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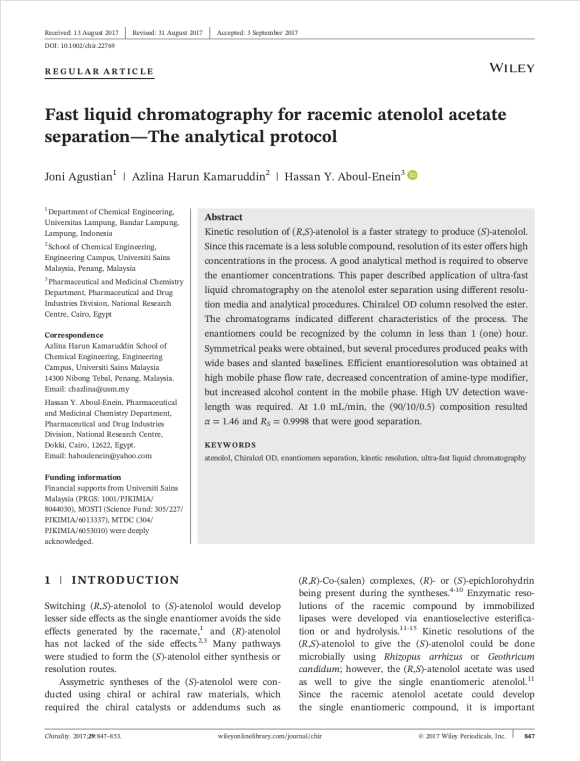

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REGULAR ARTICLE

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Fast liquid chromatography for racemic atenolol acetate separation—The analytical protocol

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Abstract
Kinetic resolution of (*R,S*)-atenolol is a faster strategy to produce (*S*)-atenolol. Since this racemate is a less soluble compound, resolution of its ester offers high concentrations in the process. A good analytical method is required to observe the enantiomer concentrations. This paper described application of ultra-fast liquid chromatography on the atenolol ester separation using different resolution media and analytical procedures. Chiralcel OD column resolved the ester. The chromatograms indicated different characteristics of the process. The enantiomers could be recognized by the column in less than 1 (one) hour. Symmetrical peaks were obtained, but several procedures produced peaks with wide bases and slanted baselines. Efficient enantioresolution was obtained at high mobile phase flow rate, decreased concentration of amine-type modifier, but increased alcohol content in the mobile phase. High UV detection wavelength was required. At 1.0 mL/min, the (90/10/0.5) composition resulted $\alpha = 1.46$ and $R_S = 0.9998$ that were good separation.

KEYWORDS
atenolol, Chiralcel OD, enantiomers separation, kinetic resolution, ultra-fast liquid chromatography

1 | INTRODUCTION

Switching (*R,S*)-atenolol to (*S*)-atenolol would develop lesser side effects as the single enantiomer avoids the side effects generated by the racemate,[1] and (*R*)-atenolol has not lacked of the side effects.[2,3] Many pathways were studied to form the (*S*)-atenolol either synthesis or resolution routes.

Assymetric syntheses of the (*S*)-atenolol were conducted using chiral or achiral raw materials, which required the chiral catalysts or addendums such as (*R,R*)-Co-(salen) complexes, (*R*)- or (*S*)-epichlorohydrin being present during the syntheses.[4-10] Enzymatic resolutions of the racemic compound by immobilized lipases were developed via enantioselective esterification or and hydrolysis.[11-15] Kinetic resolutions of the (*R,S*)-atenolol to give the (*S*)-atenolol could be done microbially using *Rhizopus arrhizus* or *Geothricum candidum*; however, the (*R,S*)-atenolol acetate was used as well to give the single enantiomeric atenolol.[11] Since the racemic atenolol acetate could develop the single enantiomeric compound, it is important

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1 | INTRODUCTION

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to estimate both enantiomers' concentration during the resolution processes.

