Application Note

Pharmaceutical Small Molecules

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Modernizing Chiral Separations with Glycopeptide-Based Chiral Columns

Chiral separation of timolol maleate with Agilent InfinityLab Poroshell 120 Chiral-T columns

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Abstract

In this application note, the development and optimization of a chiral separation for timolol maleate is presented. The method involves an Agilent InfinityLab Poroshell 120 Chiral-T column, and is contrasted with the current USP method for timolol maleate chiral separation.

Introduction

Faster separations of enantiomers have become increasingly important to the pharmaceutical industry. As increasing numbers of optically active pharmaceutical compounds are introduced, along with increasing government regulation, it is important that rapid, sensitive, and reliable methods be devised for their analysis. More than half of the drugs currently in use are chiral compounds, and nearly 90% of these are marketed as racemates consisting of an equimolar mixture of two enantiomers. Although they have the same chemical structure, most isomers of chiral drugs exhibit marked differences in biological activities.[1,2]

While many chiral separations are carried out using cellulose or amylose-based chiral selection phases (CSP) using normal-phase solvents such as hexane, other phases are frequently sought for separation based on more common solvents such as methanol, which can more easily be incorporated into a laboratory running reversed-phase methods.

The InfinityLab Poroshell 120 Chiral-T (teicoplanin) column can be used in reversed-phase and normal-phase HPLC as well as in SFC. Since it is a bonded CSP, it can be used in a wider variety of solvents than coated CSPs without the risk of phase bleed or loss.

InfinityLab Poroshell 120 Chiral-T and other teicoplanin-based CSPs have been demonstrated to work in polar ionic mode (methanol and ammonium salt-based mobile phase, reversed-phase (methanol or acetonitrile with buffer), or polar organic mode (methanol, ethanol, or another pure organic solvent). In reversed-phase mode, the retention and selectivity are controlled by the nature and concentration of the organic component as well as the mobile phase pH.[4]

Glycopeptide-based chiral columns such as InfinityLab Poroshell 120 Chiral-T (teicoplanin) can be used in a wide variety of solvents in reversed-phase and normal-phase HPLC as well as SFC. Glycopeptides are amphoteric, containing both ionizable acidic and basic groups. Thus, they can be positively charged, negatively charged, or neutral depending on the pH of the mobile phase. This allows ionic interactions involved in chiral recognition when separating ionic compounds using this class of CSP. This is thought to play a major role in chiral recognition for this class of CSPs. Other possible interactions involved with the use of antibiotics as CSPs for chiral recognition include hydrogen bonding, steric, dipole–dipole, and π–π interactions as well as hydrophobic interactions. These interactions may take place in different combinations that are determined by the properties of an individual analyte and the mobile phase mode used. Each separation mode provides simultaneous but different interactions for chiral recognition. This accounts for the large number of chiral separations and the variety of types of chiral compounds that are successfully separated with this class of CSP.

The glycopeptide teicoplanin is covalently bonded to superficially porous silica particles, creating a stable and solvent-resistant chromatographic medium. This covalently bonded phase is resistant to common HPLC mobile phases and additives, such as methanol, ethanol, IPA, THF, phosphate, formate, acetate, formic acid, TFA, TEA, and NH_4OH. The structure for the teicoplanin bonded phase is shown in Figure 1.

In this application note, a separation for timolol maleate is developed and optimized on InfinityLab Poroshell 120 Chiral-T and compared to the current USP method. The structure of this compound is shown in Figure 2.

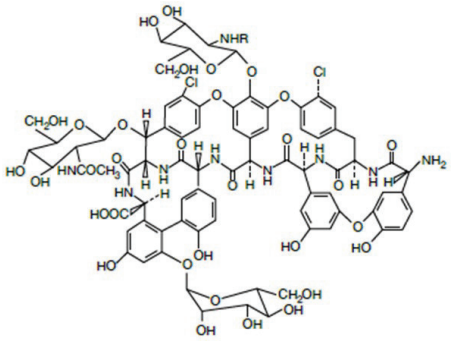

Figure 1. Structure of teicoplanin bonded phase on Agilent InfinityLab Poroshell 120 Chiral-T column.

