Chromatographic Comparison of Atenolol Separation in Reaction Media on Cellulose tris-(3,5-dimethylphenylcarbamate) Chiral Stationary Phase Using Ultra Fast Liquid Chromatography

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ABSTRACT Because chiral liquid chromatography (LC) could become a powerful tool to estimate racemic atenolol quantity, excellent enantiomeric separation should be produced during data acquisition for satisfactory observation of atenolol concentrations throughout the racemic resolution processes. Selection of chiral LC column and analytical protocol that fulfill demands of the ultra fast LC analysis is essential. This article describes the characteristics of atenolol chromatographic separation that resulted from different resolution media and analytical protocols with the use of a Chiralcel® OD column. The chromatograms showed quite different characteristics of the separation process. The single enantiomer and racemic atenolol could be recognized by the Chiralcel® OD column in less than 20 min. Symmetrical peaks were obtained; however, several protocols produced peaks with wide bases and slanted baselines. Observations showed that efficient enantioresolution of racemic atenolol was obtained at slow mobile phase flow rate, decreased concentration of amine-type modifier but increased alcohol content in mobile phase and highest ultraviolet detection wavelength were required. The optimal ultra fast LC protocol enables to reduce and eliminate the peaks of either the atenolol solvent or the buffers and provided the highest peak intensities of both atenolol enantiomers. *Chirality 24:356-367, 2012.*

KEY WORDS: racemic atenolol; Chiralcel® OD Column; enantiomers separation; analytical protocol; organic; buffers media

INTRODUCTION

Atenolol (AT, Fig.1) chemically known as 2-{4-[2-hydroxy-3-(propan-2-ylamino)propoxy]phenyl}acetamide is a famous β-adrenergic receptor blocker used for maintenance of blood pressure, angina pectoris, and arrhythmia.[1,2] This β-blocker is one of the best-selling drugs in the world [3–5] and is mainly sold in the form of racemate, which has the eudismic ratio of 46.[6] Its active isomer resides on (*S*)-enantiomer,[7] which is found to avoid side effects generated by the racemic form,[8] whereas the (*R*)-AT does not show the β-blocking activity and has not lacked of the side effects.[9,10] Because the inactive isomers of the β-adrenergic blockers bring serious health problems[11] and switching from racemate to single enantiomer develops lesser side effects,[12] many pathways have been constructed to form the optically active AT.

Highlights of the pathways to produce the (*S*)-AT are given in previous literatures.[13,14] The compound was synthesized asymmetrically from racemic or achiral raw materials that required the presence of chiral metal ligand catalysts such as (*R*,*R*)-Co-(salen) complexes to carry out the kinetic resolution processes.[15–17] The (*S*)-enantiomer was also prepared from catalytic reactions of achiral feeds without involvement of a chiral catalyst, but a chiral addendum such as (*R*)- or (*S*)-epichlorohydrin must be available.[2,18–20] Further microbial resolution of the racemic AT and its ester with the use of *Rhizopus arrhizus* and *Geothricum candidum* gave the single enantiomer in high optical purity and chemical yield.[21] Enzymatic resolution of the racemate using immobilized lipases was developed as well through enantioselective esterification and hydrolysis processes.[22,23] Although chromatographic resolution is difficult to appear in large-scale operation,[12] the chromatographic technology, which becomes a powerful tool in the development of chiral drugs,[24–26] has used various chiral selectors to separate the AT active isomer.[27,28]

Because the racemic AT resolutions have been developed, chromatography could become an effective method to estimate reduction of AT concentration/quantity during the resolving processes. Thin layer and gas chromatography[21,22,27–29] have been associated with the development, but high performance liquid chromatography (HPLC) is the most widely used technique. Many HPLC columns (Chiralcel® OD,[16–20,30,31] Amycoat,[32] Eurocel 01,[33] RegisCell,[34] Chiral-CBH,[35] Chirex 3022 (S),[36,37] and Chirobiotic™ V2[38]) have been examined under various analytical protocols to perform the AT enantiomeric separation, which resulted to various separation factors and chiral chromatograms.

Because conversion, enantiomeric excess (ee), and enantiomeric ratio (E) of the resolution processes are based on the HPLC areas of the (*R*)- and (*S*)-enantiomers, good peak shapes/chiral chromatograms must be produced during the separation. To the best of our knowledge, there has been no

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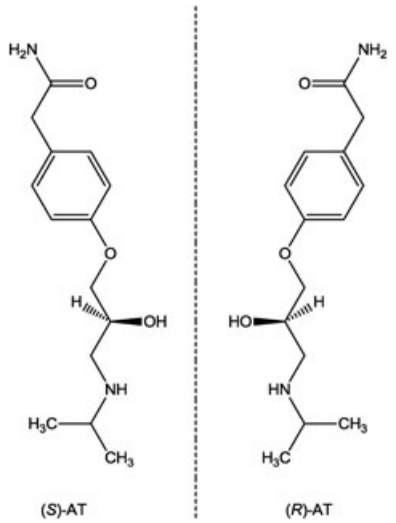

Fig. 1. A mirror image of atenolol enantiomers.

article mentioning the chiral chromatograms of the racemic AT generated by the HPLC analysis on this racemate existing in the reaction media. As part of our project in resolution of the racemate enzymatically, selection of a column and analytical protocol that can satisfy observations of the AT concentration during the reaction time is essential. Because the observations are based on the AT peaks' areas, herewith we describe characteristics of the chiral chromatograms that resulted from different reaction media and general protocols on a cellulose tris-(3,5-dimethylphenylcarbamate) chiral stationary phase (CSP, Chiralcel® OD column, Daicel Chemical Industries Ltd., Osaka, Japan) using an ultra fast liquid chromatography (UFLC) unit. Because of the racemic AT not being soluble in very hydrophobic solvents, which could risk the column, water miscible solvents are decided to be used as the reaction media. Comparisons of the analytical protocols are made to give useful description on the analytical protocols applied in the AT resolution studies.

MATERIALS AND METHODS

Materials

(*R,S*)-AT (99% pure), (*S*)-AT (97% pure), and (*R*)-AT (99% pure) were bought from Nanjing Chemlin Chemical Industry Co. Ltd (Nanjing, China), Tocris Bioscience (Bristol, UK), and Sigma Aldrich (M) Sdn Bhd (Malaysia), respectively. The racemate was dissolved directly in the reaction media according to the desired concentrations. Several standard solutions of pure enantiomers were prepared by dissolving their crystals into dimethyl sulfoxide with concentrations of 9 mg/ml. 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. The AT and other chemicals were used without purification. *Candida antarctica* lipase fraction B (Lipozyme® CALB L LCN02103 supplied by Science Technics Sdn Bhd, Malaysia), lipase basic kit (62327) from Fluka Analytical containing *Pseudomonas fluorescence* lipase (95608), hog pancreatic lipase (62300), and lipoprotein lipase from *Pseudomonas sp*. (62336) (supplied by Sigma Aldrich), and Amano lipase PS from *Burkholderia cepacia* (supplied by Sigma Aldrich) catalyzed the enzymatic reactions. All lipases were used without initial treatment.

Apparatus

Analysis of the AT enantiomers was performed on a Shimadzu UFLC LC-20A Prominence (Kyoto, Japan) system. The system consist of two units LC-20 AD dual plunger parallel flow solvent delivery pump, a SIL-20ACHT auto-sampler, a SPD-20A ultraviolet–visible (UV–vis) detector, a CTO-20 AC column oven, a DGU-A3 degasser unit, and the CBM-20A system controller. The UFLC was connected to a personal computer to operate the equipment using the Shimadzu LCsolution real time application software.

Preparation of Buffer Solution

Several 67 mM Sorensen buffers were prepared by mixing KH_2PO_4 and Na_2HPO_4 solution. A certain quantity of the KH_2PO_4 (Acros Organics, Fisher Scientific, Malaysia, 99%+) and Na_2HPO_4 (Acros Organics, Fisher Scientific, Malaysia, 98%+) were dissolved in double distilled water to form 133 mM buffers. Both solutions were then adjusted to the desired pH values and then diluted with the double distilled water to give the 67 mM buffers.