Although high-performance liquid chromatography (HPLC) is the most widely used method to quantify the atenolol enantiomers using columns such as Chiralcel OD,[6-10,16-18] Amycoat,[19] Eurocel 01,[20] RegisCell,[21] Chiral-CBH,[22] Chirex 3022 (S),[23,24] and Chirobiotic V2,[25] which were operated at various protocols, only some articles were related to the atenolol esters measurement. The racemic atenolol caproate and acetate were analyzed using infrared spectrometer or nuclear magnetic resonance or thin layer chromatography.[11,26,27] Previously, Enquist and Hermansson[28] observed atenolol diacetate concentrations using HPLC column of α1-AGP working under water-based mobile phase. As the enzymatic reactions mostly occurred in organic solvents, direct measurement of the (*R*,*S*)-atenolol acetate during the reactions would reduce the analytical time. To the best of our knowledge, there has been no article mentioning chromatograms of the (*R*,*S*)-atenolol acetate generated by ultra-fast liquid chromatography (UFLC) where the racemate exists in organic media. Hence, selection of a column and an analytical protocol that can satisfy observations of the (*R*,*S*)-atenolol acetate concentrations during the reaction using UFLC is essential.

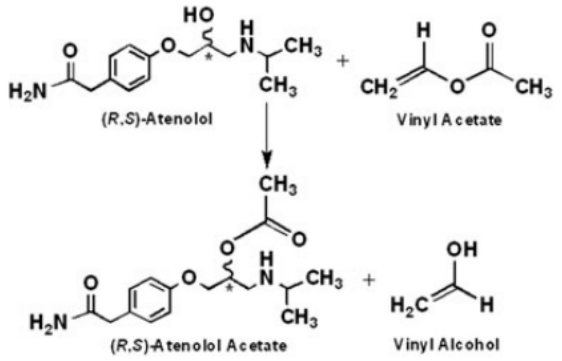

FIGURE 1 Formation of the racemic atenolol acetate

2 | MATERIALS AND METHODS

2.1 | Materials

(*R*,*S*)-atenolol (99%), (*S*)-atenolol (97%), and (*R*)-atenolol (99%) were bought from Nanjing Chemlin Chemical Industry Co Ltd (China), Tocris Bioscience (England), and Sigma-Aldrich (M) Sdn Bhd (Malaysia), respectively. All chemicals were of analytical grade except for analysis (of HPLC grade) and bought from EOS Scientific (M) Sdn Bhd, Fisher Scientific (M) Sdn Bhd, Merck Sdn Bhd, and Sigma-Aldrich (M) Sdn Bhd. Atenolols and other chemicals were used without purification.

2.2 | Synthesis of (*R*,*S*)-atenolol acetate, (*S*)-atenolol acetate, and (*R*)-atenolol acetate

Synthesis of the ester was conducted through chemical acetylation of (*R*,*S*)-atenolol (Figure 1). It was started by dissolving 8 g racemate and 3.4 mL acetic anhydride into 180 mL acetone under vigorous stirring for 5 minutes. The mixture was refluxed at 333 to 338 K for 3 hours. After acetylation, solvent was removed under vacuum. The collected residue was diluted with 25 mL deionised water and stirred for around minutes at room temperature. Later, 100 mL dichloromethane was added to the solution and the stirring was continued for 30 minutes. The mixture was transferred to a separating funnel and left for half an hour at room temperature. After the aqueous phase was removed, the organic layer was placed into 250 mL glass beaker and then extracted with 50 mL sodium carbonate solution (1% *w*/*v*) with stirring for 30 minutes. After that, the mixture was then settled for half an hour, and the organic phase was collected. These steps were repeated 5 times, but the last settling was conducted overnight. Once the aqueous phase was removed, the organic layer was then placed under constant air flows and stirring to evaporate the solvent. The crystals were finally dried overnight at 318 to 323 K. After drying, the crystals were crushed into powder and were stored at

TABLE 1 Characteristics of (*R*,*R*)-Whelk O1 chiral column

Mobile Phase	Operating Conditions	Injection (5 μL)	Peak separation	Chromatogram
Hex/EtOH/DEA (75/25/0.1)	35°C/275 nm/0.75 mL/min	(*R*,*S*)-atenolol	No	Figure 2A
Hex/EtOH/DEA (75/25/0.1)	35°C/275 nm/0.75 mL/min	(*R*,*S*)-ester	No	Figure 2B
Hex/EtOH/DEA (75/25/0.1)	35°C/254 nm/0.75 mL/min	(*R*,*S*)-ester	No	Figure 2C
Hex/EtOH/DEA (75/25/0.1)	35°C/254 nm/0.75 mL/min	(*R*,*S*)-atenolol	No	Figure 2D
Hex/EtOH/DEA (80/20/0.1)	35°C/254 nm/0.75 mL/min	(*R*,*S*)-ester	No	Figure 2E
Hex/IPA (90/10)	35°C/254 nm/0.75 mL/min	(*R*,*S*)-ester	No	Figure 2F