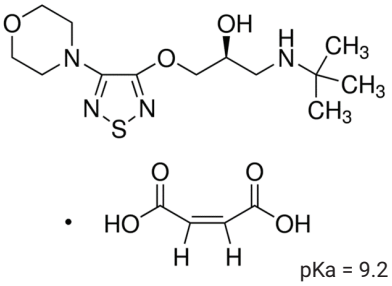

Figure 2. Structure of timolol maleate (S).

Experimental

Materials and methods

An Agilent 1260 Infinity II LC was used; the configuration is shown in Table 1. The basic chromatography conditions are listed in Table 2.

Timolol maleate, S-(−)-1-(t-butyl amino)-3-[(4-morpholino-1,2,5-thiadiazol-3-yl) oxy]-2-propanol maleate salt, European Pharmacopoeia (EP) reference standard and R-timolol European Pharmacopeia (EP) reference standard were purchased from Sigma-Aldrich. Each enantiomer was dissolved in methanol at 2 mg/mL. A sample consisting of equal volumes of each enantiomer solution was used to determine resolution. Ammonium formate LC/MS grade was also purchased from Sigma-Aldrich. HPLC-grade methanol was purchased from Honeywell. Mobile phases were prepared at 0.2% w/v by dissolving 2 g of ammonium formate in methanol. Lower concentrations were prepared using dilutions of this stock.

Table 1. Instrument configuration.

Agilent 1260 Infinity II LC	
Agilent 1260 Infinity II binary pump (G7112B)	Capable of delivering up to 600 bar, 0 to 5 mL/min
Agilent 1260 Infinity II multisampler (G7167A)	• Autosampler and heater: capillary, stainless steel, 0.075 × 220 mm (p/n 5067-4784) • Vial, screw top, amber with write-on spot, certified, 2 mL, 100/pk (p/n 5182-0716) • Cap, screw, blue, PTFE/red silicone septa, 100/pk (p/n 5182-0717)
Agilent Infinity II multicolumn thermostat (G7116A)	• Ultralow dispersion heater (G7116-60021) • Heater and column: Agilent InfinityLab Quick Connect assembly, 105 mm, 0.075 mm (p/n 5067-5961) • Column and ELSD capillary, stainless steel, 0.075 × 220 mm, SV/SLV (p/n 5067-4784)
Agilent 1260 Infinity II DAD (G7115A) equipped with a 10 mm path length	• G4212-6008, 10 mm flow cell, 1.0 µL V(σ) • 40 Hz
Agilent OpenLab CDS, version C.01.07	

Table 2. LC method conditions.

Parameter	Value
Column	Agilent InfinityLab Poroshell 120 Chiral-T, 4.6 × 100 mm, 2.7 µm (p/n 685775-603)
Mobile Phase	Methanol with ammonium formate (0 to 0.2%)
Flow Rate	1 mL/min
Temperature (Column)	30 °C
UV Wavelength	260 nm
Injection Volume	1 µL
Sample Concentration	2 mg/mL in water

Results and discussion

An initial investigation of timolol chiral separation was carried out by screening using four InfinityLab Poroshell 120 chiral columns and six mobile phases.[3,4] This testing revealed a separation of the two timolol enantiomers and the malate salt using methanol with a 0.2% w/v ammonium formate mobile phase. This separation is reproduced in Figure 3.

Polar ionic separations typically involve the use of organic solvents such as methanol or acetonitrile, with small amounts of acid or base added. Alternatively, an ammonium salt can be used, as is the case here. It was found that lowering the concentration of ammonium formate led to greater resolution and retention of the two latter peaks. These were identified as the two timolol enantiomers by comparing the