Sample Preparation and Analysis

The enzymatic reaction of the racemic AT was carried out for 36 h. During interval times, 1 ml sample was taken out from each reaction flask and placed inside a 1.5-ml vial then kept in 4 °C refrigerator prior to analysis. Before injection, all samples were either filtered through 0.45 μm membrane filter using syringe or centrifuged at 2000 g using Profuge 6k centrifuge (supplied by Interscience Sdn Bhd, Malaysia) for 15–20 min where 500–650 μl of the centrifuged samples were taken out and transferred into 1.5-ml clean bottles.

A 1–2 μl of samples were injected automatically at a time into the Chiralcel® OD column (250 × 4.6 mm) with the mobile phase as stated in Table 1. The UV–vis detector was set at the wavelength of 254–276 nm. The UFLC was operated at normal phase at 35 °C. Qualitative and quantitative analyses were conducted on the resulted chromatograms via the Shimadzu LCsolution post-run analysis software on the basis of the standard procedure for the instrumentation.[39]

Lipase-catalyzed Acetylation of (R,S)-atenolol

Reaction in organic media. A 5 mg/ml racemic AT was prepared in dimethyl sulfoxide (DMSO), dimethylformamide (DMF), and tetrahydrofurane (THF). 20 ml of each solution were transferred into 100-ml erlenmeyer flasks. Vinyl acetate was added at a ratio of 1:1.5

TABLE 1. Characteristics of Chiralcel® OD analytical protocols

Protocols [Ref.]	Mobile phase			Composition (v/v/v)	Flow rate (ml/min)	Wavelength (nm)	UFLC results	
	A	B	C				α	R_S
01[30]	Hexane	Ethanol	Diethylamine	80 A/20 B/0.6 C	1.00	254	1.33–1.37	0.73–1.07
02[**]	Hexane	Ethanol	Diethylamine	80 A/20 B/0.6 C	0.75	254	n.a	n.a
03[31]	Hexane	Ethanol	Diethylamine	75 A/25 B/0.1 C	0.70	276	1.36–1.52	0.62–0.82
04[40]	Hexane	2-Propanol	Diethylamine	60 A/40 B/0.1 C	0.50	275	1.44–1.93	0.82–0.94
05[41]	Hexane	Ethanol	Diethylamine + acetic acid	60 A/40 B/(0.2 + 0.2) C	1.00	276	n.a	n.a

UFLC, ultra fast liquid chromatography; n.a, complete data are not available; Ref., reference; α, enantioseparation factor; R_S, enantioresolution.
**Present study.

(mole/mole). The mixtures were shaken in an orbital shaker (Max Q4000 Barnstead Lab-Line, Illinois, USA) at 200 rpm and 40 °C for 30 min. Lipases were finally added to the mixtures (lipase amino from *Burkholderia cepacia*: 35 mg; *Pseudomonas fluorescence* lipase: 8 mg; hog pancreatic lipase: 8 mg; *Candida antarctica* lipase fraction B: 200 µl). Enzymatic acetylations were performed at 200 rpm and 40 °C (Protocol-01 used 35 °C and 25 ml AT solutions) in the shaker.

Reaction in phosphate buffers. A 10 mg/ml of racemic AT was dissolved in Sorensen buffer (67 mM, pH 6.6–7.8). 40 ml of the AT solutions were placed in reaction flasks (Protocols-03 and -04 used 25 ml) where vinyl acetate was added (1:5 mole/mole except Protocol-04 that used 1:1). The flasks were shaken at 40 °C and 200 rpm (Protocol-01 used 35 °C) for 30 min. Then, lipase (*Candida antarctica* lipase fraction B: 380 µl, Protocol-04 used 665 µl) was added. The enzymatic reactions were conducted in the shaker at conditions as stated previously.

RESULTS AND DISCUSSION

Characteristics of Chiralcel® OD Column

The analytical Chiralcel® OD column was supplied by Daicel Chemical Industries, Ltd. (Japan). As informed in the instruction manual,[42] the column has typical operating temperature, pressure, and flow rate of 0–40 °C, 5 MPa (maximum), and 1.0 ml/min, respectively. Its mobile phase is based on mixtures of alkane and alcohol with a limited quantity of basic or acidic modifier compound.[43] Unfortunately, this column could not handle solvents such as acetone, chloroform, ethyl acetate, methylene chloride, DMSO, DMF, and THF where presence of these solvents in small or residual quantities may destroy the cellulose tris-(3,5-dimethylphenylcarbamate) compound.[42,43] However, these solvents are usually associated with the enzymatic reactions.[44–46]

Our observation on the selection of co-solvent for enzymatic reactions of racemic AT solutions showed that racemic AT solutions in acetone, chloroform, and 2-propanol (the solvents) formed whitish suspensions when they were mixed with hexane, heptane, toluene, cyclohexane, or isooctane (the co-solvents) in proportional or higher mixing ratio as given in Table 2. It was also found that the solutions of the racemic AT in DMSO and DMF created clear bilayer mixtures when cyclohexane, heptane, or isooctane was added to the AT solutions. These combinations would influence the mobile phase composition with high hexane content in the mixture which should be considered carefully when acetone and 2-propanol are used as solvents for AT. This fact could be found on the type of the storage solvent for the Chiralcel® OD column. As informed in the instruction manual,[42,43] the column storage solvent is a mixture of the hexane/2-propanol (90/10 v/v). High column flushing with the use of the analytical mobile phase liquid is required before the column is flooded with the storage solvent (for its storage). Ethanol was found to be the best solvent because it is miscible with the co-solvent and at the same time dissolving racemic AT as described in Table 2. Because the retention times are

TABLE 2. Combination of AT solutions and their co-solvents

Solvent	AT solution concentration (mg/ml)	Co-solvent	(Solvent : co-solvent) ratio (v/v)	Condition
Acetone, 2-propanol	5	Hexane, cyclohexane, heptane, isooctane	1:1–1:3	Whitish suspension
Chloroform	9	Hexane, cyclohexane, heptane, isooctane, benzene, toluene	1:1–1:3	Whitish suspension
Ethanol	5	Hexane, cyclohexane, heptane, isooctane, toluene, benzene	1:1–1:3	Clear solution
DMSO, DMF	5	Cyclohexane, heptane, isooctane, benzene	1:1–1:3	Bilayer solution
Acetone, 2-Propanol	5	Toluene, benzene	1:1–1:3	Clear solution
DMSO, DMF	5	Toluene	1:1–1:3	Clear solution

AT, atenolol; DMSO, dimethyl sulfoxide; DMF, dimethylformamide.
Hexane, Ethanol, 2-Propanol: the Chiralcel® OD mobile phase constituents.
All mixings were conducted at room temperature.

TABLE 3. Applications of Chiralcel® OD in the atenolol analysis

Analyte(s)	Analytes' solvent	Racemic chromatogram	Selectivity (α)	Sample origin	Injection volume (µl)	Ref.
(*S*)-atenolol	Methanol	Not available	No data	Asymmetric synthesis	No data	16,17
(*R*)-, (*S*)-atenolol	Water	Not available	No data	Asymmetric synthesis	No data	18
(*R*)-, (*S*)-atenolol	Methanol	Not available	No data	Asymmetric synthesis	No data	19,20
(*S*)-atenolol	Methanol	Not available	1.30	Asymmetric synthesis	No data	30
(*R*)-, (*S*)-atenolol, (*R*,*S*)-atenolol	No information	No information	No data	Commercial drugs	No data	31
(*R*,*S*)-atenolol	Mobile phase[a]	Not available	1.86	Commercial drugs	20	40
(*R*)-, (*S*)-atenolol, (*R*,*S*)-atenolol	Mobile phase[b]	Available	4.0–4.4	Commercial chemicals	20	41
(*R*,*S*)-atenolol	No information	Not available	1.73	Commercial chemicals	8	51
(*R*,*S*)-atenolol	Mobile phase[a]	Not available	1.54	Commercial chemicals	20	52
(*R*,*S*)-atenolol	Mobile phase[a]	Available	No data	Human plasma, urine	No data	53
(*R*,*S*)-atenolol	Mobile phase[c]	Not available	1.98	Commercial chemicals	No data	54
(*R*,*S*)-atenolol	No information	Not available	1.46	Commercial chemicals	5	55

[a]Hexane-(2)-propanol-diethylamine.
[b]Hexane-ethanol-diethylamine-acetic Acid.
[c]Hexane-ethanol-diethylamine.

shorter when using ethanol in place of 2-propanol, the use of ethanol as the mobile phase constituent is highly recommended in the chiral separation of racemic AT existing in the reaction media.

