Determination of enantiomeric purity of Timolol Maleate by Supercritical Fluid Chromatography

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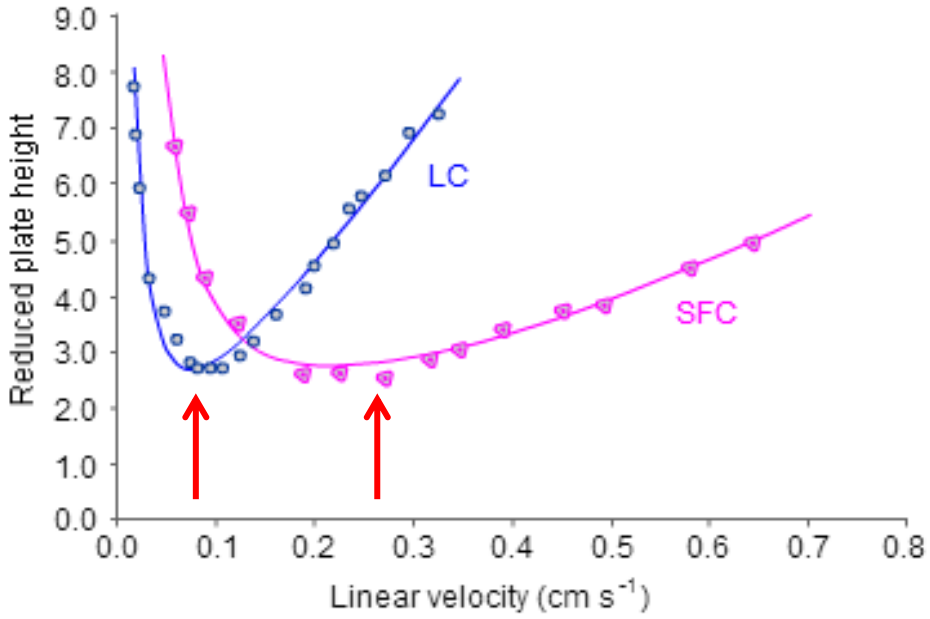
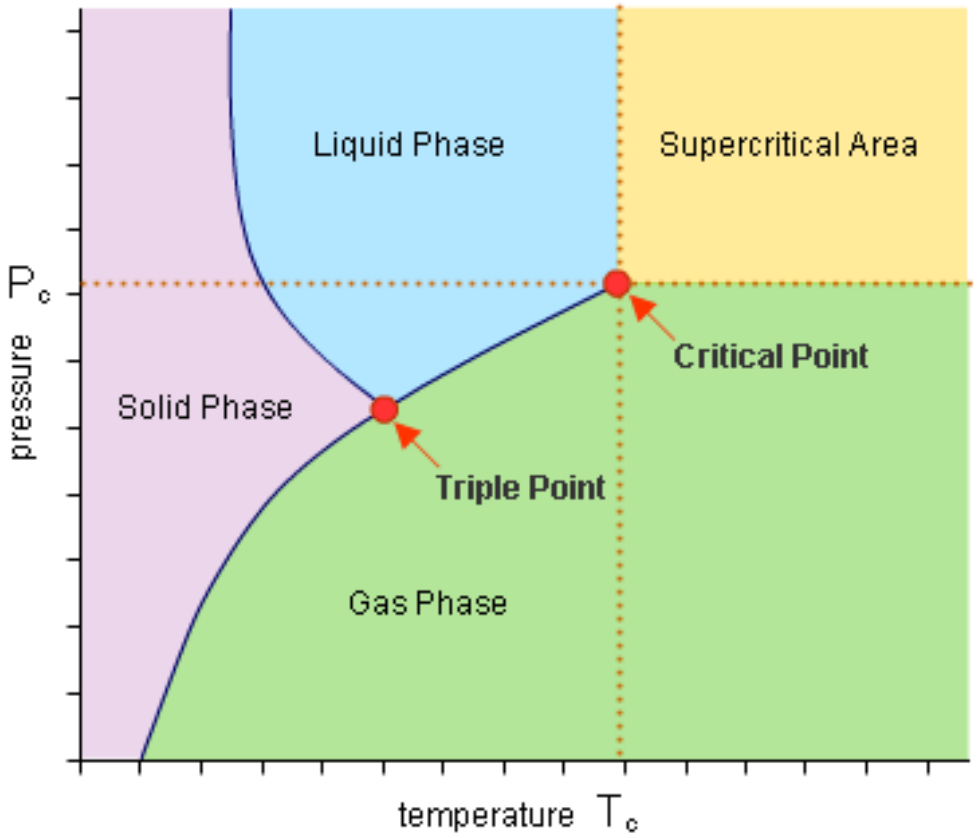
What is supercritical fluid chromatography ?

- **Intermediate chromatographic technique; properties lie between GC and HPLC**
- **Mobile phase is a “supercritical fluid”**
- **Selectivity: derivative of normal phase chromatography**
- **Added benefit of low viscosity and high diffusivity**



What is a supercritical fluid ?



Critical pressure (P_c): The highest pressure at which a liquid can be converted to a gas by increasing its temperature (sCO_2 : 73.8 bar)

Critical temperature (T_c): The highest temperature at which a gas can be converted to a liquid by increasing its pressure (sCO_2 : 31.1 ^oC)

Above its T_c a gas will not condense to a liquid regardless of pressure increase.

Above P_c a liquid will not exist in gas phase regardless of temperature increase

Neither a liquid or a gas; compressible fluids with the solvating power of a liquid but the diffusivity approaching that of gases.

Selected indicative physicochemical properties of liquids, gases and supercritical fluids

Property	Liquid	Gas	Supercritical Fluid
Density (kg/m^3)	1000	1	200 - 800
Viscosity (mPa s)	0.5 - 1.0	0.01	0.05 - 0.1
Diffusivity (cm^2/s)	10^{-5}	0.1	10^{-4} - 10^{-3}

Intermediate properties make for an interesting mobile phase for chromatography !

Why CO_2 ?

- **Low cost**
- **High purity**
- **Low toxicity**
- **Modest P_c/T_c values**

Effect of sCO_2 density (solvating power) on retention/selectivity

Adjustable eluotropic strength via organic modifiers

Can affect retention, selectivity, solubility of analytes.