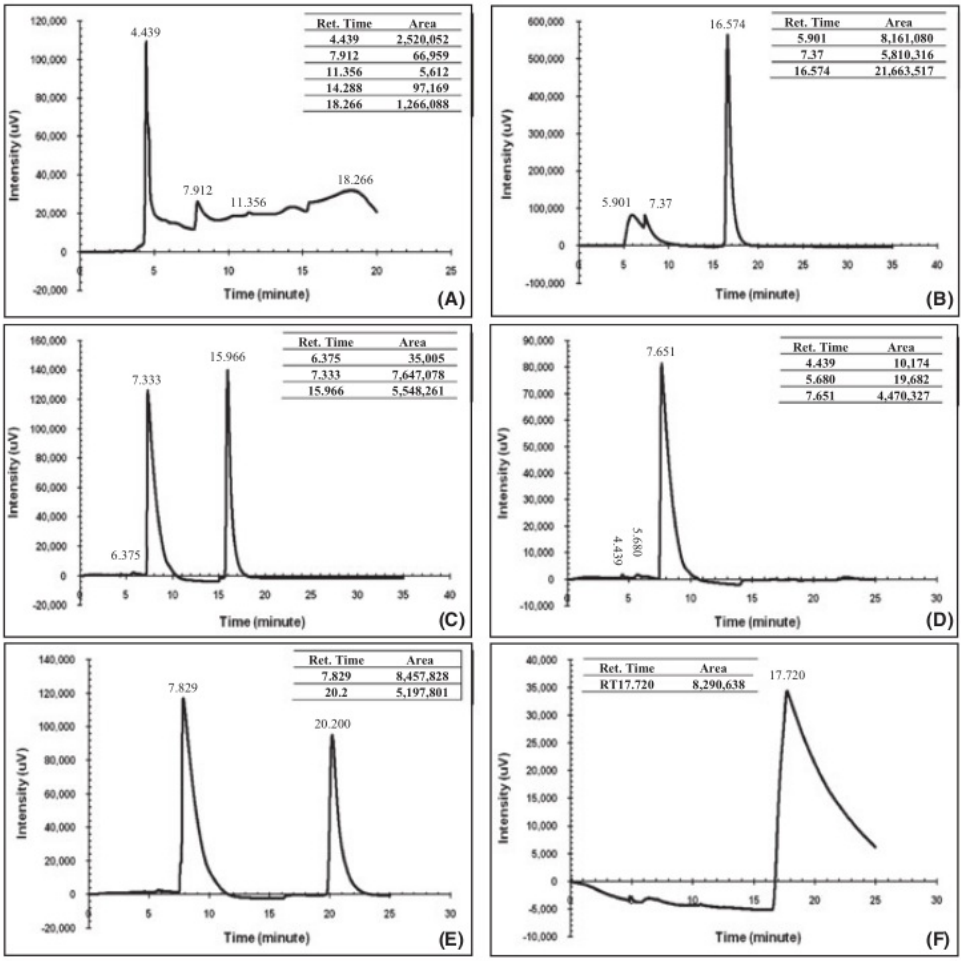

FIGURE 2 Chromatograms racemic atenolol and its ester (A: racemic atenolol; B: racemic atenolol ester; C: racemic atenolol ester; D: racemic atenolol; E: racemic atenolol; F: racemic atenolol ester. Chromatographic conditions are presented in Table 1

