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Development a chiral derivatization method for the determination of atenolol and metoprolol enantiomers in tablet preparations

N.V.T. Nguyen[1], D.K.T. Pham[1], K.N.H. Nguyen[1], T.T.T. Nguyen[1], T.L.T. Pham[1], T.K. Nguyen[1], K.H. Kim[2]*
[1] Can Tho University of Medicine and Pharmacy, Viet Nam
[2] College of Pharmacy, Kangwon National University, Chunchon, Korea

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ABSTRACT

Atenolol and metoprolol are used in the treatment of angina pectoris, certain arrhythmias, systemic hypertension, and several other cardiovascular disorders. Both compounds are produced commercially in the racemic and enantiomer, even though the S-form is responsible for the desired biological effect. The aim of this study was to development a simple, rapid chiral derivatization method for the determination of atenolol and metoprolol enantiomers in tablet preparations using chiral derivatization agent (2,3,4,6-tetraO-acetyl-β-D-glucopyranosyl isothiocyanate (GITC). The optimization of the derivatization procedure (concentration of GITC, reaction temperature and time) and UPLC conditions (pH and ionic strength of mobile phase) were investigated. With 20 times molar excess of GITC, chiral derivatization reaction was completed within 15 min at room temperature. Diastereomeric derivatives of atenolol and metoprolol were well resolved on UPLC BEH C18 column (2.1 x 100 mm, 1.7 µm) with acetonitrile - ammonium acetate buffer as mobile phase and monitored at UV 225 nm. Separation was completed in less than 10 min. The LOD and LOQ of atenolol-GITC were 0.18 µm/ml and 0.35 µm/ml, respectively. The LOD and LOQ of metoprolol-GITC were 0.06 µm/ml and 0.2 µm/ml, respectively. The recovery of two compounds was 98-102%. The results of the studies showed that the proposed RP -UPLC method is simple, rapid, precise and accurate, which can be applied for the routine assessment of atenolol, metoprolol and their enantiomers impurity in pharmaceutical dosage forms.

1. INTRODUCTION

Cardiovascular disorders are one of the main causes of mortality in the world and thus making blocking agents, especially atenolol and β-adrenergic metoprolol, is the most important and most prescribed class among pharmaceutical products[1]. Several β-adrenoceptor blocking agents, including atenolol and metoprolol, had an asymmetric carbon atom in a side chain. Both compounds are produced commercially in the racemic and enantiomer, even though the S-form is responsible for the desired biological effect. The exceptions are timolol, bunolol, and penbutolol, which are commercially available in the S-enantiomeric form in some European countries.

The difference in the activities of atenolol and metoprolol isomers necessitates an accurate method to determine the enantiomeric composition of pharmaceutical preparations containing these compounds. Liquid chromatography is now

***Corresponding author:** nguyenthingocvanct@gmail.com

the most accepted method for chiral separations, not only in the direct way, using chiral stationary phases, but also in the indirect way, using chiral derivatizing reagents[2-4]. Dinitrophenyl ether β-cyclodextrin-bonded chiralstationary phase (CSP) was used for direct enantiomeric resolution of atenolol by Cheng et al (2014); Baranowska (2015) determined metoprolol isomers and its metabolites using a Chiralcel OD-RH column packed with cellulose tris-(3,5-dimethylphenyl-carbamate) stationary phase[5,6]. Due to the difficulty of selecting the best chiral column, the higher cost and shorter lifetime of these columns are disadvantages of the direct method. The application of chiral derivatizing agent (CDA) was the first widely-used method for the enantiomeric separation of optically active molecules in liquid chromatography. In indirect method, Alwera et al (2016) used new derivatizing reagents synthesized from (S)-ketoprofen for separation of atenolol and metoprolol enantiomers, Bhusan et al (2008) determined atenolol isomers by using Marfey's reagent as chiral derivatizing agent and the reaction condition was carried out under microwave irradiation[7,8]. For amine compounds, the chemically most selective CDAs are isothiocyanates such as 2,3,4,6-tetraO-acetyl-β-D-glucopyranosyl isothiocyanate (GITC), leading to the corresponding diastereomeric thiourea derivatives[9,10].

Comprehensive documentation of the validation of an analytical method is an integral part of any data submission to regulatory agencies. For chiral drugs, enantiomeric mixtures do not escape this requirement; thus, inactive or potentially toxic isomers must be isolated and quantitated as impurities by a validated analytical method. Recently, the question of stereochemistry was addressed directly in the U.S. Food and Drug Administration guideline on the manufacture of drug substances[11,12]. Up to now, there are no monographs for the quantification of atenolol as well as metoprolol isomers in tablet preparations in United States pharmacopeia and British pharmacopeia. The aim of this study was to develop a simple, rapid chiral derivatization method for the determination of atenolol and metoprolol enantiomers in tablet preparations using chiral derivatization agent (GITC).