“Sub-critical” region

SFC instrumentation

Agilent 1260 Binary gradient system

Aurora Fusion A5 module add-on

CO_2 tank (99.9 %)

Binary pump (adjustable compressibility)

Fixed loop injection

Backpressure regulator

High-pressure flow cell

Advantages of SFC

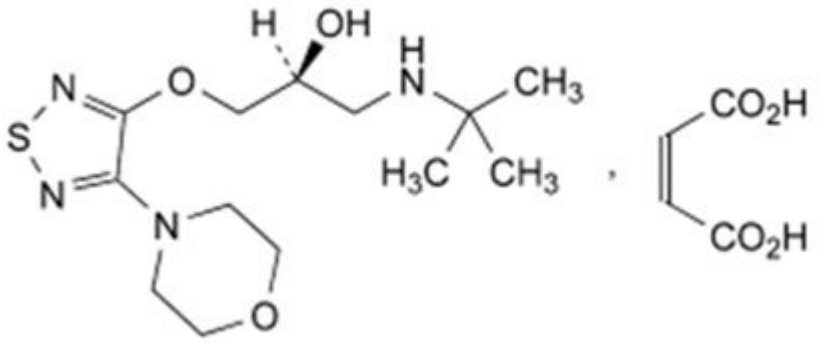
- **Higher resolution and peak capacity**
- **Less solvent waste**
- **Higher sample throughput**
- **Fast calibration**
- **Orthogonal selectivity**

Limitations of SFC

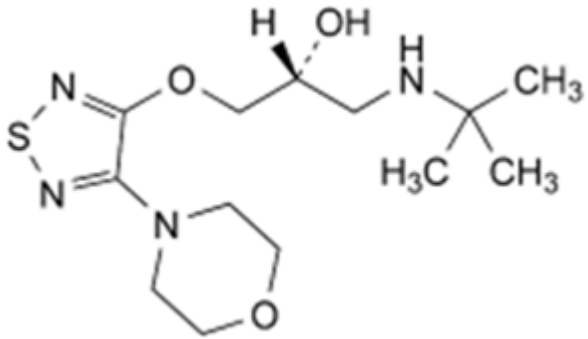
- **Limited choice of mobile phases (CO_2 + MeOH/ACN/additives)**
- **Analyte solubility**
- **Unwanted reactions (sCO_2 forms carbamic acids with $1^o/2^o$ amines)**

Timolol maleate: β-adrenergic blocker used as a single enantiomer (S-timolol).

- **hypertension,**
- **arrythmias,**
- **angina pectoris,**
- **prevention of myocardial infarctions,**
- **topical treatment of increasing intraocular pressure in patients with chronic open angle glaucoma and aphakia.**

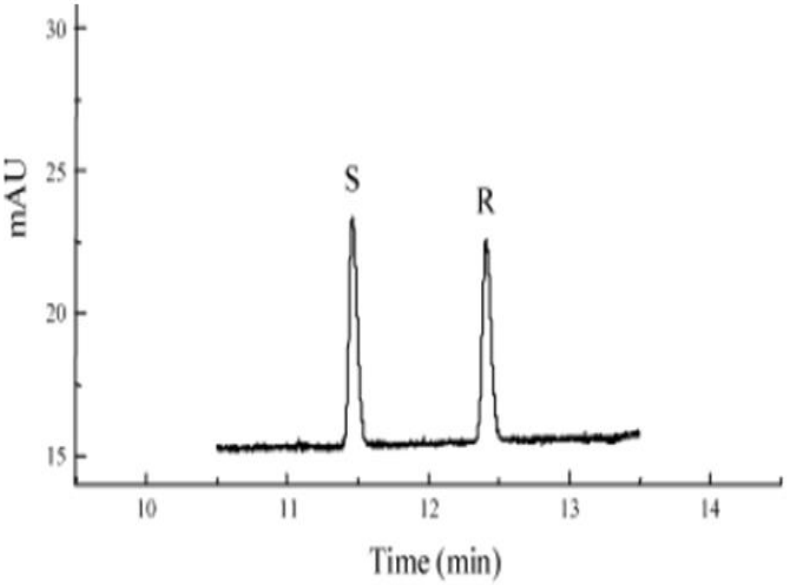


S-timolol

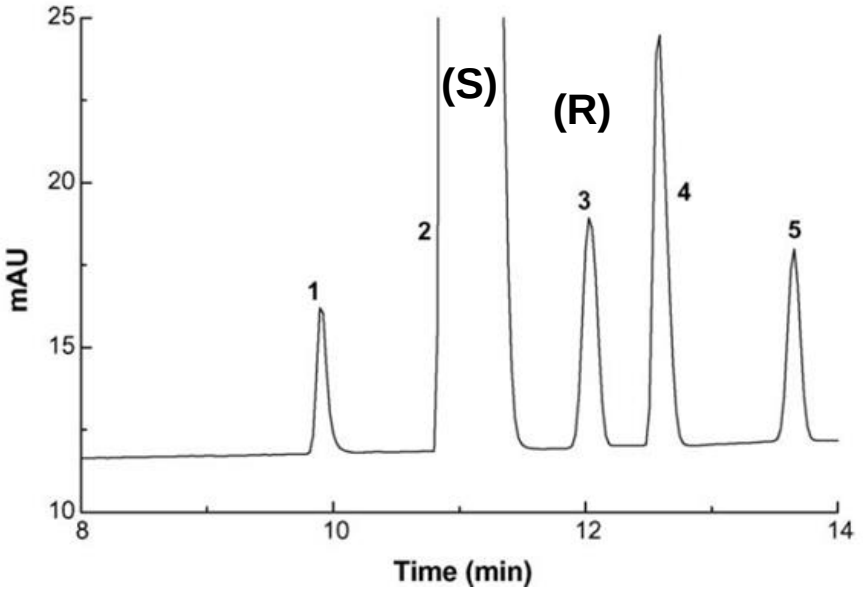


R-timolol

Enantioseparation of timolol via capillary electrophoresis



Electrophoresis 25 (2004) 2701.