TABLE 2 Protocols for (*R*,*S*)-atenolol ester

Protocol	Mobile Phase	Flow Rate, mL/min	α	R_S	Chromatogram
A	60[a]/40[b]/0.1[c]	0.50	No data	No data	Figure 3C
B*	75[a]/25[b]/0.1[c]	0.50	1.18	0.3838	Figure 3D
C	80[a]/20[b]/0.1[c]	0.50	1.14	0.3135	Figure 3E
D	85[a]/15[b]/0.1[c]	0.50	1.16	0.7163	Figure 3F
E	85[a]/15[b]/0.5[c]	0.50	1.18	0.5389	Figure 3G
F	90A/10B/0.1C	0.50	1.17	0.9260	Figure 3H
G	90[a]/10[b]/0.4[c]	0.50	1.18	0.7843	Figure 3I
H	90A/10B/0.4C	0.85	1.17	0.8013	Figure 3J
I	90[a]/10[b]/0.1[c]	1.00	1.17	0.7766	Figure 3K
J	90[a]/10[b]/0.5[c]	1.00	1.46	0.9998	Figure 3L

[a]Hexane;

[b]Ethanol;

[c]Diethylamine; UFLC: 35°C, 275 nm;

*The starting protocol.

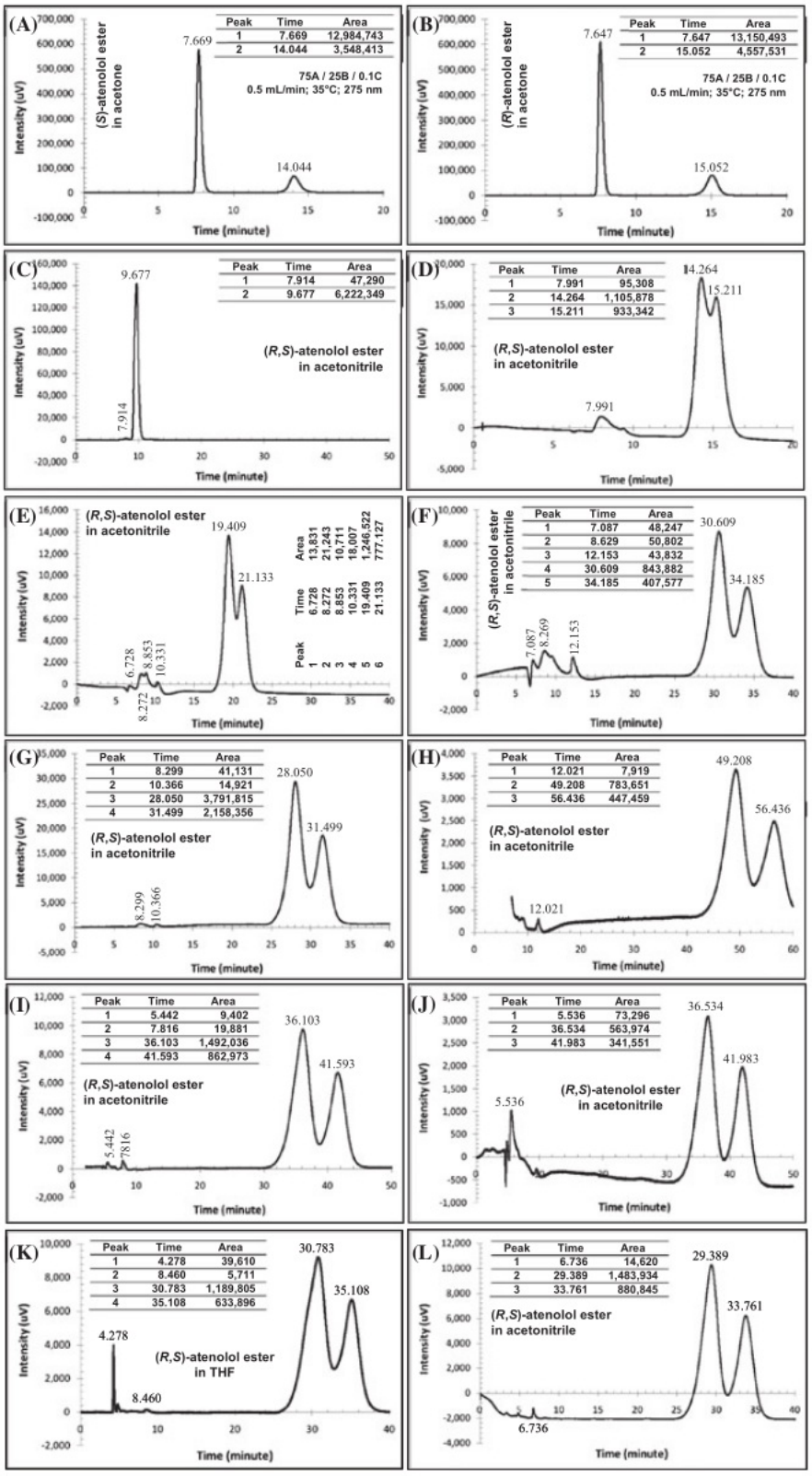

FIGURE 3 Chromatograms of atenolol acetate. Chromatographic conditions are shown in Table 2

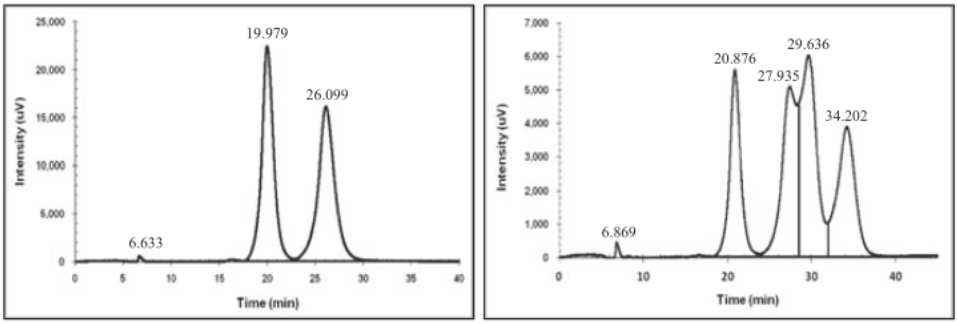

FIGURE 4 Peaks of racemic atenolol (left) and mixture of racemic atenolol and its ester (right) developed by using a mobile phase composed of hexane:ethanol :diethylamine (90:10:0.5 (v.v.v)

room temperature before used. These steps were copied for synthesis of (*S*)-atenolol acetate and (*R*)-atenolol acetate.

2.3 | Apparatus

Analysis of the enantiomers was performed on a Shimadzu UFLC LC-20A Prominence system. The system consist of 2 units LC-20 AD dual plunger parallel flow solvent delivery pump, a SIL-20ACHT auto-sampler, a SPD-20A UV-VIS detector, a CTO-20 AC column oven, a DGU-A3 degasser unit, and the CBM-20A system controller. A personal computer is linked to the UFLC using the Shimadzu LC solution Real Time Application software.

2.4 | Samples preparation and analysis

