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Separation of Beraprost sodium isomers using different cyclodextrin stationary phases

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ABSTRACT

The separation of isomers present in a new prostacyclin analogue (Beraprost sodium) on several different cyclodextrin stationary phases was investigated. Beraprost sodium, which contains six chiral centers, is a mixture of four isomers; two diastereomers each containing a pair of enantiomers. The cyclodextrin stationary phases used included: α-, β- and γ-cyclodextrins as well as several derivatized β-cyclodextrins. The mobile phase variables that were found to affect the chiral separation were: type and concentration of organic modifier, type and concentration of buffer, the cation and anion associated with the buffer, mobile phase pH, preparation of the mobile phase, and column temperature. The cyclodextrin stationary phases that were found to separate all four isomers were: the acetylated β-cyclodextrin (Cyclobond I AC) and the *para*-toluoyl ester-derivatized β-cyclodextrin (Cyclobond I PT). The selectivities of the isomers were better on the Cyclobond I AC column than on the Cyclobond I PT column. Therefore the Cyclobond I AC column was used to determine what effect each mobile phase variable had on isomer retention and resolution. Calibration curves were done for the four isomers and the correlation coefficients determined using UV detection. Plots of log peak area *versus* concentration of each isomer provided correlation coefficients of 0.998 or better. Detection limits of 75 ng/ml and quantitation limits of 500 ng/ml of each isomer were found.

INTRODUCTION

Cyclodextrins have been described as chiral, doughnut-shaped molecules composed of multiple glucose residues that are connected end-to-end in closed rings via α-1,4-glycosidic linkages [1,2]. The glucose residues are locked in the chair conformation and its orientation is constrained by the cyclic linkage so that the esterified oxygens and 3,5-hydrogens line the hole of the doughnut which creates a relatively hydrophobic core. Hydroxyl groups on the second, third and fifth carbon atoms of each glucose residue face the edges and outside of the doughnut. These hydroxyl groups produces a relatively hydrophilic rim and outer surface. Each of the glucose residues contains five carbons that are chiral. Hydrophobic molecules can bind to the cyclodextrins by entering the central hydrophobic cavity to form "inclusion complexes". Retention of the molecule depends on its size and any substituent groups that are on the molecule. The molecule must fit into the cyclodextrin cavity in order to be retained and separated from an enantiomer [1,2]. Interactions between the secondary hydroxyl groups and the enantiomers must take place for resolution to occur. Differences in the stability of the inclusion complexes that are formed for each isomer must also be great enough to allow the enantiomers to be resolved [3].

Prostacylin (PGI_2), which was discovered in 1976 [4], was found to be an inhibitor of platelet aggregation and had potent vasodilating properties [5]. This has lead to investigating its use for atherosclerosis [6], diabetes [7] and uremia [8]. The major problem with PGI_2 has been its instability in acidic or neutral aqueous solutions. The half-life of PGI_2 in an aqueous solution at pH 7.0 and 25°C is about 2 min [9]. Since PGI_2 is unstable, research has been on-