In the separation of AT enantiomers, this column gave excellent selectivity results (Table 3) as useful HPLC separation have α values of at least 1.2 and resolution factor (R_s) of 1.5.[47] Very high α values were also obtained, but the separation process required a complex mobile phase (see Ref. 41). Most of the Chiralcel® OD column were applied to analyze the AT dissolved in methanol or the analytical mobile phase. Alcohols were excellent solvents for dissolving AT especially methanol and ethanol.[48,49] However, none of these liquids have been associated with the AT in the resolution processes. Our database on enzymatic resolution of the chiral drugs showed that methanol was rarely used as solvent, whereas hexane does not dissolve the drug under study.[13,50]

All the previous AT samples originated from the commercial markets, asymmetric synthesis, and biological fluids. None of the samples were taken from the racemic resolution processes. The samples were injected directly onto the HPLC unit except the biological fluids, which required an extraction process before injection as the AT was dissolved in uncharacterized media (plasma and urine). Approximately 5–20 µl samples were injected onto the HPLC units manually. As the UFLC unit uses an auto-injection to inject the samples, the volumes could be managed lower than these values. Although the column operated under different mobile phases that already gave excellent selectivities, a complete chromatogram of the racemic AT separation was generally not presented. This fact made efforts to adopt easily an analytical protocol through consideration on the representative peak shapes/chromatograms that could not be conducted using the available analytical protocols especially for injections of the analytes existing in the reaction media.

Trial of Protocols

The highest flow rate used in the AT analysis was 1.0 ml/min[30,41] as given in Table 1. The rate was chosen as the first step in trials. Because the established quantities of diethylamine ranged between 0.1% and 0.6%, [30,31,39] the modifier content was set following these values.

High flow rate, high modifier compound (Protocol-01). This protocol was based on the study carried out by Mikuldas et al.[30] who worked on direct analysis of the (*S*)-AT produced by an asymmetric synthetic pathway. It was detailed in Table 1. This pathway did not develop the racemic ATE.

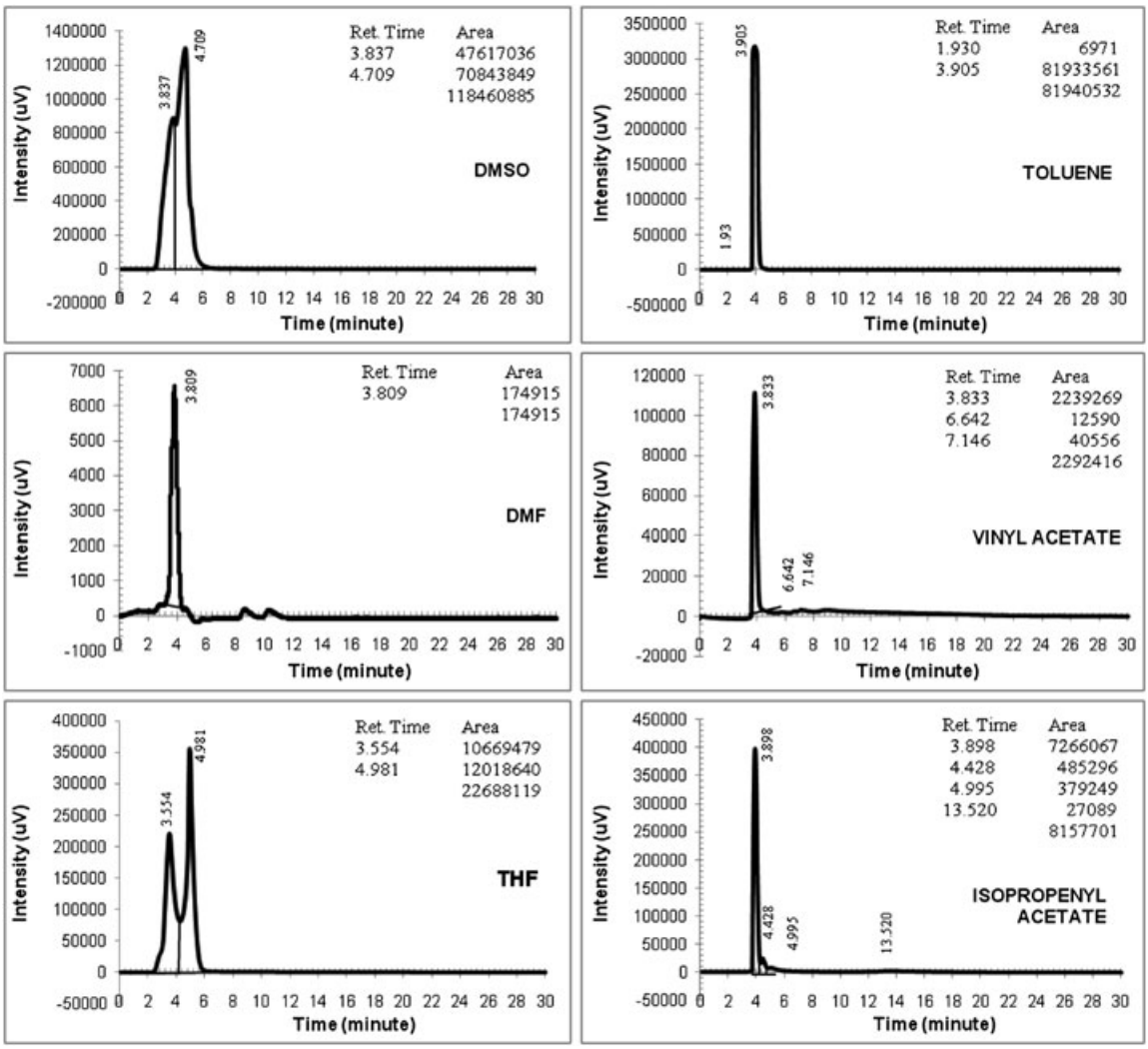

Fig. 2. Representative peaks of the experimental solvents (conditions: Protocol-01, 254 nm, 35 °C, 1 ml/min flow rate; injection volume: 5 µl; filtered samples). DMSO, dimethyl sulfoxide; DMF, dimethylformamide; THF, tetrahydrofuran.

Mikuldas et al. did not develop the protocol for the racemic AT analysis. Their chromatograms were only related to the pure AT compounds. The trials obtained the single enantiomeric AT peaks in less than 20 min.

Before separation was carried out on the racemic AT, a set of "only solvent" chromatograms was prepared. The results showed that all the "only solvent" peaks were found generally in less than 5 min (Fig. 2). Intensities of the solvents developed by the Chiralcel® OD were high except for the DMF. The column was able to recognize the peaks sharply, but the high intensities could preclude observation on the AT peaks during data acquisition. Baselines of the peaks were quite stable where they were parallel to the horizontal axis but not for the DMF and vinyl acetate, which formed slanted baselines.

