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Assay

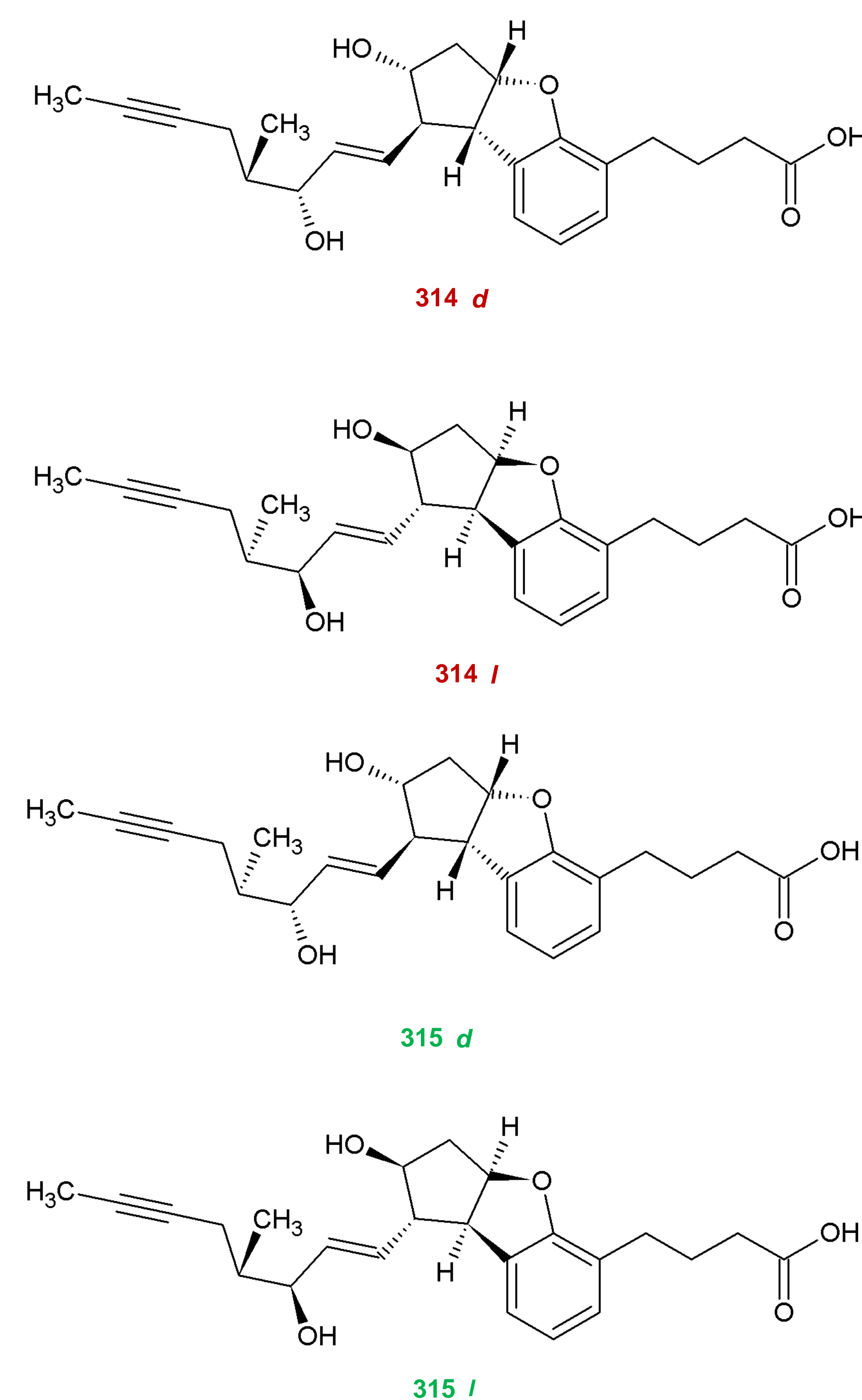


Figure 1: Beraprost Stereoisomers
314 diastereomer top, 315 diastereomer bottom. Beraprost is composed of two diastereomers and each diastereomer is a mix of two enantiomers. 314 *d* is the physiological active stereoisomer.