J. *Chromatogr. A*, 1120 (2006) 102

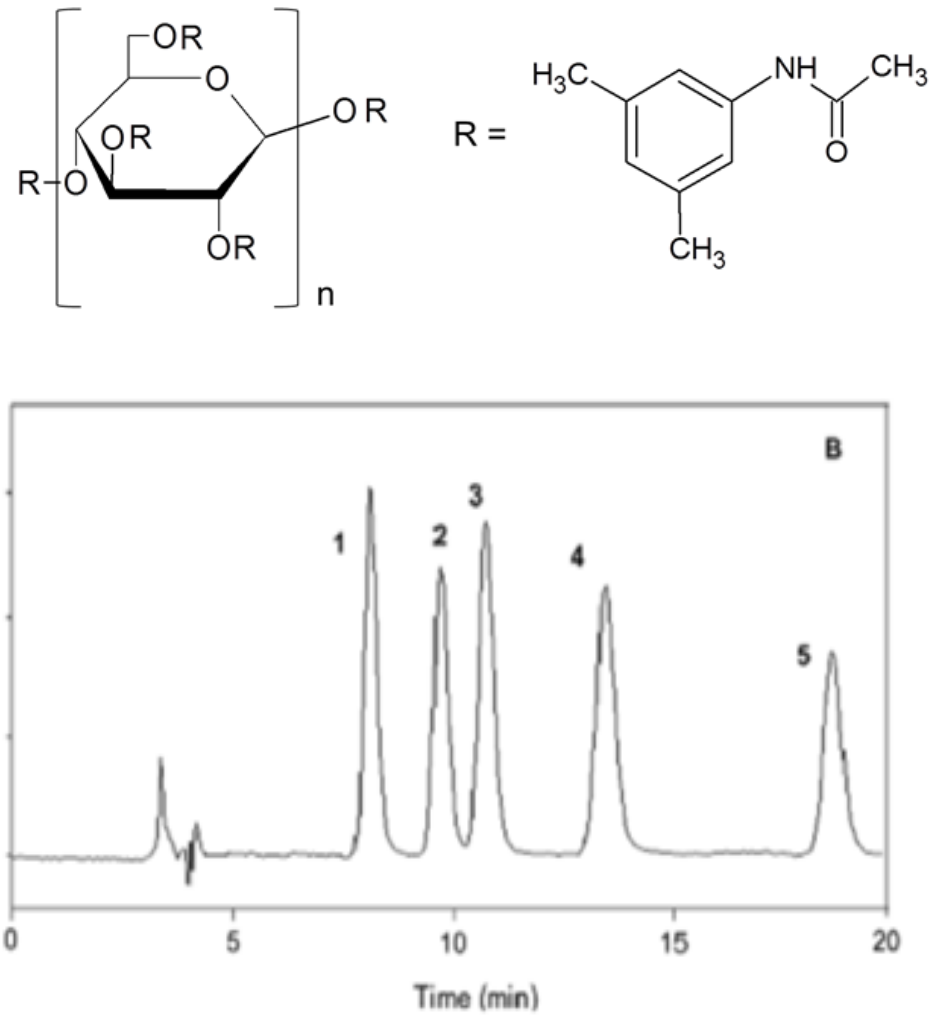


Figure 1: *HPLC separation of timolol maleate enantiomers. Column: Chiralcel OD-H 250 mm x 4.6 mm, 5 µm. Mobile phase: hexane/2-propanol/DEA (965:35:1). Flow rate, 0.7 mL.min^{-1}. Column temperature: 5 °C. Detection: UV (224 nm). Peak assignment: 1 = Dimer maleate (1.0 %). 2 = R-timolol (1.0 %). 3 = Isotimolol (1.2 %). 4 = S-timolol (1.0 %). 5 = DMTDZ (0.7 %).*

J. Sep. Sci., 26 (2003) 809.

Enantioseparation of timolol maleate: SFC and normal phase LC comparison

Solution preparation for NP-HPLC and SFC analysis

Solutions for NP-HPLC				
Diluent	**Solution**	**Analyte**	**Concentration (m*M*)[a]**	**% R-timolol w.r.t S-timolol**
Methylene chloride/2-propanol (10:30)	R-timolol Stock standard	R-timolol	7.6×10^{-2} m*M*	*n/a*
	S-timolol Stock standard	S-timolol	9.5 m*M*	
	Recovery/method precision standards	S-timolol	9.5×10^{-2} m*M*	
	CRM mixture	R-timolol S-timolol	4.7×10^{-2} m*M* 4.7×10^{-2} m*M*	
	S-timolol/R-timolol spiked standards	R-timolol	9.5×10^{-4} m*M*	1.0 %
		S-timolol	9.5×10^{-2} m*M*	
Solutions for SFC				
Diluent	**Solution**	**Analyte**	**Concentration (m*M*)[a]**	**% R-timolol w.r.t S-timolol**
MeOH	R-timolol Stock standard	R-timolol	2.3×10^{-1} m*M*	*n/a*
	S-timolol Stock standard	S-timolol	28 m*M*	
	Precision studies	S-timolol	2.8×10^{-1} m*M*	
	S-timolol/R-timolol spiked standards	R-timolol	5.7×10^{-3} m*M*, 2.8×10^{-3} m*M*, 1.4×10^{-3} m*M*, 2.8×10^{-4} m*M*, 1.4×10^{-4} m*M*,	2.0 %, 1.0 %, 0.5 %, 0.1 %, 0.05 %
		S-timolol	2.8×10^{-1} m*M*	
	CRM mixture	R-timolol S-timolol	1.4×10^{-1} m*M* 1.4×10^{-1} m*M*	50 %