2. MATERIALS AND METHODS

2.1. Chemical and materials

HPLC-grade acetonitrile and methanol was purchased from Merck Company (Darmstadt, Germany). Water prepared with a Millipore Milli-Q SP water purification system (Millipore, France) was used during sample preparation procedures and UPLC analyses. *Racemic atenolol and racemic metoprolol standards,* GITC (2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl Isothiocyanate, 98% HPLC) from Sigma-Aldrich.

Atenolol STADA tablet contains 50 mg of atenolol from STADA Vietnam Co., Ltd and Atpure-25 tablet contain 25 mg of S-atenolol from Emcure Pharmaceutical Ltd. Indian production. Betaloc® tablet contains 50 mg of metoprolol form AstraZeneca Pharmaceutical Co.,Ltd and Metpure-XL® 50 film coated tablet contains 50mg S-metoprolol tartrate from Emcure Pharmaceutical Ltd. Indian production.

2.2. Sample preparation

Atenolol solutions (200 µg/ml in methanol) and metoprolol solutions (600 µg/ml in mixture of acetonitrile – 0.1 N hydrochloric acid, 95:5) were prepared. To a 100 µl aliquot of this solution were added 100 µl of 15 mM GITC acetonitrile solution and 100 µl of 10 mM triethylamine acetonitrile solution. The resulting mixture allowed to stand at room temperature for 15 min. After reaction, aliquots were injected into the UPLC system.

2.3. Instrumentation and chromatographic conditions

The experiment was carried out by a UPLC Acquity HCLASS Waters, DAD detector (USA), the acquisition of chromatogram and integration used MassLynx SCN 85 software. The chromatographic separation was achieved using a UPLC BEH C_{18} column (2.1 x 100 mm, 1.7 µm) detected at 225 nm at room temperature, with an injection volume of 2 µl, flow rates of 0.3 ml/min. The mobile phase consisted of acetonitrile and ammonium acetate (pH=6) with ratio of 55:45 for separation of metoprolol isomers and methanol - ammonium acetate

(pH=5) with ratio of 33:67 for separation of atenolol isomers. The mobile phases were prepared fresh each day, vacuum-filtered through a 0.22 μm and degassed for 15 min.

2.4. Optimization of derivatization

The effects of the molar ratio of GITC to atenolol and metoprolol to metoprolol were analysed. The effects of time and temperature on the reaction were investigated. The solutions were added and reacted at room temperature, 45^0C and 60^0C, and 20 min, 40 min, 60 min, 90 min, 120 min, 180 min and 240 min. The resulting samples quantitated by achiral UPLC system.

2.5. Optimization of the mobile phase

Depending on a number of factors including composition of mobile phase, mobile phase ratio, the flow rate, injection volume, pH of the aqueous component of the mobile phase were investigated.

2.6. Validation of analytical procedures

For the validation of the analytical method, the guidelines of the International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use were followed[5,7]. The requirement for the drug assay follows these topics: system suitability, specificity, linearity, limits of detection (LOD) and quantification (LOQ), accuracy, precision.

2.6.1 Linearity, limits of detection and quantification

Linearity of the methods was checked using sets of up to six concentration levels. A series solution containing appropriate concentrations of two reference compounds were used for the construction of calibration curves. Limits of detection (LOD) and quantification (LOQ) for each analyte were determined at a signal-to-noise ratio (S/N) of about 3 and 10, respectively.

2.6.2 Precision and accuracy

For intra-day variability test, two β-blockers in six sample solutions were determined within one day, while for inter-day variability tests, two concentrations of β-blockers were examined in twice a day on 3 consecutive days. All the results were expressed as relative standard deviations (RSD).

The accuracy of the method was investigated by recovery studies; in particular, samples solutions were spiked with 1.0 ml aliquot of a solution containing standard compounds in sample, at three concentration levels and three samples for each level. The spiked samples were then derivatization reaction processed, and quantified in accordance with the methods mentioned above.