During analysis periods, it was difficult to record chromatographic combination of the AT and solvent peaks clearly because the solvents' intensities were high. The AT peaks appeared to be very small even almost invisible along the data acquisition. Therefore, to sharpen the AT chromatograms' view, data acquisition was started from minute 6–7 (the peak area size did not change). The UFLC representative peaks for

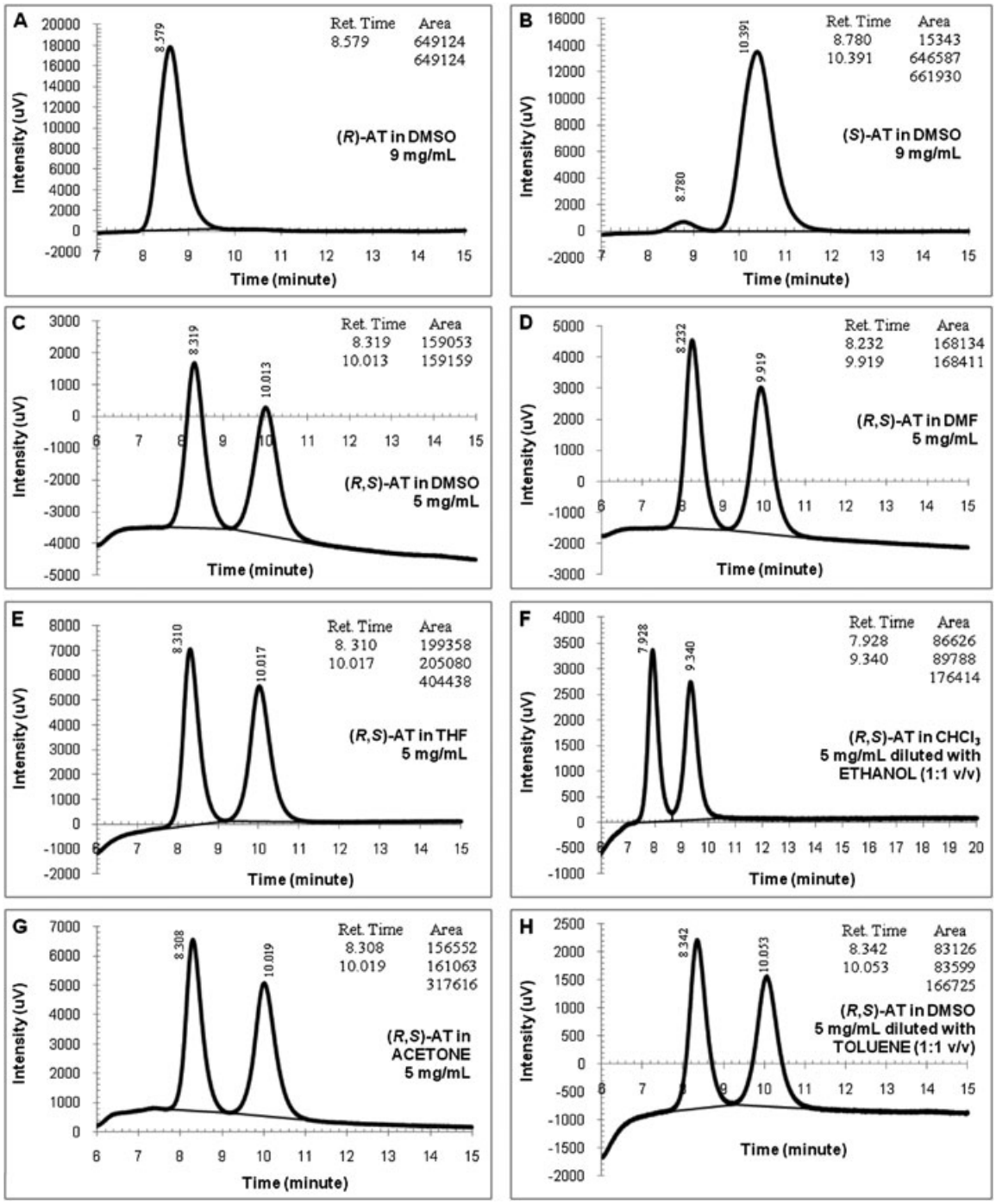

Fig. 3. Chromatograms of the atenolol (AT) in organic media (conditions: 254 nm, 35 °C, 1 ml/min; injection volume: 2 μl; centrifuged samples). (**A**) (*R*)-AT in dimethyl sulfoxide (DMSO), (**B**) (*S*)-AT in DMSO, (**C**) (*R, S*)-AT 5 mg/ml, (**D**) (*R,S*)-AT in dimethylformamide (DMF), (**E**) (*R,S*)-AT in tetrahydrofuran (THF), (**F**) (*R,S*)-AT in $CHCl_3$ 5 mg/ml diluted with ethanol (1:1 v/v), (**G**) (*R,S*)-AT in acetone 5 mg/ml, and (**H**) (*R,S*)-AT in DMSO 5 mg/ml diluted with Toluene (1:1 v/v).

the AT dissolved in some organic media resulted from the Protocol-01 were presented in Figure 3. R_S and α values were given in Table 1. Selectivity of the AT separation were moderate[56] and similar to the Mikuldas et al. results; however, the resolution factors were lower than their findings and the minimum recommended value for complete separation ($R_S > 1.20$).[47,57] Further works are needed to increase the R_S values.

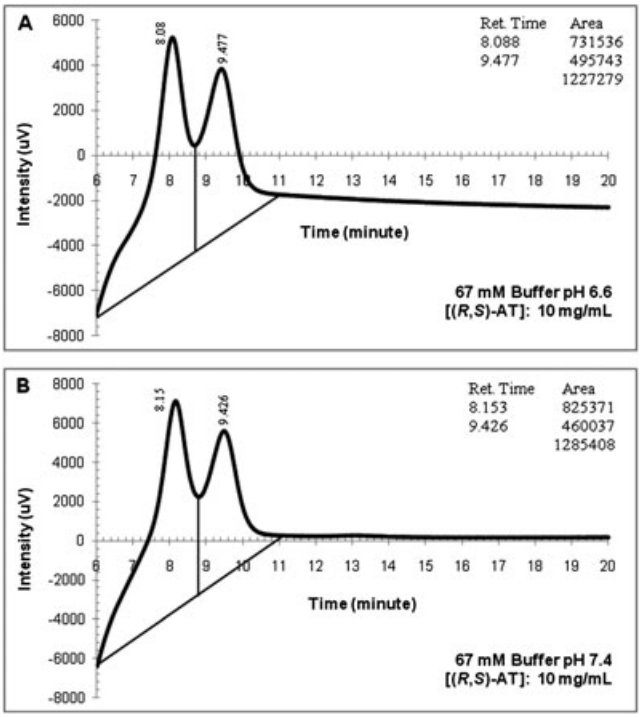

Fig. 4. Chromatographic diagrams of atenolol (AT) dissolved in Sorensen buffers (conditions: 254 nm, 35 °C, 1 ml/min; injection volume: 2 µl; centrifuged samples). (**A**) 67 mM buffer pH 6.6 [(*R,S*)-AT]: 10 mg/ml and (**B**) 67 mM buffer pH 7.4 [(*R,S*)-AT]: 10 mg/ml.

Good peaks' shapes were generated when these were injected with the pure AT enantiomers (Fig. 3(A and B)). The (*R*)- and (*S*)-AT peaks appeared faster than this protocol owner, yet the first eluted enantiomer was the same. The (*R*) enantiomer was recognized in 8.5 min, whereas the (*S*)-AT eluted at 10.3 min. The UFLC unit was proven able to generate both enantiomers peaks at faster time. Intensities of the detector created by each 9 mg/ml single enantiomer were quite high, although only 2 µl samples were injected. The cellulose-based column could recognize the AT compound at a low injection volume.