[a] Based on timolol free base M.W. 316.4 g

Effect of 0.1 % TEA on peak shape

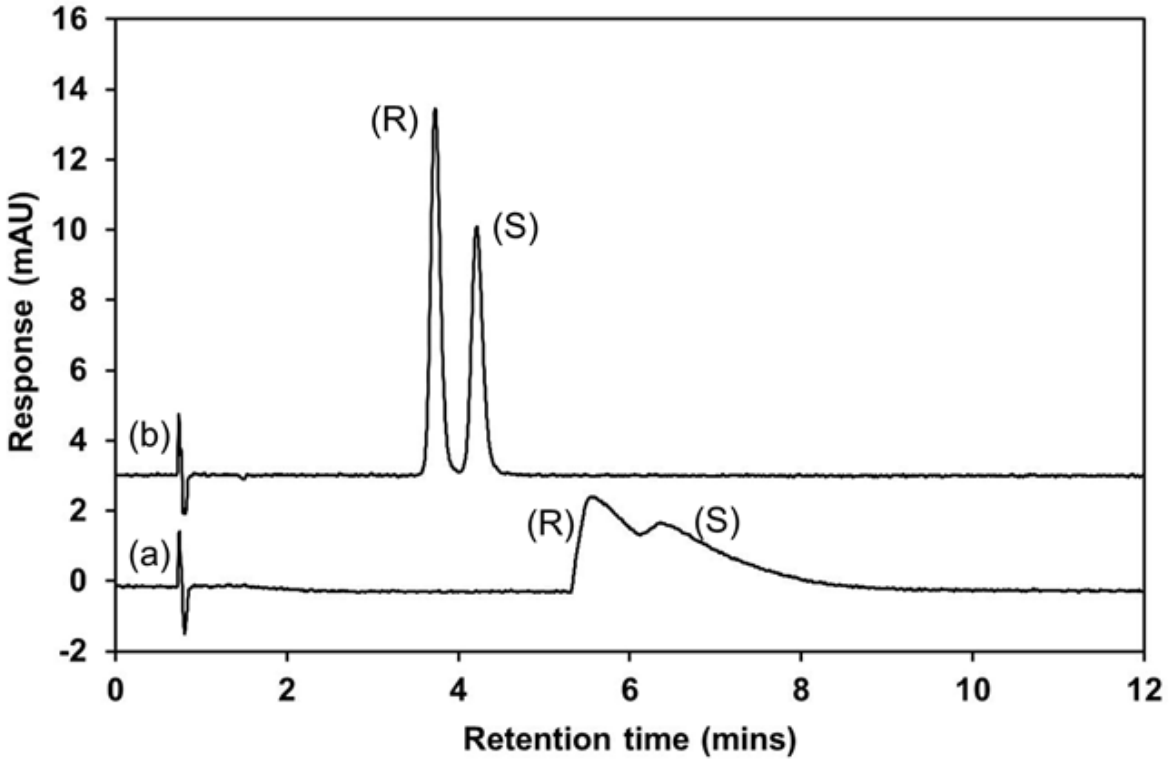


Figure 2: *Effect of 0.1 % v/v TEA on peak shape. Column: Chiralcel OD-H 250 mm x 4.6 mm, 5 µm. Mobile phase: (93:7) CO_2/MeOH (**a**) and (93:7) CO_2/0.1 % (v/v) TEA in MeOH (**b**). Flow rate: 4.0 mL.min^{-1}. Injection volume: 15 µL. Column temperature: 40 ^{o}C. Back-pressure regulation: 130 bar. Detection: 297 nm. Peak assignment: (R): R-timolol at 1.4 x 10^{-1} mM and (S): S-timolol at 1.4 x 10^{-1} mM.*

Effect of organic modifier content

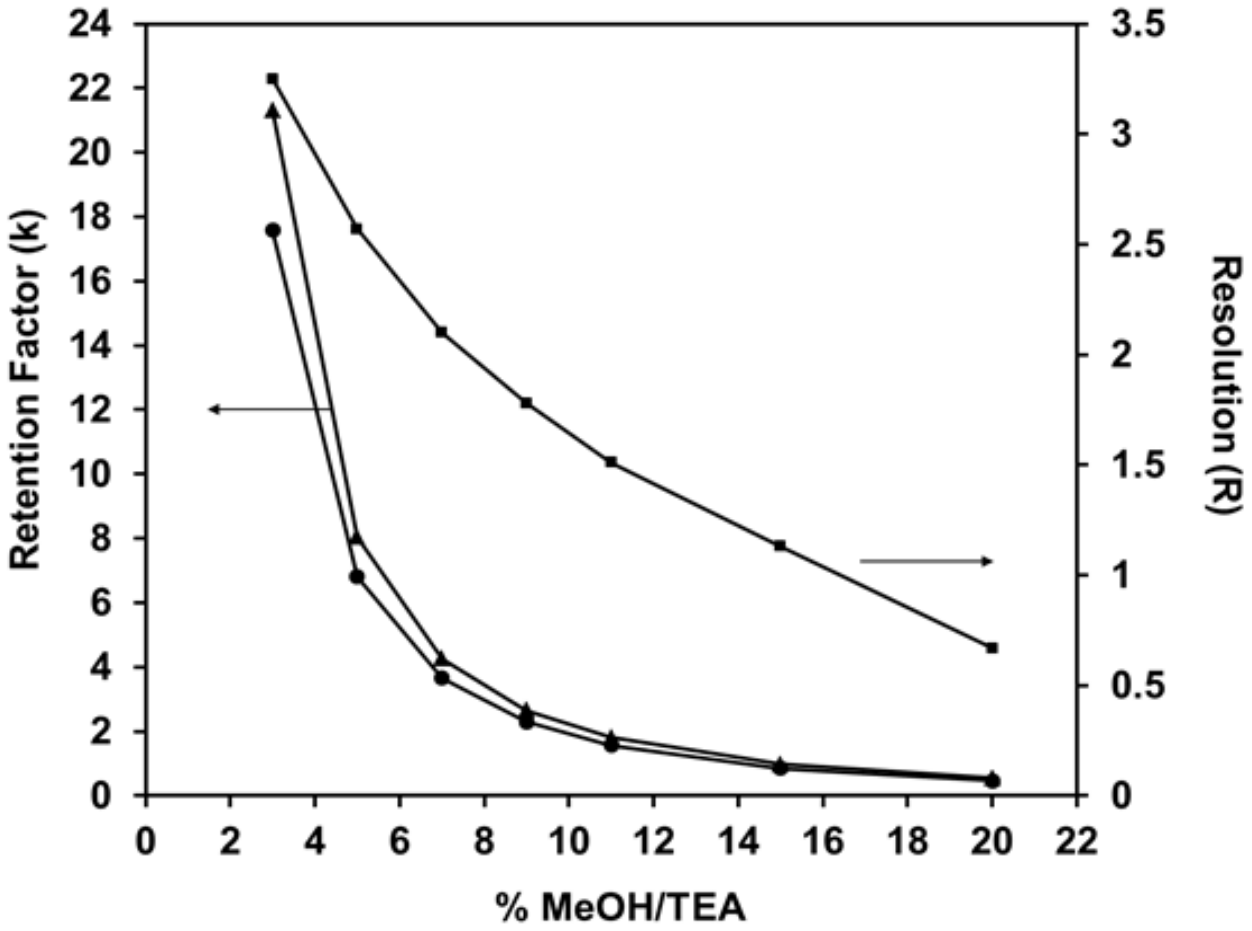


Figure 3: *Plot of retention factor (k) and R versus % MeOH. Chromatographic conditions as in Figure 2.*