Introduction

Beraprost is a synthetic prostacyclin analogue used for the treatment of pulmonary arterial hypertension.

Beraprost is composed of two diastereomers and each diastereomer is composed of two enantiomers¹ (Figure 1), for a total of 4 stereoisomers termed 314 *d*, 314 *l*, 315 *d*, and 315 *l*. A chiral method to resolve all 4 stereoisomers has been published¹. A total Beraprost method with an LLOQ of 20 pg/mL has been published².

Assays were developed to quantitate total Beraprost at an LLOQ of 5 pg/mL in human plasma and a separate assay developed to quantitate the active 314 *d* stereoisomer at an LLOQ of 5 pg/mL. The challenge was to develop a specific method, free from matrix noise, at a low LLOQ of 5 pg/mL.

Extraction:

- > 1 mL of human plasma is diluted and applied to a polymeric mixed-mode strong cation exchange SPE cartridge.
- > Internal standard is beraprost-d6.
- > Successive washes of the SPE cartridge with H₂O, 0.5% HOAc, 45:55 MeOH:50 mM KH₂PO₄, pH 2.7, and 50:50 MeCl₂:hexane.
- > Eluted with ACN
- > Dried and reconstituted with 200 µL of 20:80 MeOH:5 mM NH₄OAc

Chromatography: (see Figure 2)

- > 4 pumps, two valves, and two sets of binary mobile phases are used. MP C and MP D are used for the chiral column and MPA and MPB used for the analytical column. 80 µL of extract is injected.
- > Chiral (BPS-314 *d*) method: the 314 *d* peak is resolved from the other 3 stereoisomers using a ChromTech chiral AGP column, 4 x 100 mm, 4 µm. MP C: (0.5:0.5:99 ACN:IPA:H₂O) with 50 mM NH₄HCO₃; MP D: (40:60 IPA:H₂O) with 50 mM NH₄HCO₃ (Figure 3).
- > Total Beraprost method: the chiral AGP column is used as a preliminary column without stereoisomer resolution: MP C: 10 mM NH₄OAc, pH 4.1; MP D: MeOH. (Figure 5)
- > For the chiral method the BPS-314 *d* peak is heart-cut onto a trapping column, a Luna 2x30 C8. In the total Beraprost method a single peak of all stereoisomers is heart-cut onto this trapping column.
- > MP A and MP B, same for both methods, elutes analyte off the trapping column and onto an Ascentis Express C18, 2.1 x 100 mm, 2.7 µm analytical column and into the mass spectrometer. MP A is (95:5 H₂O:MeOH) with 2 mM NH₄OAc and MP B is (85:10:5 MeOH:H₂O:ACN) with 2 mM NH₄OAc. Flow rate is 0.6 mL/min using a ramp from 45%B to 50%B over 4 minutes. (Figures 4 and 6). These MP's and LC conditions provide optimal peak shape and sensitivity.

Mass spectrometry:

TIS source on an API-5000 operated in negative ion mode. Transitions used are *m/z* 397.3 → 269.3 for analyte and *m/z* 403.3 → 269.3 for Beraprost-d6.