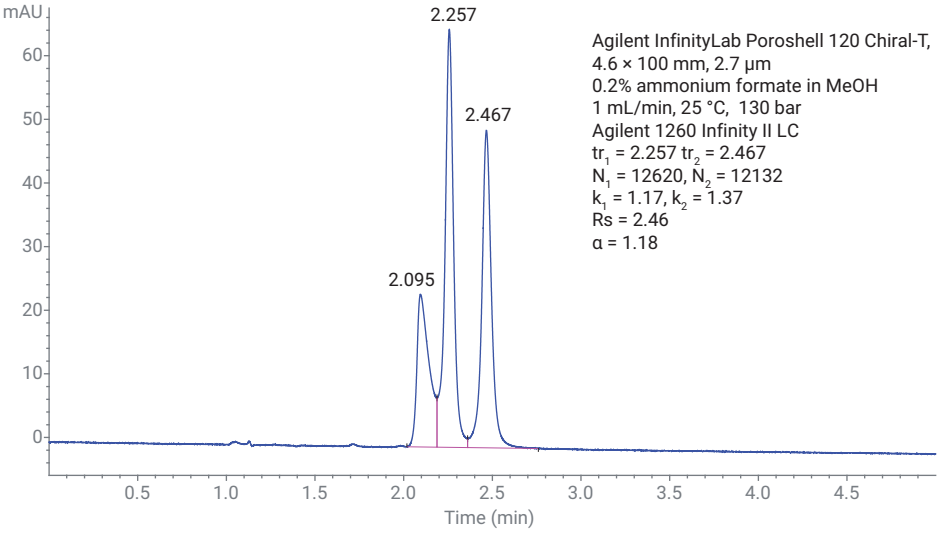

Figure 3. Separation of timolol maleate using 0.2% ammonium formate in methanol.

UV spectra. This behavior is typical of separations carried out using polar ionic separation mode. When applied to the teicoplanin or vancomycin phases, only molecules without ionizable groups demonstrate fast separations in a mobile phase consisting of just methanol, ethanol, acetonitrile, or combinations of these anhydrous solvents. A good example of a polar organic separation is the 5-methyl-5-phenylhydantoin or thalidomide shown in reference (chiral compendium).

When, however, the compound has an ionizable group, it is sometimes necessary to add small amounts of acid and base or a volatile salt. This is due to the presence of ionizable groups present within the macrocyclic glycopeptide itself. This latter mobile phase condition is referred to as the polar ionic mode to differentiate it from the polar organic mode.

For polar ionic mode selectivity, the compound usually has at least two functional groups, one of which must be ionizable. These functional groups can include alcohols, halogens, nitrogen in any form (primary, secondary, tertiary), carbonyl, carboxyl, oxidized forms of sulfur, or phosphorus. Many acids and bases as well as volatile salts can be used on glycopeptide phases. Ammonium acetate is typical for acidic molecules, and ammonium trifluoroacetate and ammonium formate are used for basic molecules. The pKa for timolol is approximately 9.2, and so ammonium formate, an MS compatible salt, is favored.

The first peak was identified as maleate, the co-salt. Figure 4 shows the separation at three ammonium formate concentrations, 0.2, 0.15, and 0.1%. The first timolol peak was identified as the "S" enantiomer by injecting the European Pharmacopeia standard. Table 1 lists the concentration of ammonium formate, the retention of the two timolol enantiomers, and the resolution of the timolol enantiomers for six concentrations. These data are plotted in Figures 5A and 5B. A chromatographic overlay of this data set is shown in Figure 6. This data shows the resolution of the timolol enantiomers increasing with lower ammonium formate concentrations; however, the retention

Table 3. Timolol maleate separation on Agilent InfinityLab Poroshell 120 Chiral-T at varied ammonium formate concentrations.

% Ammonium Formate	Rs	t1	t2
0.0	–	–	–
0.025	4.12	8.585	9.917
0.05	3.71	5.073	5.798
0.1	3.17	3.204	3.597
0.15	2.78	2.577	2.848
0.20	2.46	2.257	2.467