SFC versus NPLC: Direct comparison

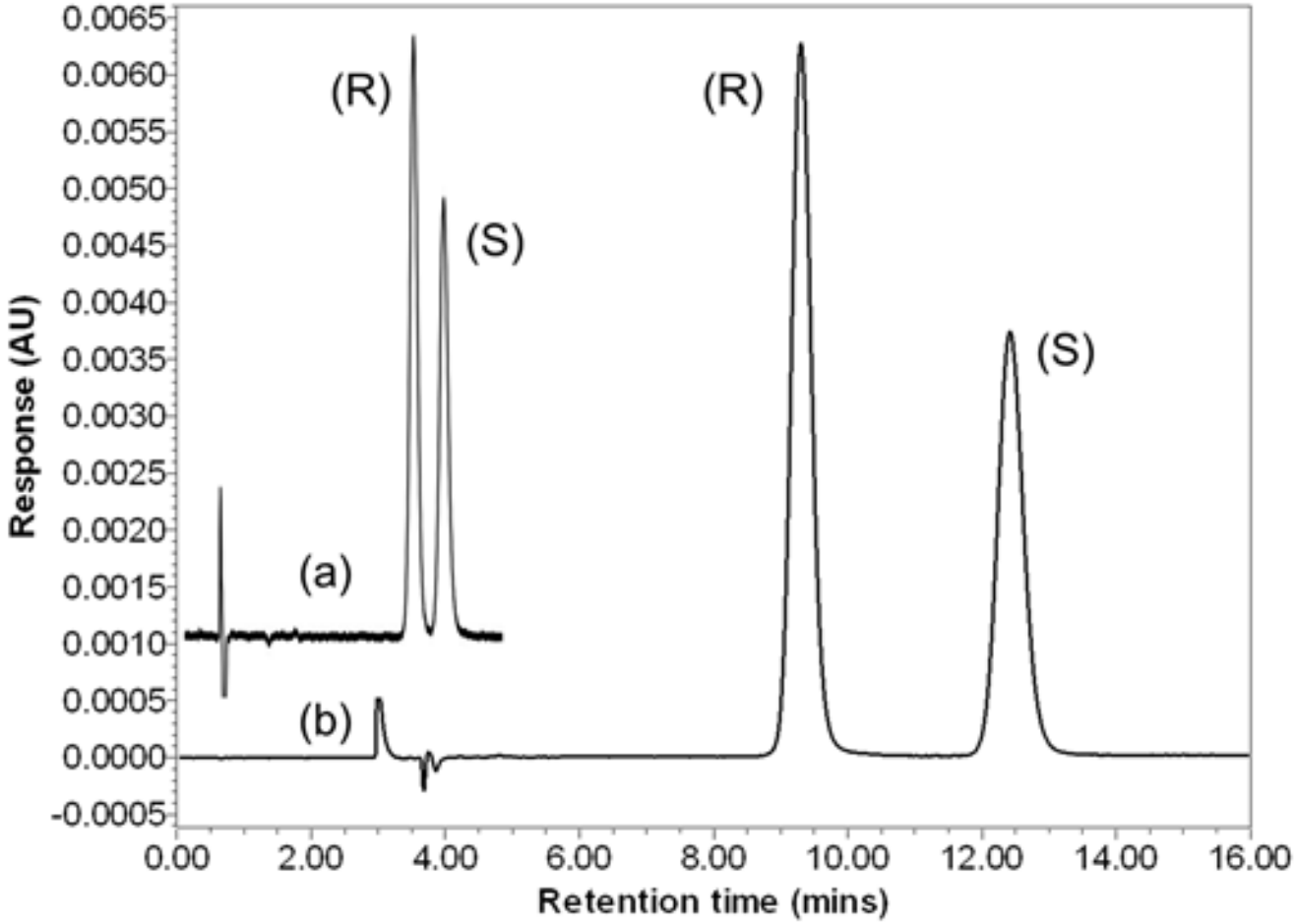


Figure 4: *Comparison of optimised SFC separation (a) and normal phase separation (b) of timolol enantiomers. Chromatographic conditions for (a) are as given in Figure 2(b). Chromatographic conditions for (**b**); Column: Chiralcel OD-H, 4.6 mm x 250 mm, 5 µm. Mobile phase: hexane/2-propanol/DEA (960:40:2). Flow rate: 1.0 mL.min^{-1}. Injection volume: 5 µL. Column temperature: Ambient. Detection: UV at 297 nm.*

