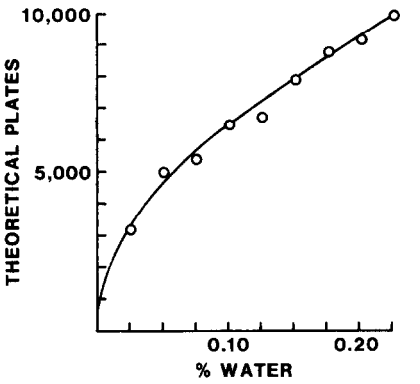


Fig. 2. Capacity factor for methyl carboprost (A) and medroxyprogesterone acetate (B) with a mobile phase of 75% hexane, 25% dioxane and various amounts of water with a Zorbax-CN column.

Fig. 3. Theoretical plates for methyl carboprost with a mobile phase of 75% hexane and 25% dioxane and various amounts of water up to saturation using a Zorbax-CN column.

Only small amounts of acetonitrile were soluble in hexane without adding a less polar solvent such as 2-propanol which then decreased retention and resolution. Switching to the less polar Zorbax-CN column produced an improvement in the efficiency of the separation and the use of dioxane instead of acetonitrile further improved the resolution. Because polar modifiers improved the resolution, the effect of water on the separation was investigated using a mobile phase containing 75% hexane and 25% dioxane. The solubility of water in the mobile phase was *ca.* 0.22% and increased with larger concentrations of dioxane. As the water concentration was increased from 0 to 100% of saturation the capacity factor decreased initially and then increased to twice the initial value (Fig. 2). Over the same range of water concentration the number of theoretical plates increased from 500 to 10,000 plates (Fig. 3). A summary of the chromatographic parameters obtained with the optimum mobile phase is given in Table I. A chromatogram showing the separation of the three isomers with the optimized mobile phase is shown in Fig. 4.

Linearity and precision

The assay was shown to be linear for samples between 0.5 and 2 mg/ml by both peaks heights and peak areas relative to the internal standard (correlation coef-

TABLE I

CALCULATED CHROMATOGRAPHIC PARAMETERS FOR OPTIMIZED SEPARATION

Compound	*Retention time (min)*	*k'*	*Resolution*
Medroxyprogesterone acetate	10.8	6.2	11.5
15-(*R*) Epimer	17.1	9.8	1.65
Methyl carboprost	18.2	10.4	
5,6-*trans* Isomer	19.5	11.3	1.95

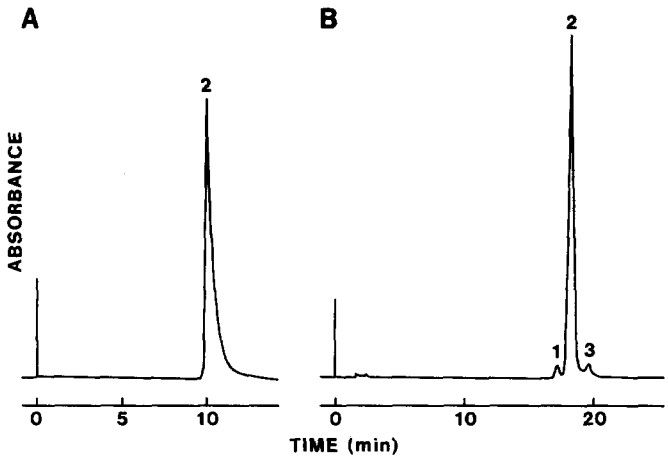


Fig. 4. Chromatograms of methyl carboprost isomers (peaks 1 = 15-(*R*) epimer; 2 = methyl carboprost; 3 = 5,6-*trans* isomer) with Zorbax-CN column and a mobile phase of (A) 75% hexane and 25% dioxane and (B) 75% hexane, 25% dioxane and 0.2% water.

ficient greater than 0.999). Precision data for six separate sample preparations gave a 0.5% relative standard deviation (R.S.D.) by peak heights and a 0.7% R.S.D. by peak areas.

Detection limit

Detection was at 214 nm using a zinc lamp because it was advantageous to have the line source detector when normalizing response factors to methyl carboprost's side band absorption. The UV cutoff for the mobile phase was *ca.* 210 nm. The detection limit at 214 nm with a signal-to-noise ratio of 3 was found to be 20 ng on-column or equivalent to a 1 μg/ml solution. The detection limit for integrating either of the isomers on the side of the methyl carboprost peak was slightly higher at 50 ng on-column. This is equivalent to 0.2% of either isomer in 1.2 mg/ml sample. Sensitivity can be increased by using lower wavelengths on a variable-wavelength detector or injecting more than 20 μl.