About 1 to 5 μL of 25 mM (*R*,*S*)-atenolol acetate dissolved in organic solvents was injected automatically at a time into the HPLC column with certain a mobile phase composition (described later). U*V*/Vis detector was set at the wavelength of 254 to 276 nm. The UFLC was operated at normal phase at 35°C. Qualitative and quantitative analysis were conducted on chromatograms via Shimadzu LC solution postrun analysis software based on the standard procedure for this instrumentation.[29]

3 | RESULTS AND DISCUSSION

The first column tested to identify the racemic atenolol ester was (*R*,*R*)-Whelk O1 column made by Regis Technologies Inc (Illinois). The column has been used extensively to measure the nonsteroidal anti-inflammatory drugs such as ibuprofen and ketoprofen and has the same mobile phase component as Chiralcel OD. The experimental results using the column were shown in Table 1. The first mobile phase used to identify the racemic atenolol, and its ester was similar with the mobile phase applied in the transesterification process.

The chromatogram for the racemic atenolol was not high, but the column provided a good measurement of the racemic atenolol ester (Figure 2A,B. When the wavelength was reduced to 254 nm, the column identified the racemic atenolol better than the previous method and no change on the ester peak was observed. Similar results were found when the mobile phase was changed to a lower alcohol content (80/20/0.1% (*v*/v)). But the mobile phase with the lowest alcohol content gave a peak with a very wide base. In general, the racemic atenolol and its ester appeared at minute 7 and minute 16, respectively. The column can identify the racemic atenolol and its ester but cannot separate the enantiomers.