Formation of good peaks was also observed when the Chiralcel® OD column was used to separate the racemic AT dissolved in some common organic solvents as shown in Figure 3(C–F). Compared with the single enantiomers, a slight difference in retention time was noted where the racemic AT peaks appeared slightly quicker than the single enantiomers. It was found that the detected intensities of the racemate were not high (around 3.5–7 mAU). The racemate concentration in the reaction media was set at 5 mg/ml, which was around half of the individual AT enantiomer concentration. Because the AT does not dissolve in hexane, low concentrations of the drug were used in the analysis to prevent sedimentation of the AT inside the Chiralcel® OD column. Further observations on the peaks' characteristics indicated that the baselines of each enantiomer peak were almost parallel to the horizontal axis, but most of the baselines deviated from the horizontal zero point. Chromatographic readings of the racemic AT gave almost the same values for both enantiomers as can be observed from tables inserted in each diagram of the racemate. This means the Chiralcel® OD can recognize the AT concentrations even at lower concentrations than the single enantiomers.

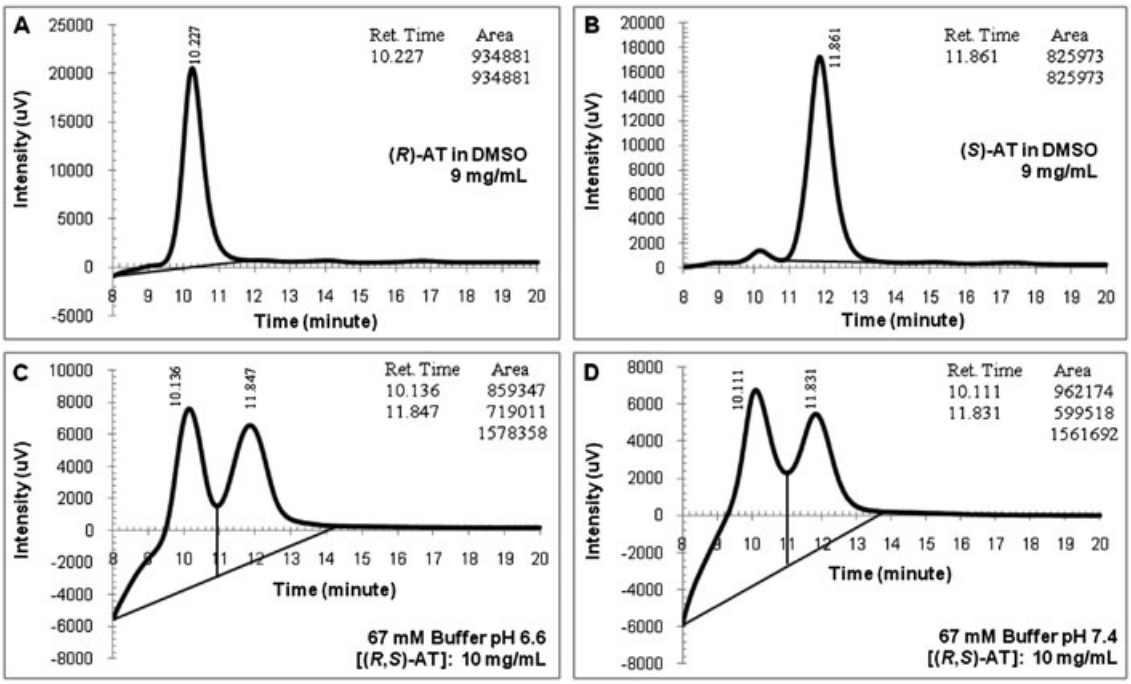

Fig. 5. Representative peaks of atenolol (AT) dissolved in dimethyl sulfoxide (DMSO) and Sorensen buffers (conditions: 254 nm, 35 °C, 0.75 ml/min; injection volume: 2 µl; centrifuged samples). (**A**) (*R*)-AT in DMSO 9 mg/ml, (**B**) (*S*)-AT in DMSO 9 mg/ml, (**C**) 67 mM buffer pH 6.6 [(*R,S*)-AT]: 10 mg/ml, and (**D**) 67 mM buffer pH 7.4 [(*R,S*)-AT]: 10 mg/ml.

Because the racemic AT has high solubility in water, Sorensen phosphate buffers were also selected as alternative media for the enzymatic reaction. The aqueous solutions were injected directly to the UFLC unit without purification or pretreatment. Chromatograms of these racemic AT were given in Figure 4(A and B).

The chiral column separated and recognized the AT enantiomers. Higher intensities than the racemic AT in the organic solvents were obtained. Both peaks' shapes were good, but the enantiomeric peaks had wide bases and were very close to each other. Compared with the pure AT chromatograms (Fig. 3(A and B), the results differed greatly, although the racemic AT had only slightly higher concentrations than the single enantiomers. Two factors could possibly cause this problem: (1) effect of the water-based analytes where the buffers assisted in the sample separation[56–59] and (2) more racemic AT concentrations were diluted by the mobile phase although only 2 µl samples were injected. Despite that the analytical mobile phase had high hexane content (80% v/v), the buffer-based analytes were resolved