L.O.D determination

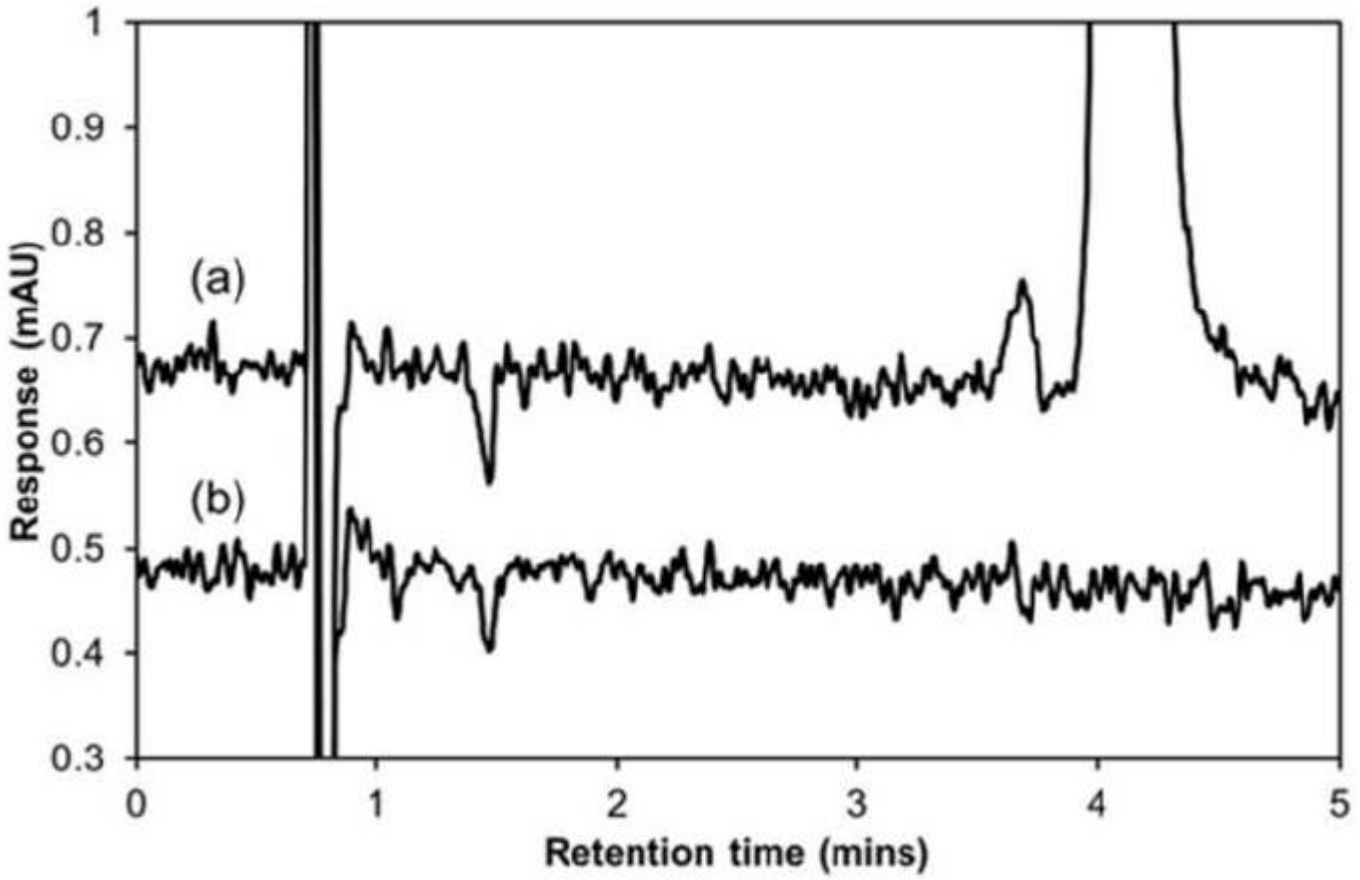
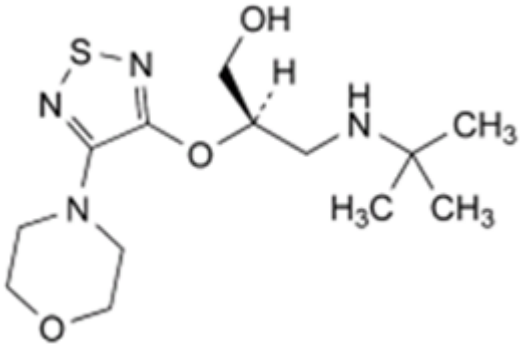
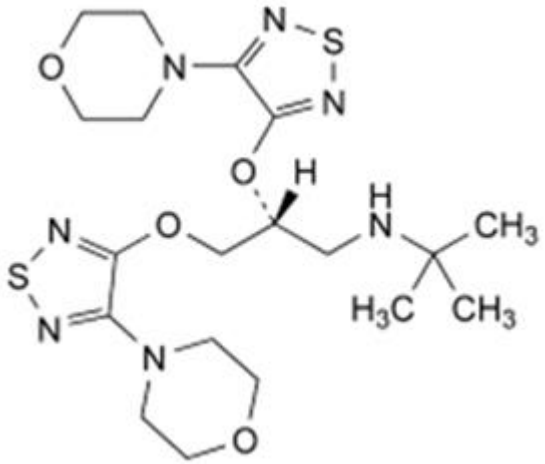


Figure 5: *R-timolol at 0.5 % w.r.t S-timolol maleate. S/N ratio: 3.0. Chromatographic conditions as in Figure 4a.*

European Pharmacopoeia specified impurities in Timolol Maleate



IMP B



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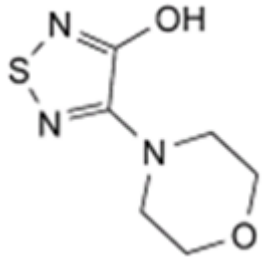

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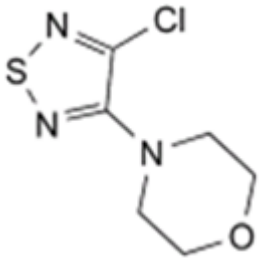

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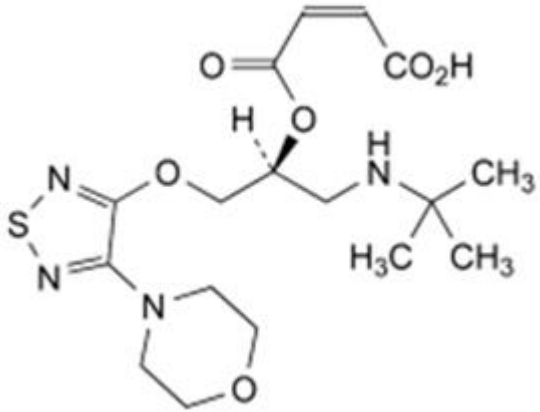