Since no peak separations were found with the (*R*,*R*)-Whelk O1 column, Chiralcel OD was used to separate the racemic atenolol ester in the same manner as the racemic atenolol. Comparison of α and R_S developed by various mobile phase compositions and flow rates using the available UFLC unit were given in Table 2.

In general, each mobile phase composition and flow rate gave a moderate selectivity. The resulted values were found in the range of 1.14 to 1.18. Although the alcohol content had been decreased to the same with the storage mobile phase composition and the flow rate was increased to the typical column flow rate (1.0 mL/min), the selectivity values were almost the same. But the big difference was observed from the R_S.

As described in Table 2, the R_S differed greatly. The separation factor for the starting protocol produced the lowest R_S value amongst the tested protocols ($\alpha = .3838$). However, this protocol was better than the protocol that used the highest alcohol content (40%), which gave no racemic ester separation (Figure 3C). Lower alcohol content indeed developed higher R_S than the starting method under the same mobile phase flow

rate (0.5 mL/min, Protocol A-G). At this flow rate, the highest R_S was found at the mobile phase composition of 90%-hexane + 10%-ethanol (Protocol G). This composition was applied during all the racemic ester analysis as it will risk the column if the mobile phase has lower alcohol content than the column storage composition because the atenolol does not dissolve in hexane.

Although the flow rate of 0.5 mL/min offered higher R_S, the typical flow rate for the column was chosen as the analytical mobile phase flow rate (Protocol I, J) since this flow rate gave faster enantiomeric separation time (shown in Figure 3) and higher flow rates tended to provide better enantiomeric resolution factors. Observation on the modifier compound indicated that lower modifier quantity developed higher R_S at low flow rate, but higher flow rate produced higher R_S.

Based on the starting protocol, the (*S*)-atenolol ester appeared faster than its opponent as given in Figure 3A, B. Its retention time was 14.044 minutes, while the (*R*)-atenolol ester was detected at 15.052 minute. The mobile phase with higher alcohol content than the starting protocol indeed gave quicker retention time as shown in Figure 3C, but no racemic atenolol ester separation was developed. Other UFLC protocols produced the racemic atenolol ester retention times slower than the starting protocol, but some of these protocols gave better enantiomeric separation.

The starting protocol developed an overlapping situation of enantiomeric peaks similar to the Protocol C, although both enantiomers' peaks appeared faster. This was not good as the racemic ester could not be separated finely. Although the alcohol content of 15% (Protocol D, E) provided faster retention time than the storage mobile phase composition under the same flow rate, the later protocol developed better racemic ester separation. Hence, the (90/10) content was selected as the mobile phase composition. This content was able to provide peaks of the racemic atenolol and its ester vmixture (Figure 4). Separation of the racemic atenolol enantiomers produced by this mobile phase composition using 0.5% DEA content gave a bigger difference on the peaks retention time than the mobile phase used in the transesterification stage (ie, the starting protocol for the hydrolysis stage). Further observations on this content suggested that the flow rate of 1.0 mL/min offered faster retention time than its rivals (0.5 and 0.85 mL/min). Under this flow rate, the (90/10/0.5) composition resulted $\alpha = 1.46$ and $R_S = 0.9998$ that were good for enantiomers separation. But overlap peaks were observed between the (*S*)-atenolol and (*S*)-atenolol ester when both racemates were mixed. The modifier content also influenced chromatographic intensity where higher modifier created higher peaks area.

4 | CONCLUSION

The Chiralcel OD column could separate the (*R*,*S*)-atenolol acetate enantiomers, which required almost 1 hour separation time. Both enantiomeric peaks appeared symmetrically, but several protocols gave peaks with wide bases and slanted baselines. The experiments indicated that efficient enantioresolution of (*R*,*S*)-atenolol acetate was obtained at high mobile phase flow rate, decreased concentration of amine-type modifier, but increased alcohol content in mobile phase. High UV detection wavelength was required. At 1.0 mL/min, the (90/10/0.5) mobile phase composition resulted $\alpha = 1.46$ and $R_S = 0.9998$ that were good for enantiomers' separation.

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The authors declare no conflict of interest.

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