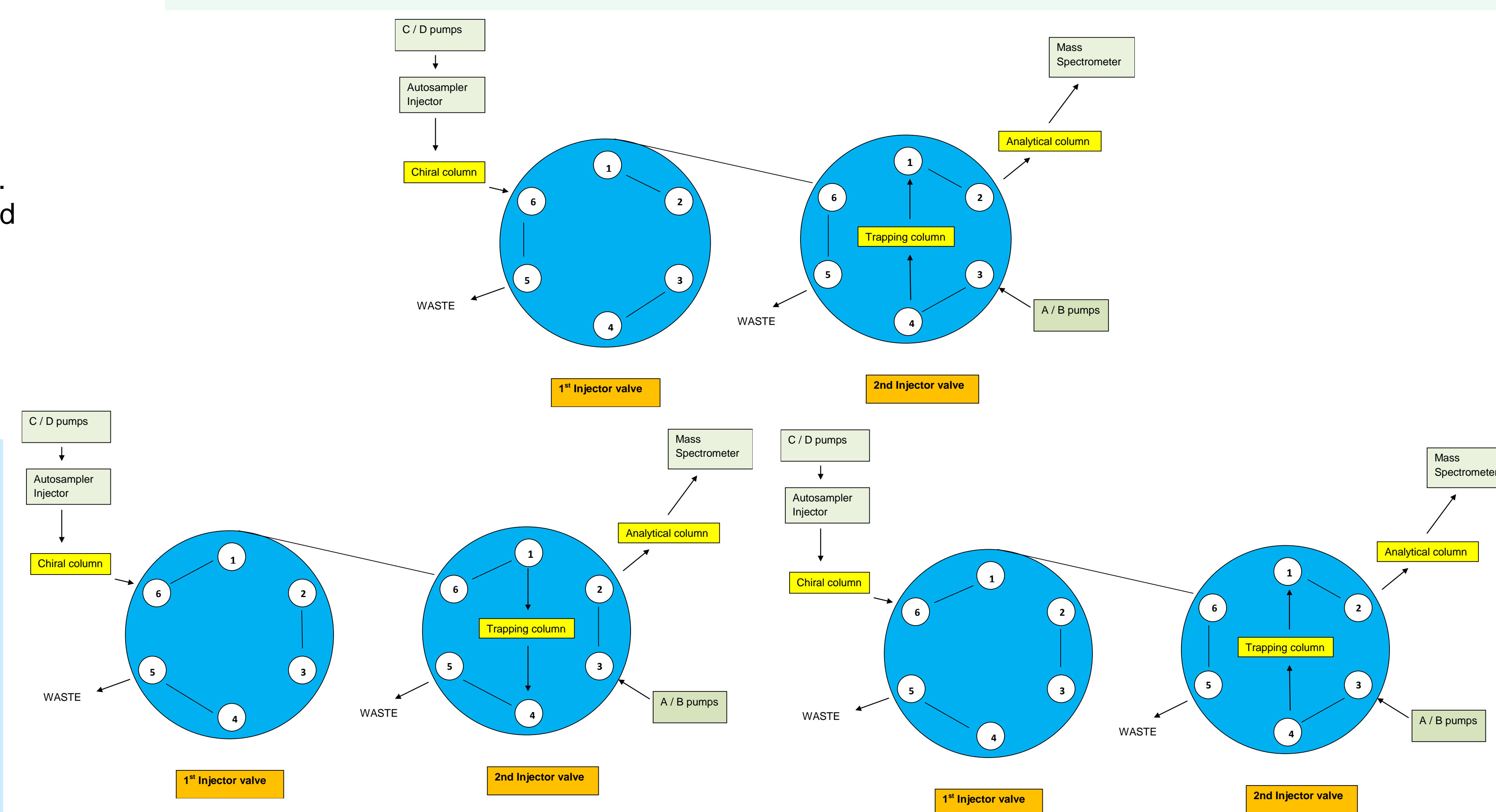


Figure 2

Top: First two stereoisomers and matrix components sent to waste

Bottom Left: From about 2.7 to 4.3 minutes (Figure 3) the 314 *d* peak is heart-cut onto the trapping column. In the total Beraprost method the different mobile phases produce a single peak off the chiral column that is heart-cut onto the trapping column from about 2.7 to 3.6 minutes (Figure 5).

Bottom Right: Elution of the trapping column onto the analytical column and into the mass spectrometer. The chiral column is washed during this time.