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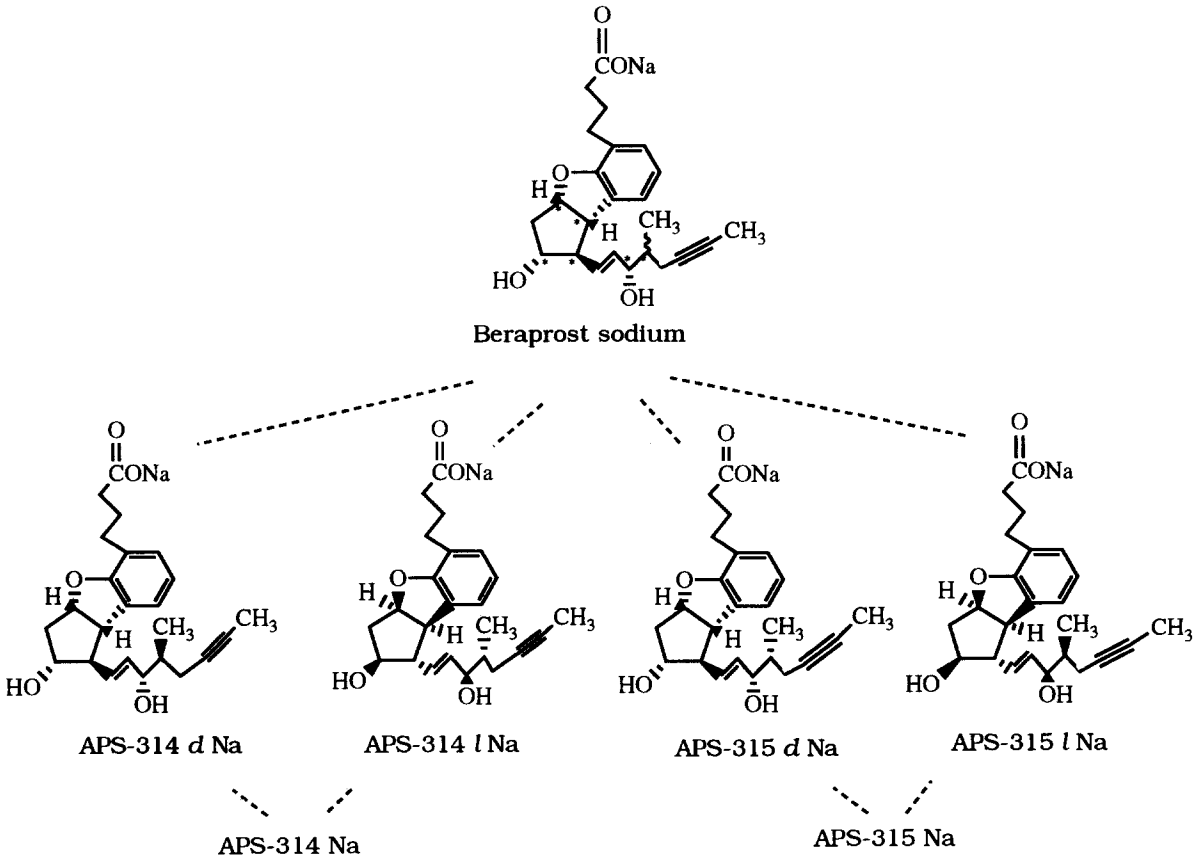

Fig. 1. The chemical structure of Beraprost sodium and the four isomers.

going to produce an analog that is more stable yet maintains the same anti-platelet and vasodilating properties.

The compound used in this study, Beraprost sodium {Sodium (1*R**,2*R**,3*aS**,8*bS**)-2,3,3*a*,8*b*-tetrahydro-2-hydroxy-1-[(*E*)-(3*S**,4*RS*)-3-hydroxy-4-methyl-1-octen-6-ynyl]-1*H*-cyclopenta[*b*]benzofuran-5-butyrate}, is a prostacylin analogue that is significantly more stable in aqueous solutions and maintains the same properties as those found for the orginal prostacylin [10–14]. Beraprost sodium (Fig. 1) is composed of two diastereomers with each diastereomer containing a pair of enantiomers. The ability to separate and quantitate the isomers present in a racemic mixture is important in pharmaceutical products, especially in terms of both bulk drug and formulation stability and purity [15].

This paper will discuss the separation of the isomers present in Beraprost sodium using different cyclodextrin stationary phases. The two stationary phases that were able to separate all four isomers were the acetylated β-cyclodextrin (Astec Cyclobond I AC) and the *para*-toluoyl ester-derivatized β-cyclodextrin (Astec Cyclobond I PT). The mobile phase parameters that had an affect on the chiral separation were identified and studied. The acetylated β-cyclodextrin stationary phase was used to determine the effect that the different mobile phase variables had on the separation of the isomers and was also used to optimize the Beraprost sodium isomer separation.

EXPERIMENTAL

Instrumentation

The instrumentation used in this study includes a

Spectra-Physics IsoChrom HPLC pump, Spectra-Physics Model 8810 autosampler, Spectra-Physics Model 100 variable-wavelength UV-visible detector, and a Spectra-Physics ChromJet integrator. The stationary phases that were studied included: a 250 × 4.6 mm I.D. Astec Cyclobond I AC column (acetylated β-cyclodextrin), a 250 × 4.6 mm I.D. Astec Cyclobond I column (β-cyclodextrin), a 250 × 4.6 mm I.D. Astec Cyclobond I RSP column (*R,S*-hydroxypropyl ether substituent), a 250 × 4.6 mm I.S. Astec Cyclobond I SN column (*S*-naphthylethyl carbamate substituent), a 250 × 4.6 mm I.D. Astec Cyclobond I RN column (*R*-naphthylethyl carbamate substituent), a 250 × 4.6 mm I.D. Astec Cyclobond I DMP column (3,5-dimethylphenyl carbamate substituent), a 250 × 4.6 mm I.D. Astec Cyclobond I PT column (*para*-toluoyl ester substituent), a 250 × 4.6 mm I.D. Astec Cyclobond III (α-cyclodextrin) and a 250 × 4.6 mm I.D. Astec Cyclobond II column (γ-cyclodextrin) all purchased from Astec (Whippany, NJ, USA). Injection sizes of 50 μl were used for all of the studies except for the calibration data where 100-μl injections were used. A 1.0 ml/min flow-rate was used for all studies. A detection wavelength of 282 nm was used throughout the study.