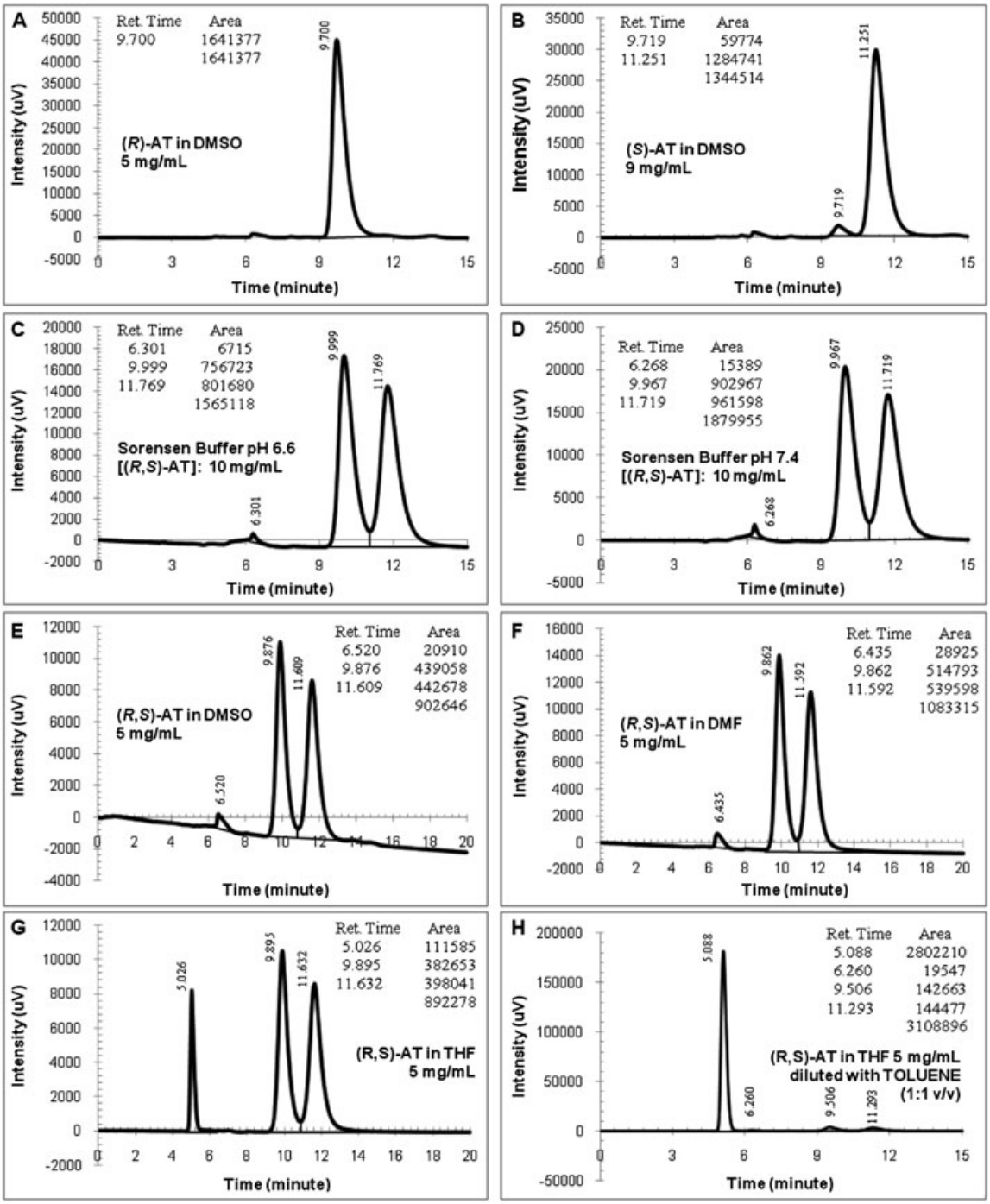

Fig. 6. Peaks of pure and racemic atenolol (AT) obtained by Protocol-03 (conditions: 276 nm, 35 °C, 0.70 ml/min; injection volume: 1 µl; centrifuged samples). (**A**) (*R*)-AT in dimethyl sulfoxide (DMSO) 9 mg/ml, (**B**) (*S*)-AT in DMSO 9 mg/ml, (**C**) Sorensen buffer pH 6.6 [(*R*,*S*)-AT]: 10 mg/ml, (**D**) Sorensen buffer pH 7.4 [(*R*,*S*)-AT]: 10 mg/ml, (**E**) (*R*,*S*)-AT in DMSO 5 mg/ml, (**F**) (*R*,*S*)-AT in dimethylformamide (DMF) 5 mg/ml, (**G**) (*R*,*S*)-AT in tetrahydrofuran (THF) 5 mg/ml, and (**H**) (*R*,*S*)-AT in THF (5 mg/ml) diluted with toluene (1:1 v/v).

highly by the cellulose tris-(3,5-dimethylphenylcarbamate) chiral stationary phase.

However, the peaks' baselines were not parallel to the horizontal axis. These lines deviated severely. The lines were generated automatically by the UFLC unit, albeit modification could be carried out using the existing software, but it seems difficult to change the baselines as the peak areas would also change. These slanted lines influenced the chromatographic readings. As shown in the inserted tables, areas of the (*R*)- and (*S*)-AT differed greatly for both buffer types (area of the (*R*)-enantiomers was almost twice of the (*S*)-AT area).

Low flow rate, high modifier compound (Protocol-02). The protocol was a modified form of the first protocol. Same operating conditions were used, but the mobile phase flow rate was lower than the original value (reduced by 25%). As the Protocol-01 produced the enantiomeric peaks with wide bases for the buffer solutions, it was decided to reduce the flow rate as it has a dramatic influence on retention time and selectivity.[44] Protocol-02 was examined on the single enantiomeric and the racemic AT dissolved in the Sorensen buffers. Results of the racemic AT chromatograms are presented in Figure 5.

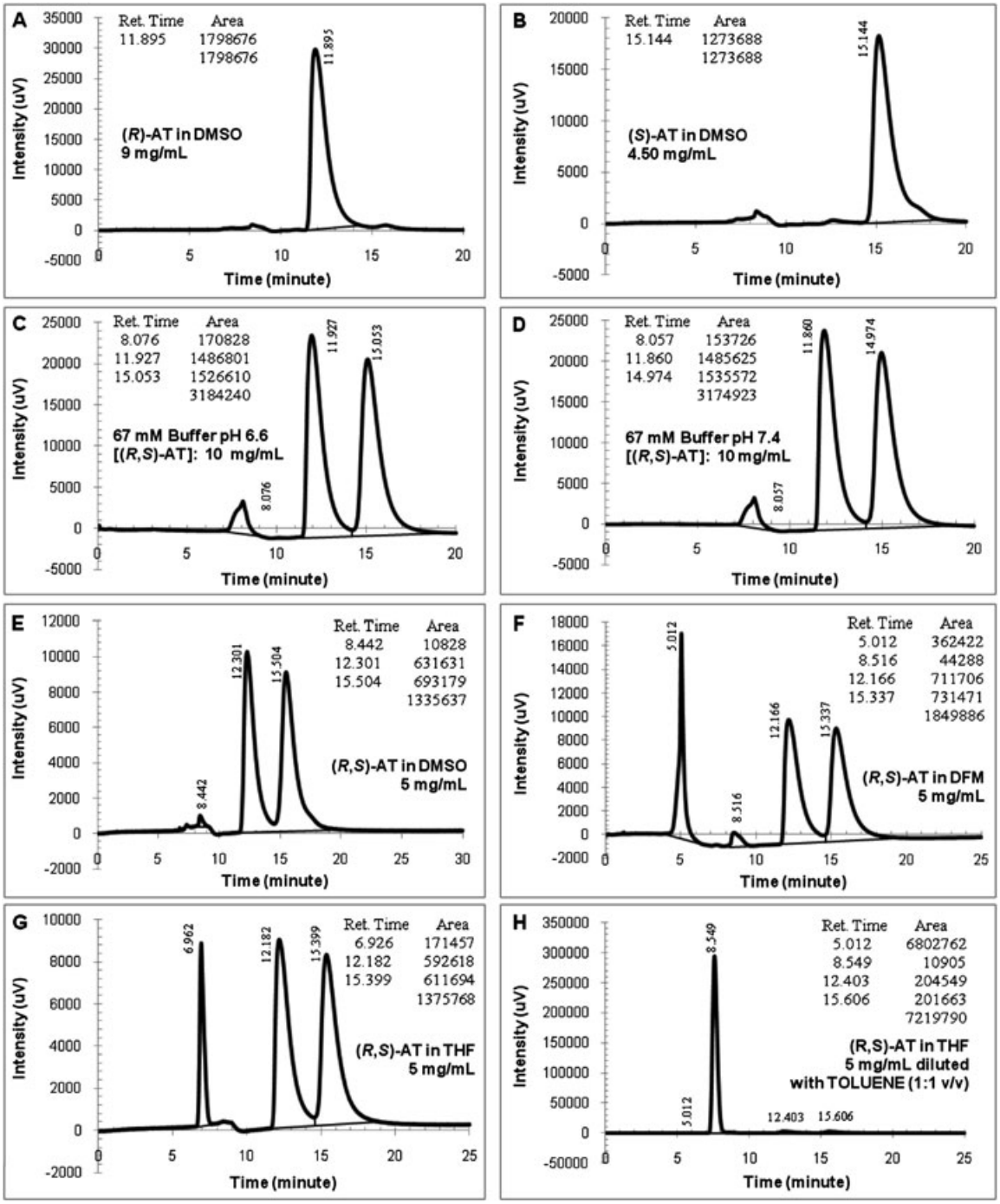

Fig. 7. Chromatograms of single enantiomers and racemic atenolol (AT) produced by Protocol-04 (conditions: 275 nm, 35, 0.5 ml/min flow rate; injection volume: 1 μl; centrifuged samples). (**A**) (*R*)-AT in dimethyl sulfoxide (DMSO) 9 mg/ml, (**B**) (*S*)-AT in DMSO 4.50 mg/ml, (**C**) 67 mM buffer pH 6.6 [(*R*,*S*)-AT]: 10 mg/ml, (**D**) 67 mM buffer pH 7.4 [(*R*,*S*)-AT]: 10 mg/ml, (**E**) (*R*,*S*)-AT in DMSO 5 mg/ml, (**F**) *R*,*S*)-AT in dimethylformamide (DMF) 5 mg/ml, (**G**) (*R*,*S*)-AT in tetrahydrofuran (THF) 5 mg/ml, and (**H**) (*R*,*S*)-AT in THF (5 mg/ml) diluted with toluene (1:1 v/v).