IMP E

Specificity study

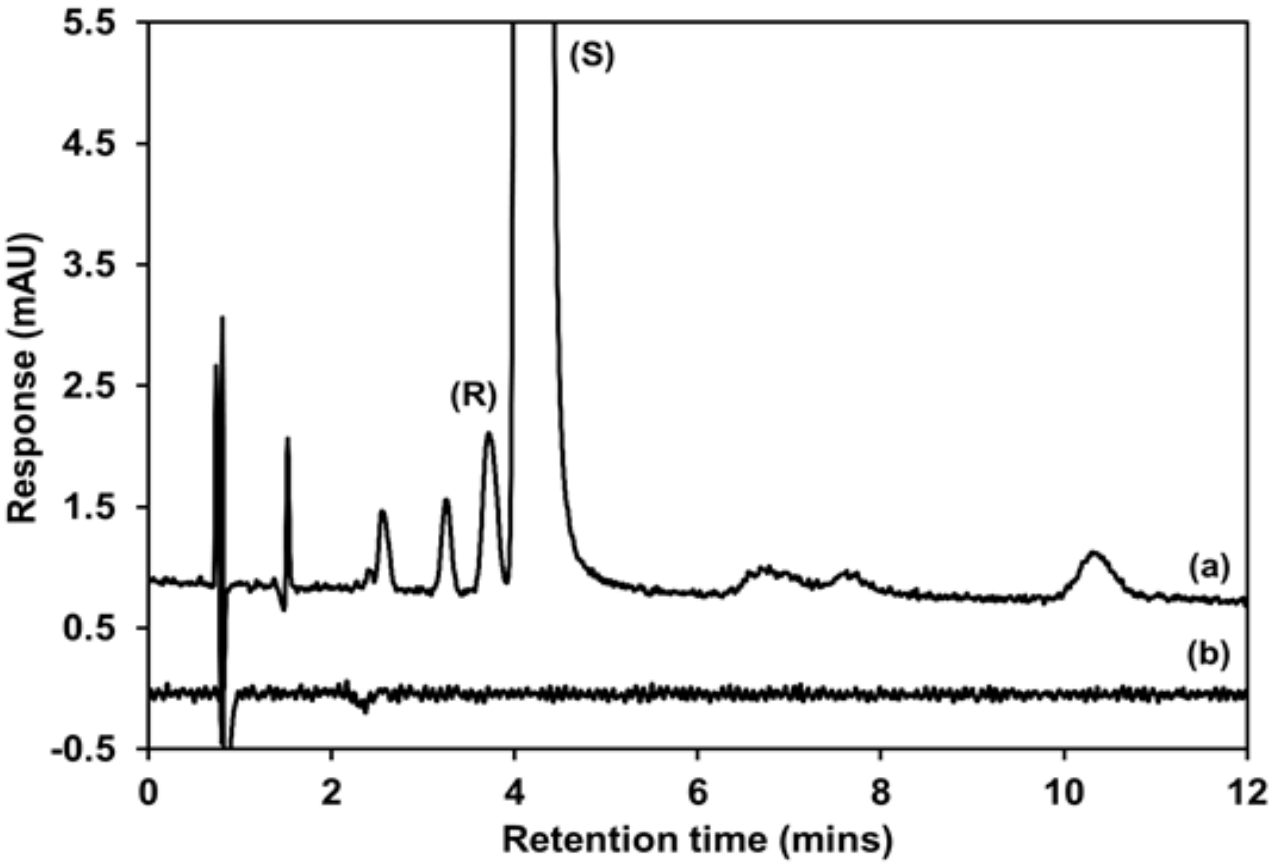

Figure 6: *(a) Resolution mixture spiked with known EP impurites. (b) MeOH blank. Chromatographic conditions in Figure 4a.*

Method robustness studies

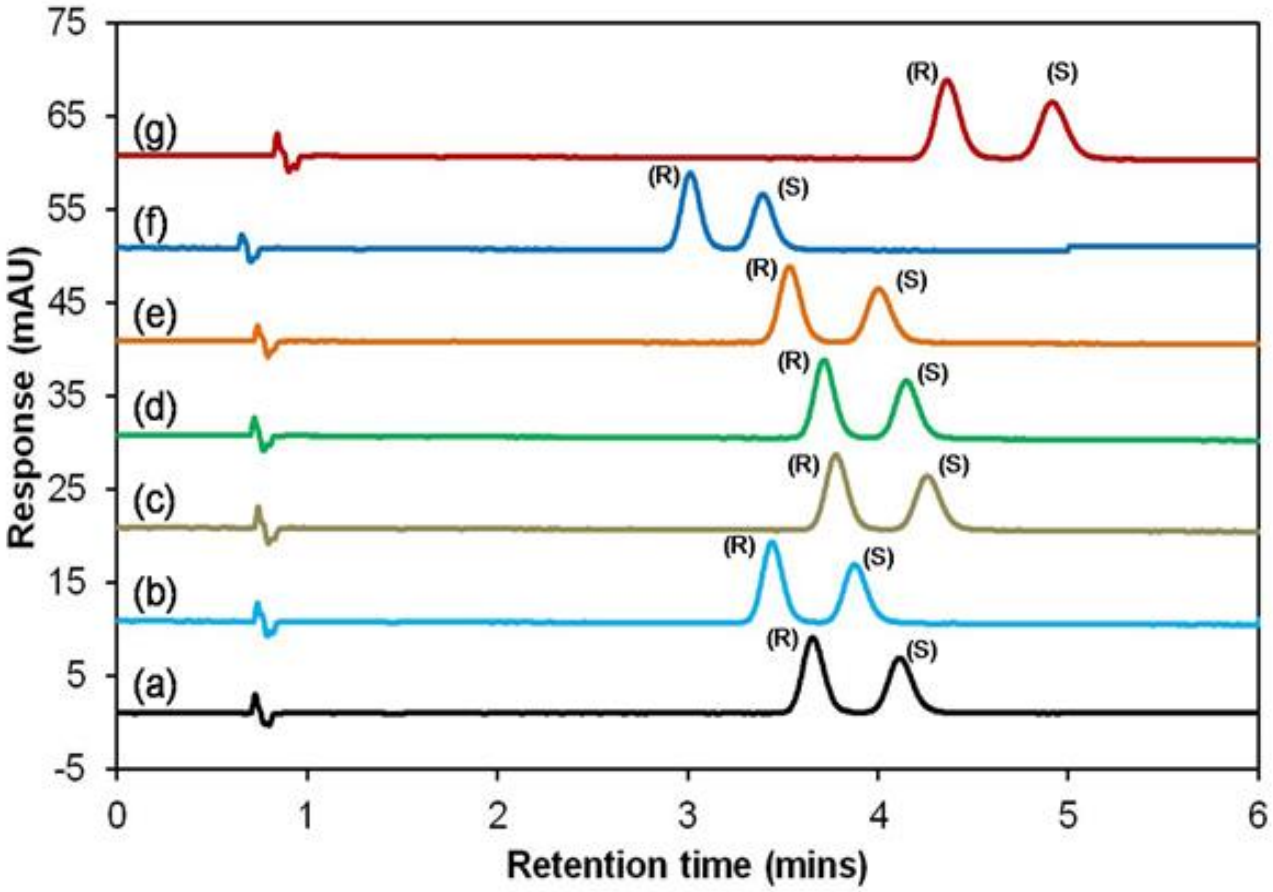

Figure 7: *Typical chromatograms of the resolution mixture containing R-timolol and S-timolol maleate at 0.0045 % (w/v). (**a**): Optimised conditions. (**b**): 140 bar (**c**): 120 bar (**d**): 45 °C (**e**): 35 °C (**f**): 4.5 mL.min^{-1} and (g), 3.5 mL.min^{-1}.*

Analytical performance criteria

Robustness

Condition	R-timolol RRT[b]	Rs[b,c]	Selectivity[b]	R-timolol peak symmetry[b]	S-timolol peak symmetry[b]	R-timolol % area[d]	Equivalency[e]
Optimum conditions[a]	0.89	2.0	1.12	0.89	0.86	1.05	1.0
Flow rate: 4.5 mL.min^{-1}	0.89	1.9	1.13	0.90	0.86	1.03	1.0
Flow rate: 3.5 mL.min^{-1}	0.89	2.2	1.13	0.86	0.85	1.05	1.0
Column temperature: 35 °C	0.88	2.0	1.13	0.90	0.83	1.07	1.0
Column temperature: 45 °C	0.90	2.0	1.12	0.92	0.86	1.05	1.0
Backpressure: 120 bar	0.89	2.1	1.13	0.88	0.85	1.08	1.0
Backpressure: 140 bar	0.89	2.0	1.13	0.90	0.84	1.08	1.0