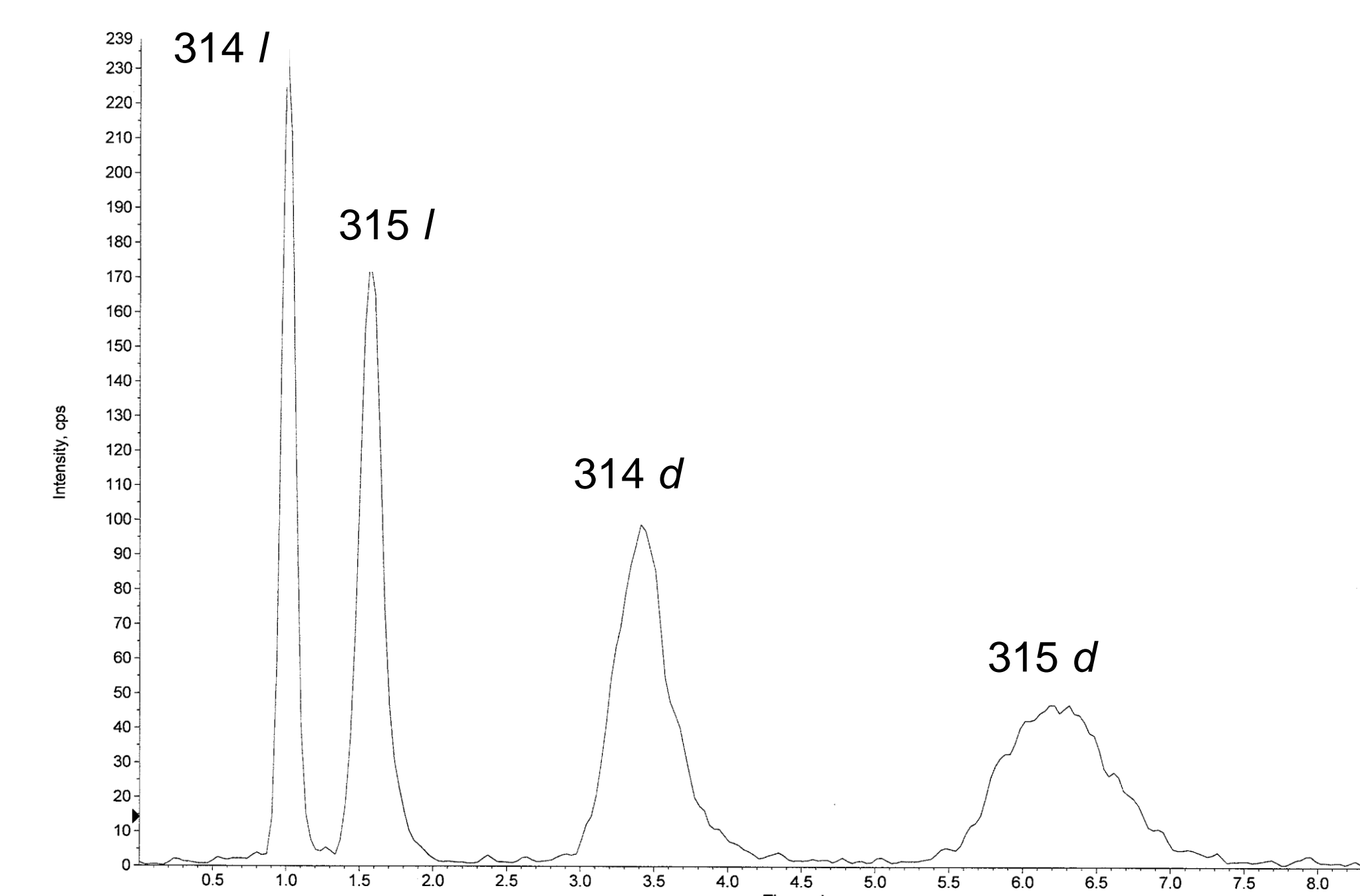


Figure 3: Chiral (314 *d*) method. The 4 stereoisomers of Beraprost eluting off the chiral AGP column. The chiral column was connected directly to the mass spectrometer; a 2000 pg/mL neat solution was injected.