Single enantiomeric peaks were formed excellently (Fig. 5 (A and B)) by the cellulose-based CSP. It recognized the (*R*)- and (*S*)-AT areas highly. The peak intensities obtained by Protocol-02 for both enantiomers were higher than the previous observations (Protocol-01). A large difference in the peak areas between the single enantiomers was observed. But a slanted baseline was found on the (*R*)-enantiomer.

The buffers-based separation produced similar results as the previous protocol. The peaks' shapes were good but wide at their bases. The baselines were heavily nonparallel to the horizontal axis, which indeed influenced the peaks' sizes. Areas of the (*R*)-AT were found higher than the (*S*)-enantiomer as can be observed from tables in Figure 5(A and B). Several differences to the Protocol-01 results were also observed.

Reduction of the mobile phase flow rate by 25% indeed had significant effects on the separation process of the AT enantiomers. The new protocol produced both enantiomeric peaks slower than the Protocol-01. The (*R*)- and (*S*)-AT appeared at minutes 10.1–10.2 and 11.8, respectively, which differed approximately 2 min from the Protocol-01 results. However, compared with the previous protocol peaks' areas, the later protocol peaks' sizes were higher (130,000–280,000 peak areas). These results proved that lowering the flow rate did increase interaction between the analyte and CSP.

Low flow rate, low modifier compound (Protocol-03). This procedure was applied because lower flow rate gave quite higher peaks' areas. The protocol was adopted from Santoro et al.[31] that used the Chiralcel® OD column to separate and quantify the AT content in drug tablets. Compared with the previous protocols, Protocol-03 was not only lower in the flow rate but also significantly lower in the modifier quantity (see Table 1). But it used higher alcohol content and a higher UV detection wavelength. Results of the data acquisition of the single enantiomers (in DMSO) and racemate (in Sorensen buffers) are shown in Figure 6(A–D).

The peaks appeared at minutes 9.9 and 11.7, which were not too different from the previous procedures. Although concentrations of the enantiomers were the same, this protocol gave higher enantiomeric peak intensities (2–3 times) than Protocol-01. These peak intensities were obtained from smaller injection volumes (only 1 μl). This fact indicates that the used mobile phase gave good environment to the CSP and analyte so that the CSP recognized the presence of AT enantiomers even at a small injection volume. It is likely that the high alcohol content increased solubility of the AT in the mobile phase. Lower quantity of the diethylamine compound still provided good peak shapes of the separation process.

The results obtained from Protocol-03 were better than those from Protocols-01 and −02. High intensities obtained using Protocol-03 could eliminate or reduce the peaks of either the solvent or the buffers. As described in each diagram, the DMSO peak disappeared from the chromatographic detection, whereas the Sorensen buffers gave low peak areas. The peak baselines were parallel to the horizontal axis. Values of the peak area of the racemic AT were almost the same (see tables in Fig. 6(C and D)), similar to the chromatographic characteristics obtained by Protocol-01 for the AT dissolved in the organic solvents. The separation factor, as given in Table 1, showed moderate to easy process.[56] But this protocol produced uncompleted chiral separation as the R_S values were less than 1.2.

Low flow rate, high alcohol content (Protocol-04). Protocol-04 was developed to determine the optical purity and separate β-blocking substances available in commercial drug chemicals. The protocol used 2-propanol instead of ethanol as the mobile phase constituent. It has higher alcohol content than the previous protocols, but the modifier composition was the same as the third protocol. Because maximum allowable flow rate of 2-propanol is lower than ethanol,[43] the Chiralcel® OD column was operated using the lowest mobile phase flow rate (Table 1). Symmetrical peaks were produced during the chiral separations (Fig. 7). Quite stable peaks' baselines and high peaks' intensities were observed. This protocol gave the highest enantioselectivity factors than the methods described previously (Table 1). However, similar to Protocols 01–03, the produced R_S were lower than the minimum

TABLE 4. Performance of the enzymatic processes

Protocol	Media	Enzyme	Initial peak area (*R*)-AT	Initial peak area (*S*)-AT	Reaction time (h)	Final peak area (*R*)-AT	Final peak area (*S*)-AT	Conversion (%)	UFLC result
01	Buffer pH 6.6	CALB	404,565	336,419	4	575,084	550,784	[a]	Figure 8(A)
	Buffer pH 7.4	CALB	365,272	311,406	4	479,770	550,784	[a]	Figure 8(B)
	DMSO	LAPS	364,639	405,557	6	283,940	295,047	X_R:19.36; X_S:26.12	
	DMF	LAPS	386,184	387,844	6	273,886	275,667	X_R:23.11; X_S:27.67	
02	Buffer pH 6.6	CALB	859,347	719,011	4	834,251	656,905	X_R:2.88; X_S:8.41	Figure 8(C)
	Buffer pH 7.4	CALB	962,174	599,518	4	811,644	692,252	X_R:15.48; X_S: [a]	Figure 8(D)
03	Buffer pH 7.0	CALB	937,008	1,001,900	4	848,275	821,430	X_R:9.25; X_S:18.59	
	Buffer pH 7.4	CALB	902,967	961,598	4	869,367	825,555	X_R:3.63; X_S:14.62	
	THF	PFL	382,653	398,041	4	59,744	70,809	X_R:84.22; X_S:91.78	Figure 8(E)
	DMF	HPL	514,793	539,598	4	393,648	375,060	X_R: 22.09; X_S:31.67	Figure 8(F)
04	Buffer pH 6.6	CALB	1,486,801	1,526,610	10–12	1,405,513	1,339,520	X_R:5.41; X_S:10.92	
	Buffer pH 7.4	CALB	1,485,625	1,535,572	10–12	1,522,099	1,125,854	X_R: [a]; X_S:23.79	
	THF	Lipoprotein	592,618	611,694	6	0	0	X_R:100; X_S:100	Figure 8(G)
	DMF	CALB	711,706	731,471	6	152,396	255,771	X_R:77; X_S:51.82	Figure 8(H)

UFLC, ultra fast liquid chromatography; CALB, *Candida antarctica* lipase fraction B; LAPS, Lipase amino from *Burkholderia cepacia;* PFL, *Pseudomonas fluorescence* lipase; HPL, hog pancreatic lipase; DMSO, dimethyl sulfoxide; DMF, dimethylformamide; THF, tetrahydrofuran.
[a]Conversions cannot be calculated.