Chemicals

Trimethylamine, tripropylamine, tributylamine and triethanolamine were purchased from Aldrich (St. Louis, MO, USA). Ammonium hydroxide, triethylamine, acetic acid, formic acid, nitric acid, sulfuric acid and tartaric acid were purchased from Mallinckrodt (Paris, KY, USA). HPLC-grade acetonitrile, methanol, ethanol and isopropanol were purchased from Burdick & Jackson (Muskegon, MI, USA).

RESULTS AND DISCUSSION

The major mobile phase parameters that were found to affect isomer retention and selectivity on the acetylated β-cyclodextrin column were: preparation of the mobile phase, type and concentration of organic modifier, type and concentration of buffer, mobile phase pH, type of cation and the type of

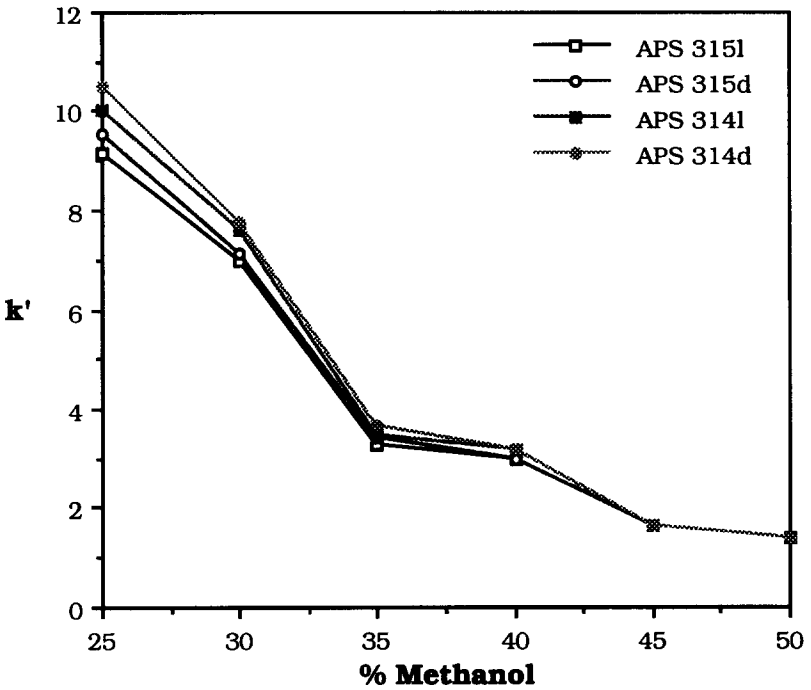

Fig. 2. The effect of methanol concentration using a mobile phase composed of 0.05% triethylamine (v/v), pH 3.5 adjusted with acetic acid.

anion present in the mobile phase, and column temperature. Each of these mobile phase parameters was studied and the effect that each had on the separation and selectivity of the Beraprost sodium isomers was determined.

Mobile phase preparation

Preparation of the mobile phase was found to influence the retention and resolution of the Beraprost sodium isomers. The mobile phase must be prepared so that the base (*i.e.*, triethylamine) is accurately measured first and then the buffer pH adjusted with an appropriate acid (*i.e.*, acetic acid). The isomer separation was not successful if an acid was measured out and then the buffer pH adjusted with a base. All of the studies done were prepared with the base being accurately measured out and then the pH of the buffer solution adjusted using an appropriate acid.

Organic modifier

Retention and resolution of the isomers were found to decrease as the concentration of organic modifier was increased. Methanol provided the best separation for the four isomers while very little or no resolution of the isomers was observed when stronger organic modifiers, such as acetonitrile, ethanol and isopropanol were used. Fig. 2 shows the relationship between the concentration of methanol and isomer retention. Resolution of the isomers was the best when the mobile phase was composed of 25% methanol. Higher concentrations of methanol resulted in loss of resolution. The isomers were more highly retained at lower concentrations of methanol, however, signicant band broadening and poor resolution occurred.