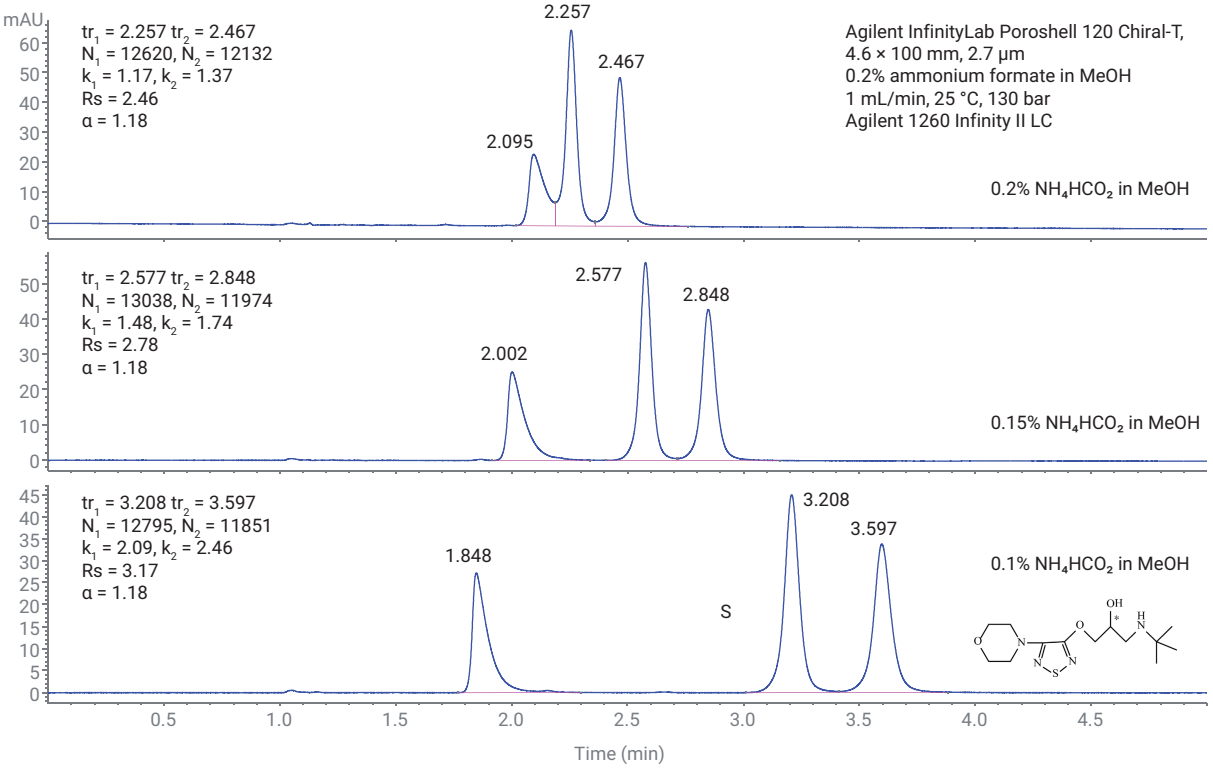

Figure 4. Separation of timolol maleate using 0.2, 0.15, and 0.1% ammonium formate in methanol.

of the peaks increases. Additional resolution was achieved by lowering the ammonium formate concentration. At a concentration of 0.025% w/v ammonium formate, a resolution of 4.12 is achieved. At 0% ammonium formate, the peaks are retained by the column to the point where no timolol peak is apparent.

The USP method for timolol maleate utilizes a 5 µm, 4.6 × 250 mm normal-phase method, using a USP-classified L40 (cellulose *tris*-3,5-dimethylphenyl carbamate-coated porous silica) column with hexane/isopropanol/dimethylamine, 960:40:2 mobile phase. This method is also listed in the European Pharmacopeia. This mobile phase is not MS compatible due to the presence of the dimethylamine.[5,6,7] Another problem with the USP/EP method is that coated chiral columns are used. Coated columns, compared to immobilized chiral columns, have a relatively limited lifetime, in some cases can be as little as 200 injections. Many of the solvents commonly used in HPLC eluents, such as acetone, chloroform, DMF, dimethyl sulfoxide, ethyl acetate, methylene chloride, and THF may destroy the chiral stationary phase if they are present, even in residual quantities, in the system. In general, a system using this normal-phase solvent is dedicated to normal-phase applications.[8] The InfinityLab Poroshell 120 Chiral-T is a fully bonded column resistant to commonly used chromatography solvents.

A reference showing the separation using the European Pharmacopeia method on a 4.6 × 250 mm column shows retention times for the two timolol enantiomers between 9 and 14 minutes, while meeting the requirements for resolution of 4.8. This method is compared with a new method, using SFC on the same column. While the new method is considerably faster, less resolution is reported.[9]