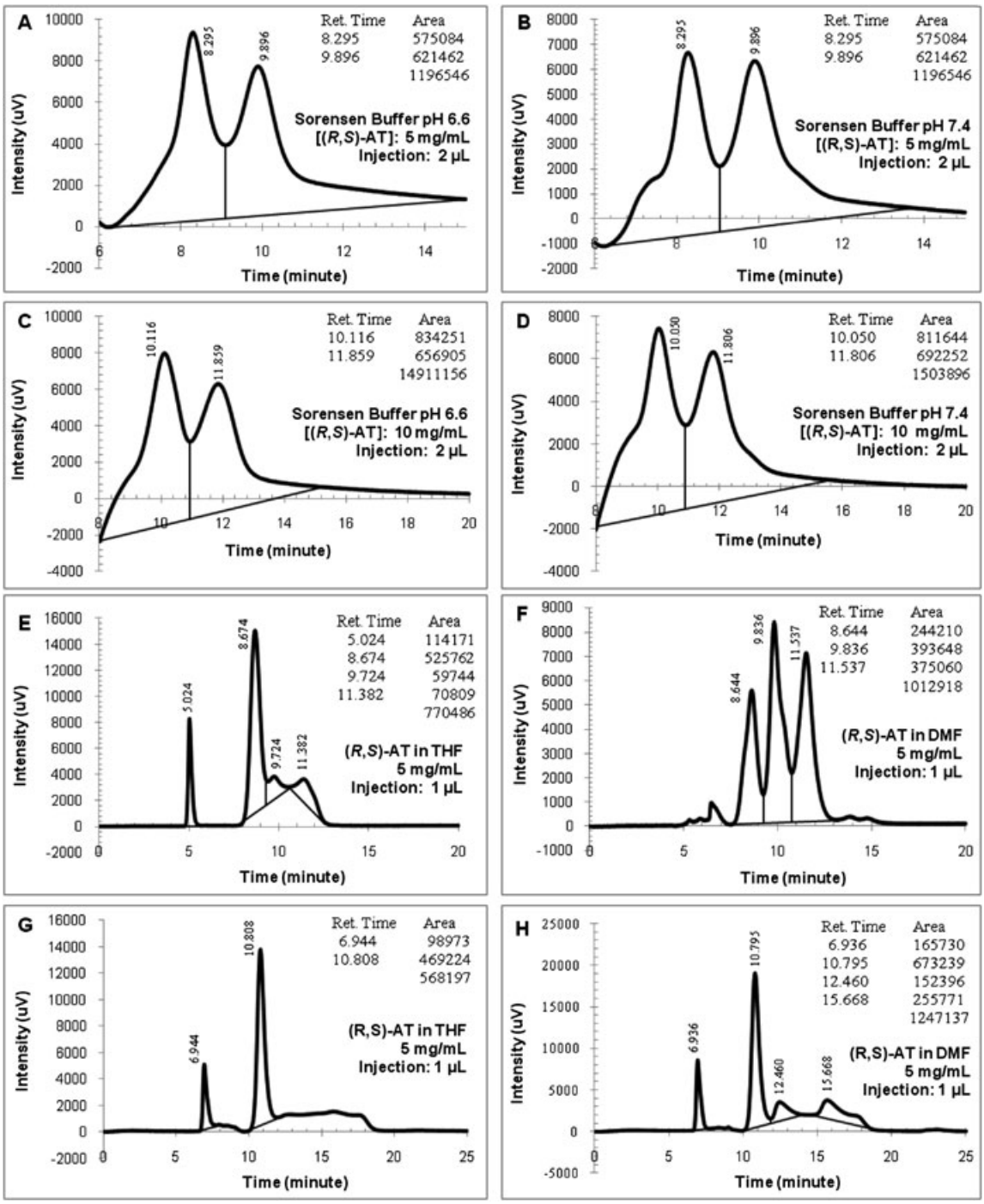

Fig. 8. Ultra fast liquid chromatography chromatograms of the enzymatic process solutions. (**A**) Sorensen buffer pH 7.4 [(*R*,*S*)]-atenolol (AT): 5 mg/ml; injection: 2 µl, (**B**) Sorensen buffer pH 7.4 [(*R*,*S*)]-AT: 5 mg/ml; injection: 2 µl, (**C**) Sorensen buffer pH 6.6 [(*R*,*S*)]-AT: 10 mg/ml; injection: 2 µl, (**D**) Sorensen buffer pH 7.4 [(*R*,*S*)]-AT: 10 mg/ml; injection: 2 µl, (**E**) (*R*,*S*)-AT in tetrahydrofuran (THF) 5 mg/ml; injection: 1 µl, (**F**) (*R*,*S*)-AT in dimethylformamide (DMF) 5 mg/ml; injection: 1 µl, (**G**) (*R*,*S*)-AT in THF 5 mg/ml; injection: 1 µl, and (**H**) (*R*,*S*)-AT in DMF 5 mg/ml; injection: 1 µl.

recommended chiral resolution for a complete chromatographic separation.

Compared with the earlier protocols, the AT peaks appeared slower. The (*R*)-AT appeared at minute 12, whereas the (*S*)-enantiomer was eluted at minute 15. However, this flow rate developed the highest time difference between the AT enantiomers during the separation process. Excellent peaks' areas were also generated by this protocol. As described in Figure 7(A), the (*R*)-AT peak area was slightly higher than the result produced by Protocol-03 (Fig. 6(A)) and was three times higher than the first protocol for the same enantiomer (Fig. 3(A)). The racemic AT dissolved well in the mobile phase containing high 2-propanol composition. Although Protocol-04 produced higher peak areas, the protocol was limited by maximum allowable working pressure (5 MPa) in which 2-propanol tends to develop higher column pressures than ethanol.[43] With the use of this protocol, the Chiralcel® OD column recognized a very hydrophobic compound highly as drawn in Figure 7(H).

Protocol-05. Because high flow rate and alcohol content develop high pressure in the column, it is decided not to observe the chiral AT separation operated by the last protocol. Although Protocol-05 has high ethanol composition, which is useful to clean-up the Chiralcel® OD column,[42] this compound is related to low mobile phase flow rate. As described in the instruction manual, the maximum flow rate for 100% ethanol is 0.5 ml/min.[42,43] Our observation on Protocols 01–04 indicated that when the cleaning process was carried out at this flow rate, the column pressure could increase to more than its maximum allowable value. Hence, same as to Protocol-04, high alcohol composition would risk the column, especially when it is operated under the typical flow rate for the Chiralcel® OD column (1.0 ml/min).

Enzymatic acetylation of racemic AT. The enzymatic processes were aimed to resolve the (*R*,*S*)-AT to yield the active isomer of the AT compound. Because its racemic compound shows hydrophilic behavior, it cannot be processed in hydrophobic solvents. Hence, water miscible compounds (DMSO, DMF, and THF) and buffer solutions were chosen as the reaction media. Results of the processes are given in Table 4, whereas the UFLC chromatograms are presented in Figure 8. It was observed that the AT enantiomers reacted with the acetate compound in both media. Some reactions still required longer reaction times to increase their conversions. High conversions were obtained once the reactions were conducted in organic media using *Pseudomonas fluorescence* lipase (X_R: 84.22%, X_S: 91.78%), Lipoprotein lipase (X_R: 100%; X_S: 100%), and *Candida antarctica* lipase fraction B (X_R: 77%, X_S: 51.82%) during certain reaction times (4–6 h). Several conversions cannot be calculated as the final enantiomers' peak areas were found higher than the initial values. Protocol-04 indeed gave the highest peaks' areas for the organic and buffer media as stated previously.

As described in Figure 8, all UFLC chromatograms showed good enantiomeric peaks where Protocols-03 and −04 gave better chromatographic results. Most chromatograms formed slanted baselines except the result from the enzymatic reaction of (*R*,*S*)-AT in DMF, which formed almost parallel baselines to the horizontal line. The enantiomeric peaks with wide baselines were observed on the organic and buffer media. Interferences from the product peak could cause this matter. Comparisons resulted from Figure 7(F and G) and Figure 8(E–H) proved that the product peaks, which appeared before the enantiomeric peaks, were very close to the (*R*)-AT peak. However, the buffer media did not show the same phenomenon. The initial and final enantiomeric peaks had the same baselines conditions as can be seen from Figures 8(A–D), 4(A and B), and 5(C and D).

CONCLUSION

Different characteristics of the AT separation were resulted by various reaction media and protocols using a Chiralcel® OD column. Either single enantiomeric or racemic AT dissolved in organic solvent or Sorensen buffer media could be separated by this column in less than 20 min by using the UFLC unit. Good peaks were developed, but several protocols produced peaks with wide bases and slanted baselines. The protocols gave moderate to easy separation factors, but the resolution values required further improvement.

High mobile phase flow rate and increased concentration of modifier compound in mobile phase but low alcohol content were used by Protocol-01, which gave smooth peaks for the solvents and AT. At these conditions, separation of the AT (racemate and single enantiomers) dissolved in organic solvents was found better than the buffer-based analytes. Although Protocol-02 had lower flow rate but higher area than the first protocol, the column still could not separate the racemic AT peaks satisfactorily. Increased peak intensities of the organic solvents were produced by both protocols.

Reduced flow rate and decreased concentration of modifier compound but high alcohol content and increased UV detection wavelength (Protocols -03 and −04) gave the best separation results. These protocol produced higher intensities obtained from smaller injection volumes than the previous protocols, although the AT concentrations were the same. It reduced or eliminated peaks of either the solvent or buffer and provided peaks with baselines parallel to the horizontal axis.

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