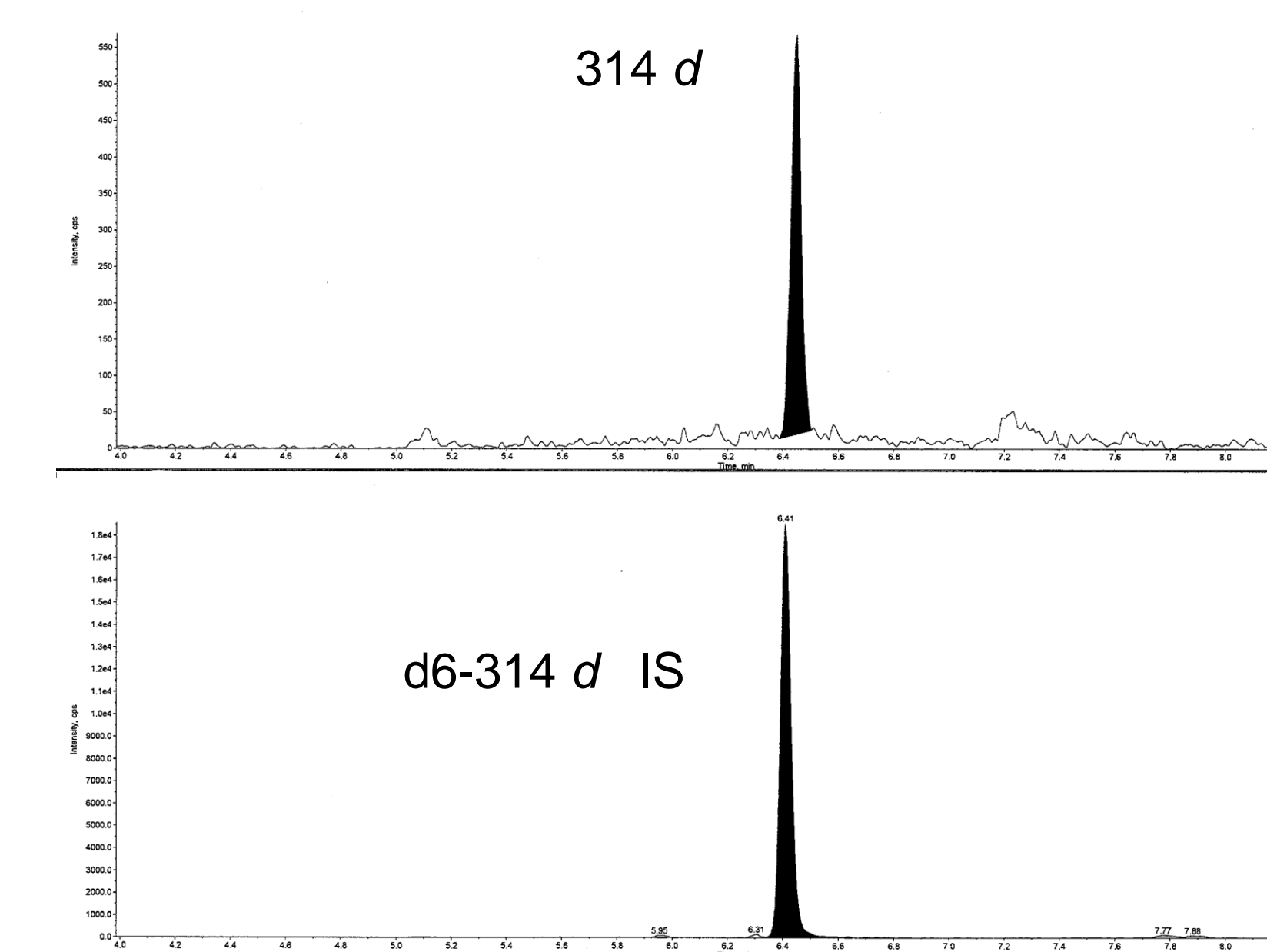


Figure 4: Chiral (314 *d*) method. The 314 *d* stereoisomer at an LLOQ of 5 pg/mL.