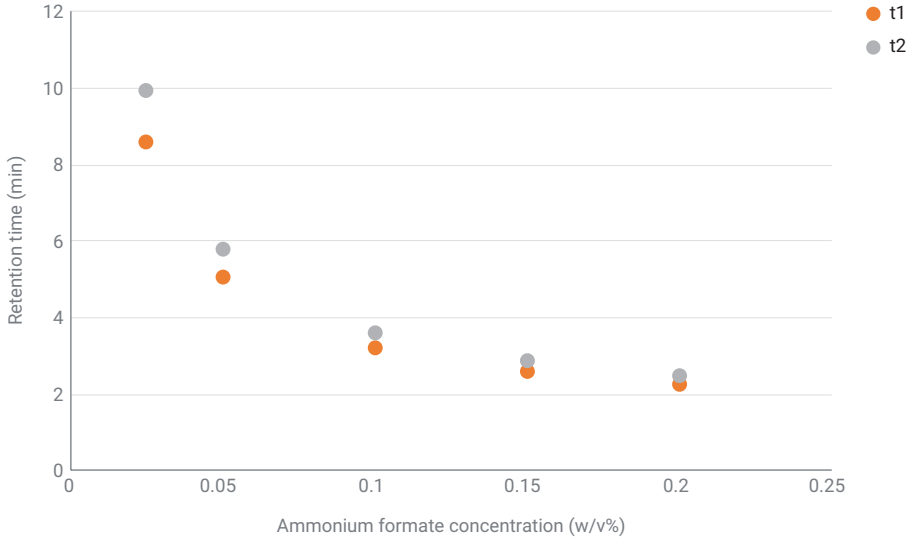

Figure 5A. Retention time of timolol enantiomers as a function of ammonium formate concentration.

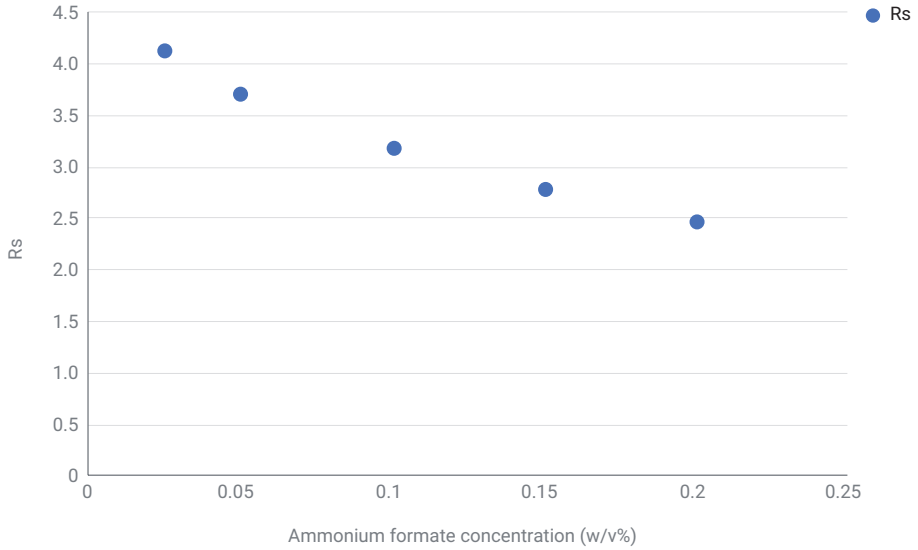

Figure 5B. Resolution of timolol enantiomers as a function of ammonium formate concentration.

Conclusion

The separation of timolol enantiomers is demonstrated using a polar ionic mobile phase (methanol with ammonium formate) with an InfinityLab Poroshell 120 Chiral T-column. The method shown uses 220 nm UV detection, but the mobile phase is fully compatible with MS detection. This would enable easy adaption to a troubleshooting process method or forensic analysis. This method can be interchangeably run on any HPLC system.