Mobile phase pH

The β-cyclodextrin stationary phases have a pH range of 3.5 to 7.0. If the mobile phase pH is lower than 3.5 or higher than 7.0, column life can rapidly decrease. Fig. 3 shows the effect that mobile phase pH had on isomer retention. At pH 5.0, the diastereomers were separated, however the enantiomers were not resolved whereas at pH 3.5 the enantiomers were resolved although not baseline re-

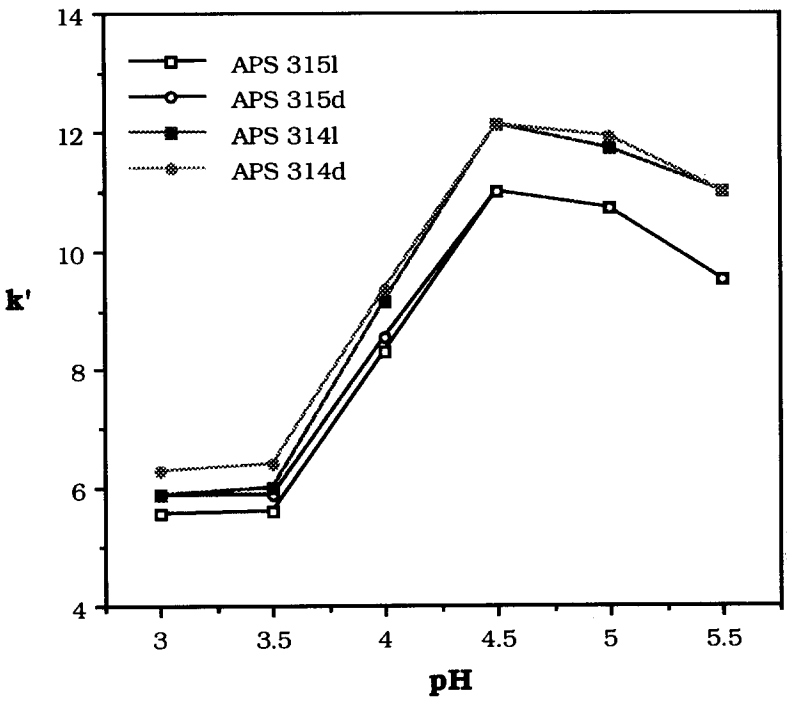

Fig. 3. The effect of mobile phase pH using a mobile phase composed of 0.05% triethylamine (v/v), the pH adjusted with acetic acid and 30% methanol.

solved. It is interesting to note that as the mobile phase pH was increased, resolution between the diastereomers increased while resolution between the enantiomers decreased.

Buffer concentration

Table I shows the results that were obtained when the concentration of buffer (triethylamine) in the mobile phase was increased. Isomer retention, resolution and selectivity decreased as the concentration of buffer was increased. It has been reported that as the concentration of triethylamine in the mobile phase was increased, a corresponding decrease in analyte retention was observed as well as an increase in the efficiency of the separation [16]. The decrease in analyte retention and the improvement in efficiency may be attributed to the triethylamine binding to the silanol groups on the silica backbone and/or sites on the β-cyclodextrin functional group.

Effect of cations and anions

The cation used in the mobile phase as part of the buffer was also studied to determine what effect it might have on the chiral separation. The following amine modifiers were studied: ammonia, trimethylamine, triethylamine, tripropylamine, tributylamine, and triethanolamine. Table II shows the affect that each cation had on isomer retention. When triethanolamine, triethylamine or ammonia were used in the mobile phase, the four isomers were resolved. However, the best resolution was observed when triethylamine was used.

Several inorganic and organic anions were studied to determine what effect the different anions would have on the separation. The five different anions that were studied were; sulfate, nitrate, tartrate, formate and acetate. Table III shows how the different anions affected isomer retention. Acetate provided the best resolution for the isomers. Formate also separated the isomers, however peak shape was not acceptable. The other anions did not adequately separate the isomers; poor peak shape and poor selectivities typically were observed.

TABLE I

EFFECT OF BUFFER CONCENTRATION ON ISOMER RETENTION

Mobile phase: methanol–triethylamine, pH 3.5 (adjusted with acetic acid) buffer (25:75, v/v).

Triethylamine (%)	Capacity factor (k')			
	APS 315*l*	APS 315*d*	APS 314*l*	APS 314*d*
0.01	14.8	15.0	16.4	16.6
0.02	11.2	11.6	12.4	12.8
0.04	10.3	10.7	11.3	11.8
0.05	9.4	9.8	10.3	10.8
0.075	9.6	9.9	10.5	10.9
0.10	7.7	8.1	8.3	8.9
0.20	5.8	6.2	6.2	6.8
0.50	3.1	3.3	3.3	3.6

TABLE II

EFFECT OF DIFFERENT CATIONS ON THE CHIRAL SEPARATION

Mobile phase: methanol–0.05% cation, pH 3.5 (adjusted with acetic acid) buffer (30:70, v/v).