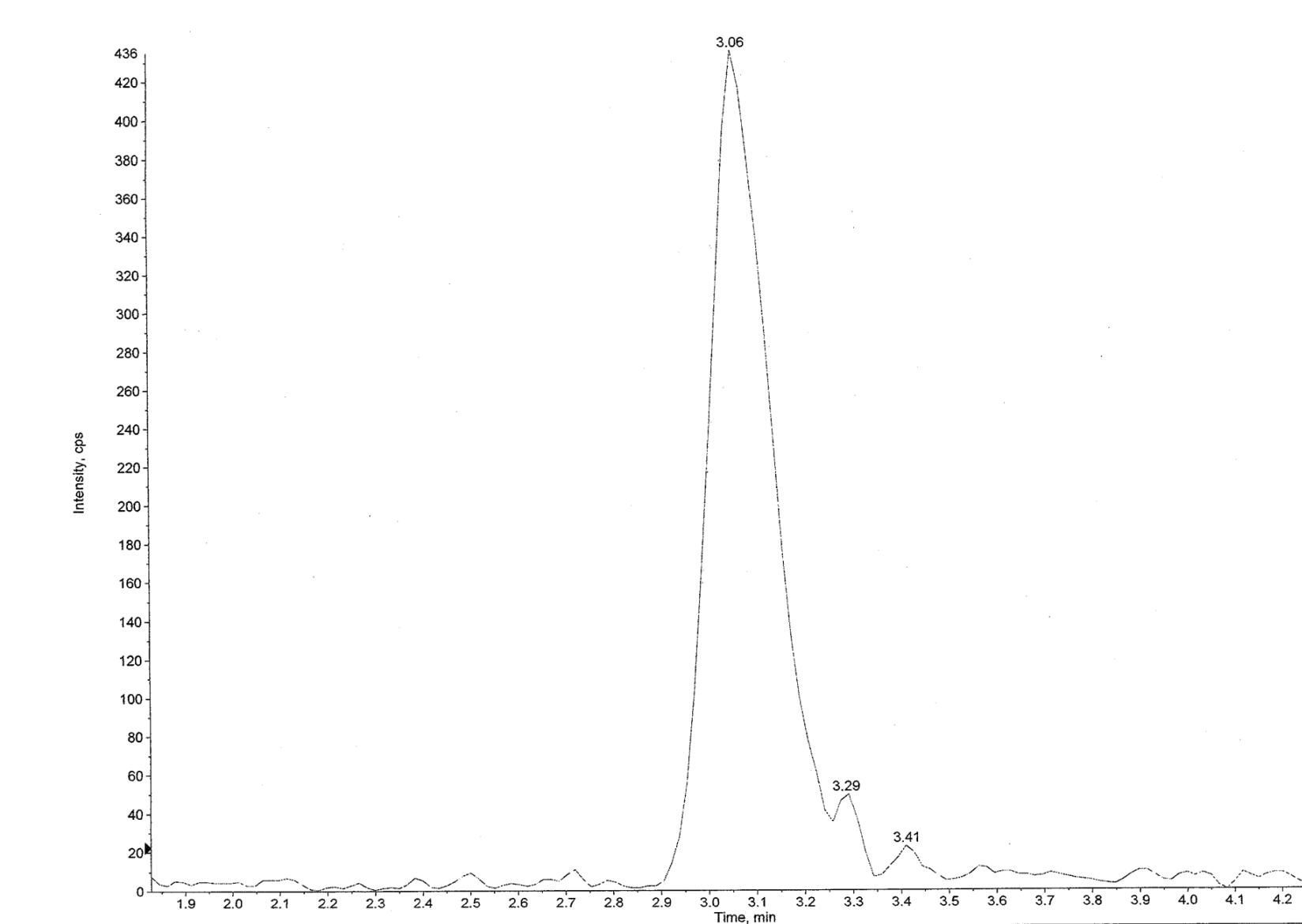


Figure 5: Total Beraprost eluting off the chiral column. The chiral column is used as a preliminary column without stereoisomer resolution.