Factors affecting resolution

The retention of these prostaglandins under the optimized chromatographic conditions was highly dependent on the concentration of water in the mobile phase. Retention increased with increasing water concentration (Fig. 2). Efficiency increased substantially as saturation of the mobile phase with water was approached (Fig. 3). The improvement in efficiency is expected from the effect of water on the unmodified silica surface. Initially, with no water in the mobile phase, the prostaglandins are subject to strong interactions with a small concentration of highly adsorptive sites, presumably silanols, in the stationary phase. Retention with poor efficiency is observed (Fig. 4). As the water concentration increases in the mobile phase, water becomes an integral part of the stationary phase[10,13–15]. As the highly adsorptive sites are masked by the water a uniformly polar stationary phase begins to form. The equalization of the strengths of the interactions of 15-methyl $PGF_{2\alpha}$ methyl esters

with the stationary phase leads to a greater efficiency as measured by the number of theoretical plates (Fig. 4). For the retention to increase as shown in Fig. 2, the concentration of water in the stationary phase must be greater than that in the mobile phase. The dependence of retention and efficiency on water concentration indicates that the hydroxyl groups on the prostaglandins play a major role in the interaction with the mobile and stationary phase. The internal standard, medroxyprogesterone acetate, is structurally incapable of interacting through hydroxyl groups like the prostaglandins, so its retention is unaffected by the relative water concentrations in the mobile and stationary phases.

The relative contributions of the mobile phase and stationary phase to the resolution of the isomers were investigated by using the same mobile phase with columns of relatively greater and lesser polarity (*i.e.* silica and Zorbax-TMS columns). The chromatograms for the isomers using the silica and Zorbax-TMS columns are shown in Fig. 5. The isomers are separated on the silica column although the retention has increased 150% over the Zorbax-CN column and the efficiency of the separation is significantly less than for the Zorbax-CN column. Also, a silica column with significantly less capacity had to be used (10-μm packing, Waters μPorasil). With the silica column a more polar effective stationary phase is established but the interaction of the isomers with the silica column is not as efficient as that for the Zorbax-CN column presumably because of incomplete deactivation of the highly adsorptive sites. With the Zorbax-TMS column, the 15-*R* epimer was not resolved from the 15-(*S*) isomer and the 5,6-*trans* isomer was only poorly resolved. Retention was *ca.* 75% of that obtained with the cyanopropyl column. For the TMS column, there is no polar functionality (equivalent to that of Zorbax-CN column) to serve as an anchor for the formation of an effective stationary phase. As a result, the Zorbax-TMS stationary phase did not provide the specific interactions necessary for resolution of methyl carboprost and its isomers. Optimum resolution of the isomers

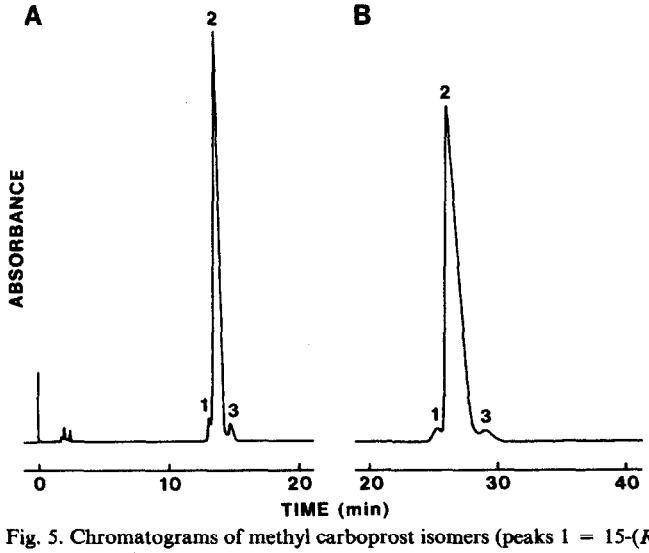


Fig. 5. Chromatograms of methyl carboprost isomers (peaks 1 = 15-(*R*) epimer; 2 = methyl carboprost; 3 = 5,6-*trans* isomers; concentrations of 1 and 3 are at 3% of 2 with a mobile phase of 75% hexane, 25% dioxane and 0.2% water with (A) Zorbax-TMS column and (B) μPorasil column.

was a result of both proper selection of the stationary phase and further modification of the effective stationary phase by the mobile phase.

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