Cation	Capacity factor (k')			
	APS 315*l*	APS 315*d*	APS 314*l*	APS 314*d*
Triethylamine	6.7	7.0	7.2	7.6
Tributylamine	9.4	9.5	10.2	10.5
Ammonia	10.5	11.1	11.4	12.4
Triethanolamine	11.5	12.0	12.7	13.4
Tripropylamine	15.5	15.9	17.1	17.7
Trimethylamine	17.5	17.7	19.3	19.8

TABLE III

EFFECT OF DIFFERENT ANIONS ON THE CHIRAL SEPARATION

Mobile phase: methanol–0.05% NH_4, pH 3.4 (adjusted with appropriate acid) buffer (30:70, v/v).

Anion	Capacity factor (k')			
	APS 315*l*	APS 315*d*	APS 314*l*	APS 314*d*
Acetate	9.7	10.1	10.5	11.2
Sulfate	9.6	10.4	10.4	11.4
Tartrate	9.8	10.5	10.5	11.5
Nitrate	9.8	10.1	10.6	11.2
Formate	11.7	12.0	12.8	13.4

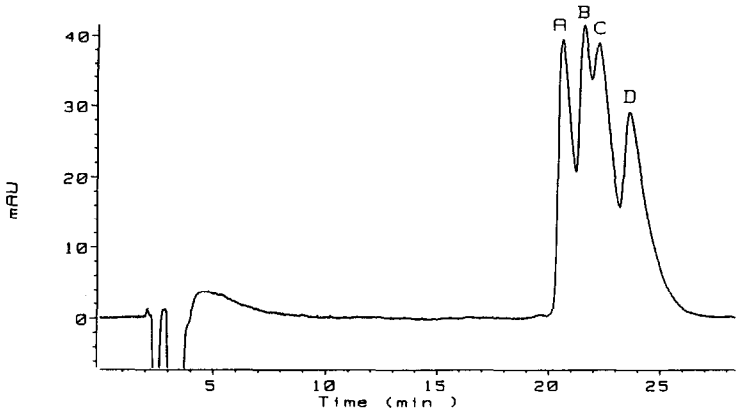

Fig. 4. The separation of the Beraprost sodium isomers: a mobile phase composed of methanol–0.05% (v/v) triethylamine, pH 3.5 adjusted with acetic acid) buffer (25:75, v/v). Peaks: A = APS 315*l*; B = APS 315*d*; C = 314*l*; D = APS 314*d*.

Temperature

The effect of temperature over the range of 0 to 30°C was studied. As the column temperature is lowered, kinetic rates are decreased and interactions between the isomers and the stationary phase are slowed down. As the column temperature was decreased isomer retention was found to increase. (The mobile phase concentration of organic modifier was adjusted so that the isomers would elute within 30 min.) However, no significant differences were observed for the resolution and retention of the isomers at the different temperatures. Therefore, a column temperature of 30°C was used.

Separation

Fig. 4 shows the separation of the four isomers on the acetylated β-cyclodextrin column with a mobile phase consisting of 0.05% (v/v) triethylamine, a pH of 3.5 adjusted with acetic acid and methanol–water (25:75). The column temperature was maintained at 30°C.

Quantitation

Calibration curves and detection limits were determined for the isomers. Isomer standards were prepared that covered a range of 0.049 μg/ml to 98.9 μg/ml. Linear calibration curves of log peak area *versus* ppm of isomer were obtained for 100-μl injections. The correlation coefficients obtained were greater than 0.998 for all four isomers. A detection limit of 75 ng/ml and a limit of quantitation of 500 ng/ml for each isomer was found.

CONCLUSIONS

The separation of the four isomers present in Beraprost sodium was studied using different derivatized and underivatized cyclodextrin stationary phases. The cyclodextrin stationary phases that were found to separate all four isomers were: the acetylated β-cyclodextrin (Cyclobond I AC) and the *para*-toluoyl ester-derivatized β-cyclodextrin (Cyclobond I PT). The mobile phase parameters that influenced the separation were identified and studied. The preparation of the mobile phase was also found to influence the isomeric separation. Calibration data were obtained for each isomer. Detection limits for each isomer were found to be 75 ng/ml with a quantitation limit of 500 ng/ml.

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