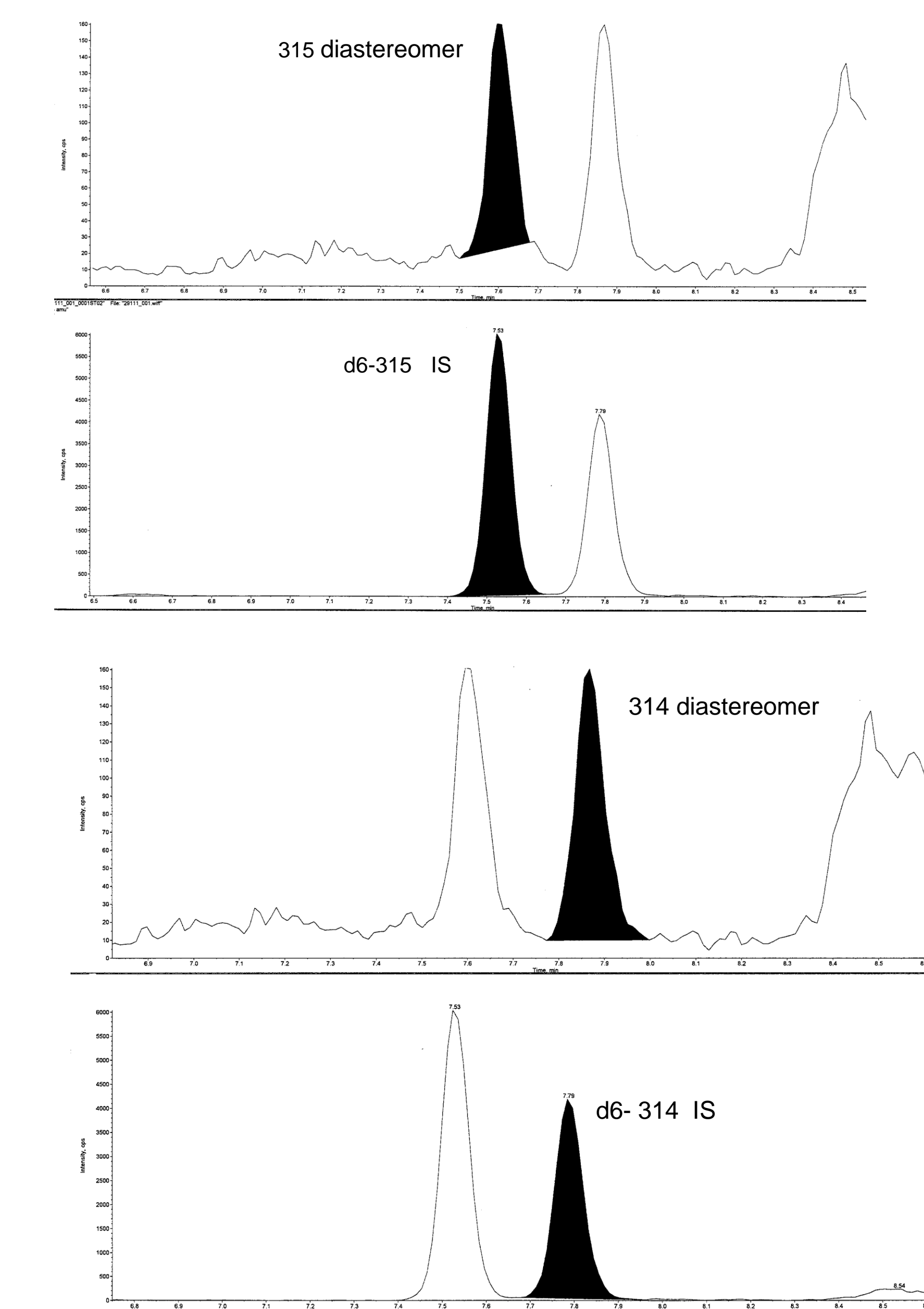


Figure 6: Total Beraprost method displaying the two diastereomers of Beraprost at the 5 pg/mL LLOQ. Diastereomer resolution occurs on the C18, 2.1 x 100, 2.7 µm analytical column. The areas of both diastereomer peaks are summed for quantitation.

Results and Discussion

Two dimensional chromatography (Figure 2) is used to achieve the required specificity from matrix noise, given the low LLOQ of 5 pg/mL, for both the total Beraprost method and the chiral 314 *d* method.

As a consequence of the chromatography required to resolve analyte from matrix noise the two diastereomers in the total method are resolved with a resolution of approximately 1.2 or higher (Figure 6). To quantitate total Beraprost the two peaks are individually integrated and the area counts summed.

While Beraprost is a carboxylic acid, it was found that the use of a polymeric mixed-mode strong cation exchange SPE cartridge lowered the matrix effects and improved specificity. Extraction efficiency averaged 78%. Matrix effects were assessed with spikes of individual plasma lots at the 5 pg/mL LLOQ. The average (n=6) %Dev was -2.5% with a CV of 7.8% or less. Precision at the 15 pg/mL low QC level was a 6.3% CV or less and a 10% CV at the 5 pg/mL LLOQ. In the chiral 314 *d* method the S/N peak to peak at the 5 pg/mL LLOQ is approximately 65:1.

References

- Sly, L.A. et al., J. Chromatography, 641 (1993) 249-255.
- Lee, J. et al., J Chrom B, 859 (2007) 229-233.