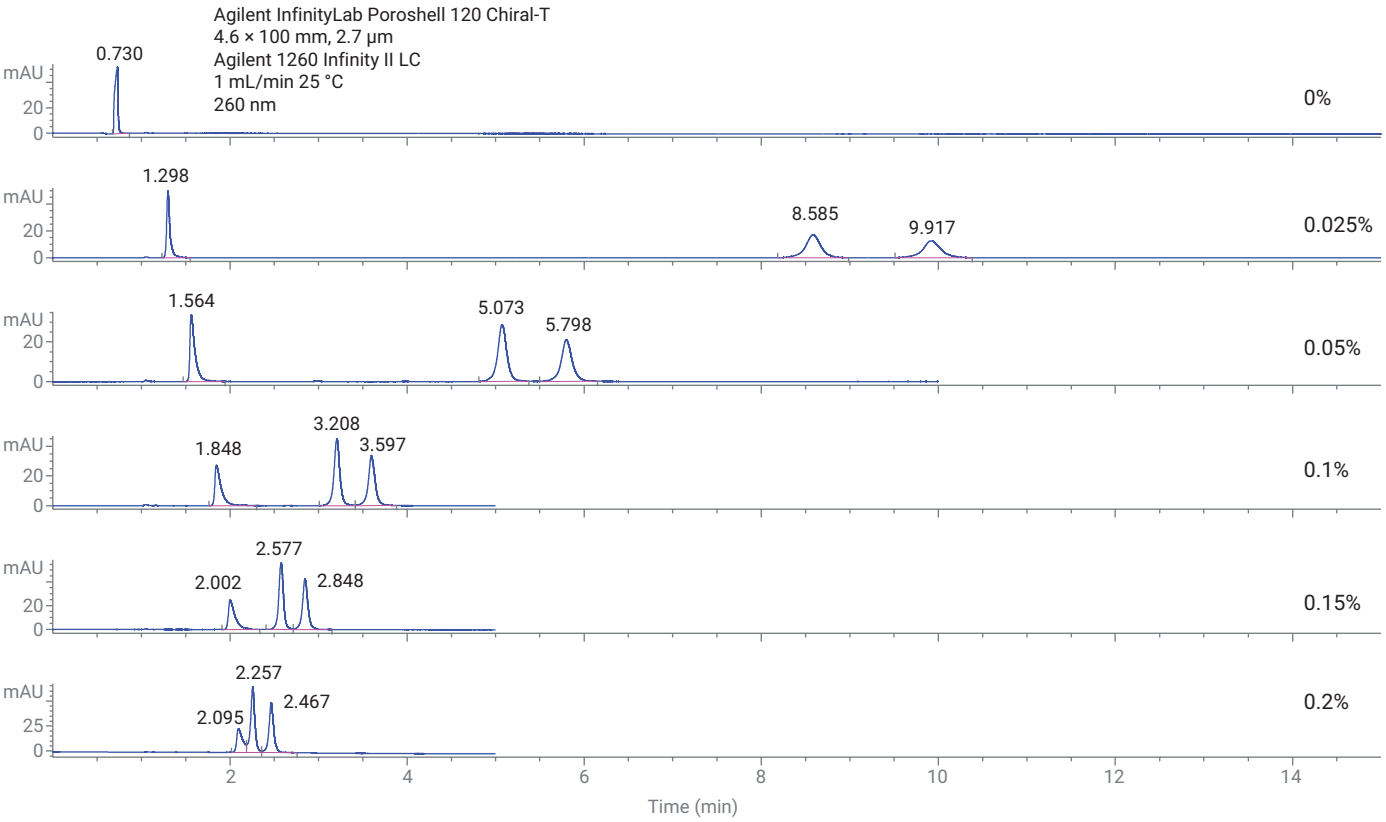

Figure 6. Separation of timolol maleate as a function of ammonium formate concentration.

References

1. Bonner, W. A. Parity Violation and the Evolution of Biomolecular Homochirality. *Chirality* **2000**, *12*, 114–126.
2. Food and Drug Administration, FDA's Policy Statement for the Development of New Stereoisomeric Drugs. **1992**, *57* Fed. Reg. 22249.
3. Long, W. J.; Mack, A. E. Screening Chiral Compounds Using Superficially Porous Based Chiral Columns and a Method Development Solution. *Pittcon* **2018**, CO-1479.
4. Put Agilent InfinityLab Poroshell 102 Chiral Innovations to Work on Your Challenging Separations. *Agilent InfinityLab Poroshell 120 Chiral application compendium*, publication number 5991-8450EN, **2017**.
5. United States Pharmacopeia USP 40 Timolol Maleate Monograph.
6. European Pharmacopoeia, 4th edition, Addendum 2002, Council of Europe, Strasbourg.
7. European Pharmacopoeia (Version 7.5): Timolol Maleate Monograph.
8. Instruction Manual for Chiralcel OD-H and OJ-H Chiral Technologies 07/2013.
9. Marley, A.; Connolly, D. Determination of (R)-Timolol in (S)-Timolol Maleate Active Pharmaceutical Ingredient: Validation of a New Supercritical Fluid Chromatography Method with an Established Normal Phase Liquid Chromatography Method. *J. Chromatogr. A* **2014**, *1325*, 213– 220.

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