Repeatability and intermediate precision

	Standard 1		Standard 2		Standard 3	
	Day-1	Day-2	Day-1	Day-2	Day-1	Day-2
R-timolol retention time % RSD	< 0.1 %	< 0.1 %	< 0.1 %	< 0.1 %	< 0.1 %	< 0.1 %
R-timolol peak area % RSD	2.1 %	1.8 %	2.7 %	2.0 %	2.0 %	2.7 %

Limit of detection

	S/N	% area
R-timolol	3.4	0.52

Accuracy

R-Timolol	101 %

[a] Flow rate: 4.0 mL.min^{-1}, Column temperature: 40 °C, backpressure regulation: 130 bar.
[b] n=3.
[c] Rs calculated as $R = 2(t_{r1} - t_{r2})/(W_{B(1)} - W_{B(2)})$
[d] n=6
[e] Ratio of analytical performance criterion versus performance under optimum conditions

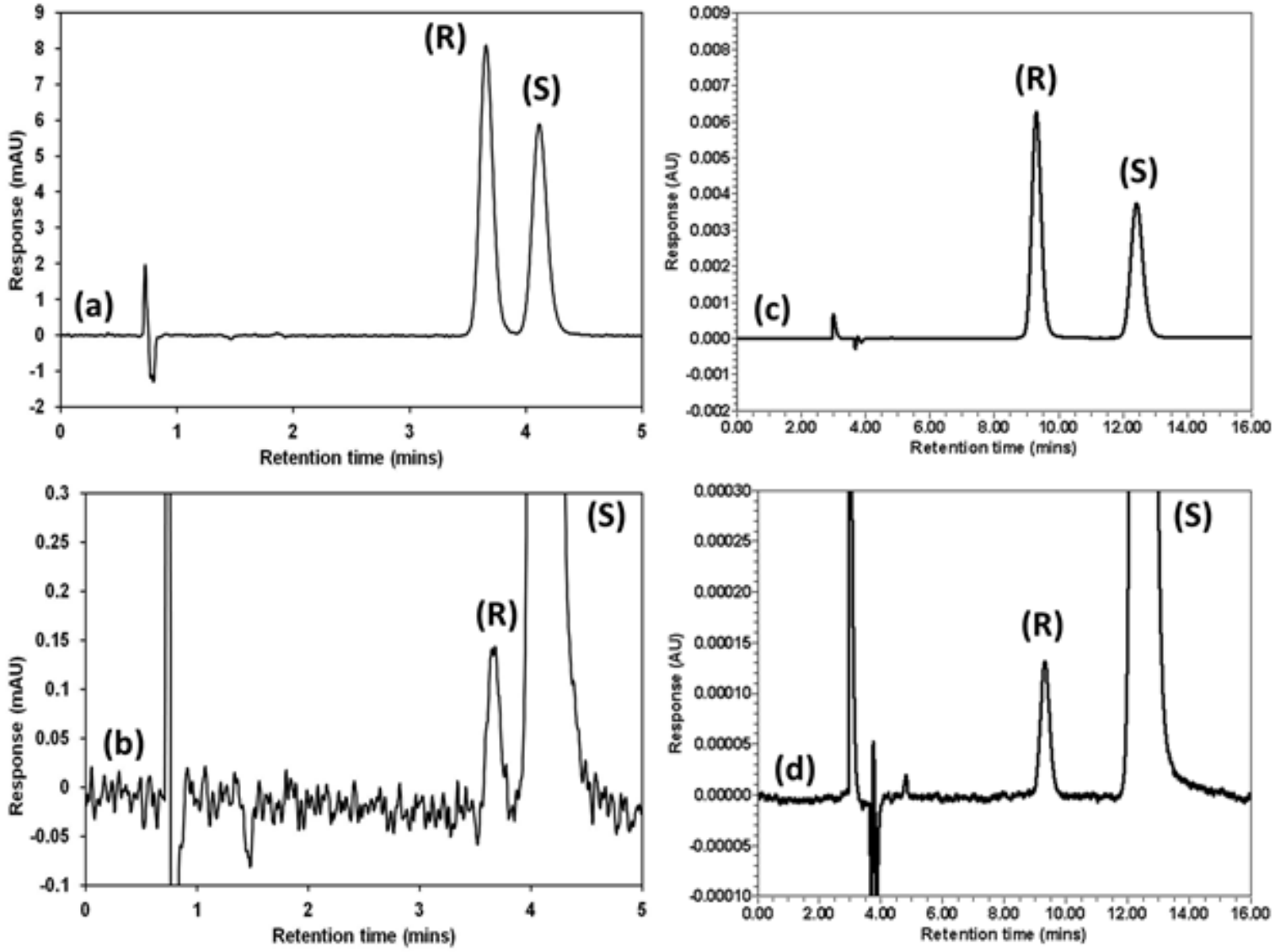

Figure 8: *Typical chromatograms comparing SFC (**a,b**) and NP-HPLC (**c,d**) for the analysis of R-timolol in the presence of S-timolol maleate. R-timolol present at 1.0 % w.r.t S-timolol.*

Comparison of analytical performance criteria: SFC vs NPLC

Parameter	SFC	NP-HPLC
R-timolol relative response factor (RRF)	0.83	0.80
R-timolol relative retention time (RRT)	0.89	0.75
R-timolol, S-timolol tailing factor	1.1, 1.1	1.2, 1.3
R-timolol, S-timolol plate count	18,464 N/m, 18,324 N/m	18,064 N/m, 18,676 N/m
Resolution	2.0	4.8
S-timolol peak area repeatability[a]	0.2 %	0.4 %
R-timolol peak area repeatability[b]	2.1	2.5
% recovery of R-timolol[c]	101 %[c]	98 %[c]
S-timolol working standard concentration	0.009 % (w/v)	0.003 % (w/v)
Analysis time per sample (min)	5	16
Solvent usage per sample (mL)	1.4	16

[a] (n=6)
[b] 1.0 % R-timolol in S-timolol, n=6
[c] 1.0 % R-timolol in S-timolol

"Determination of (R)-timolol in (S)-timolol maleate active pharmaceutical ingredient: Validation of a new SFC method with an established NPLC method", Adrian Marley and Damian Connolly, ***Journal of Chromatography A*** *(2013) accepted.*

Conclusions

- NPLC chiral method transferred to SFC
- 3-fold reduction in runtime
- (4-fold increase in flow rate)
- 11-fold reduction in solvent consumption

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Agilent Technologies