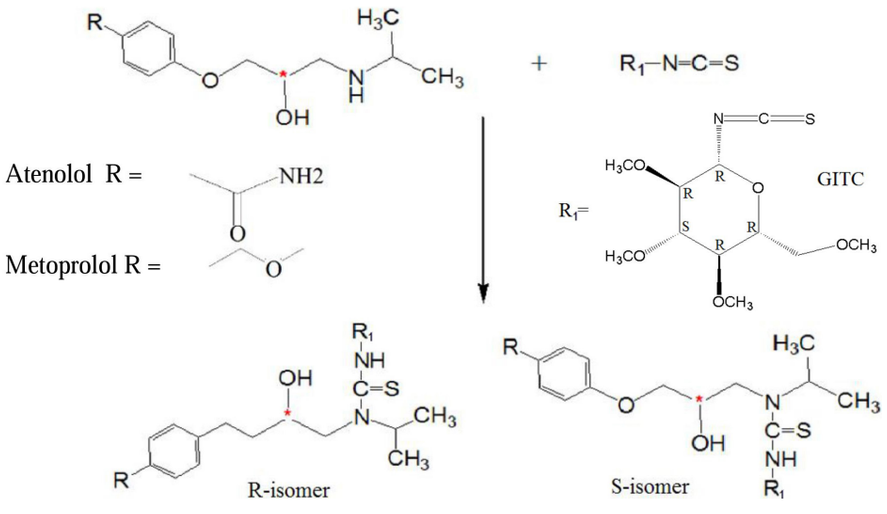

Figure 1. System suitability parameters

3. RESULTS AND DISCUSSION

3.1. Optimization of derivatization

The effect of the concentration of GITC added was shown in Figure 2. An increase of the concentration of GITC led to a general increase in formation of the diastereomers. Derivatization of atenolol and metoprolol were increased upto 20 times molar excess of GITC. In the final analytical conditions, the GITC concentration was chosen 20 times molar excess in order to provide an adequate excess of reagent.

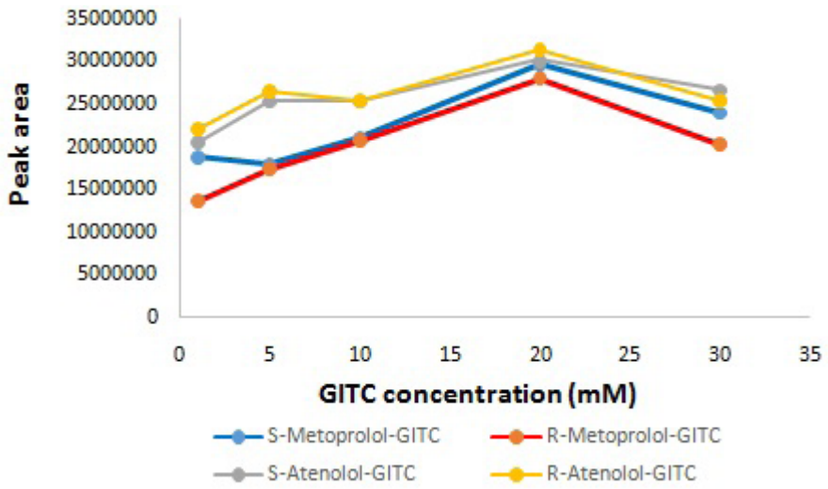

Figure 2. The effect of GITC concentration on derivatization reaction

As the reaction temperature was increased from room temperature to 45^{0}C or 60^{0}C, the peak areas of diastereomers were increased but the range of change was narrow. The formation of the derivatives of atenolol and metoprolol increased with the reaction time up to 20 min at room temperature and reached a plateau (Figure 3). This chiral derivatizing agent is suitable for the indirect resolution of chiral primary and secondary amines and amino alcohols using RP-UPLC, during reaction process, no racemization. The optimization reaction formed 2 diastereomers with the same ratio (50% of R-isomer-GITC and 50% of S-isomer-GITC).

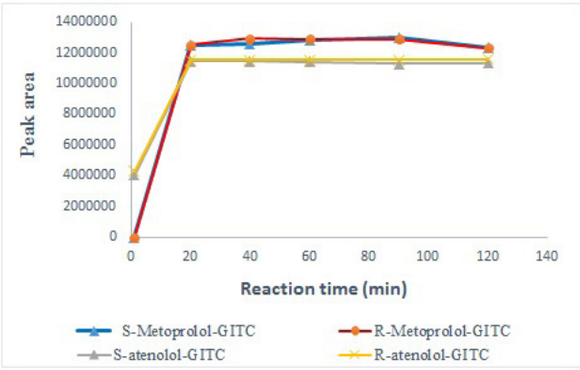

Figure 3. The effect of reaction time on derivatization reaction

3.1.1 Chromatographic behavior of the derivatives

The chirality of drugs is an important issue from the pharmacological, pharmacokinetic, toxicological, and regulatory points of view. The ample difference between the activities of the isomers of atenolol and metoprolol requires an accurate method to determine the enantiomeric composition of pharmaceutical dosage forms. GITC reacted selectively with β-blocker (atenolol and metoprolol) to form the corresponding diastereomeric thiourea. These diastereomers were well separable by RP-UPLC. Figure 4 show chromatograms of β-blockers derivatized with GITC. The results show that the diastereomeric thiourea product from GITC and β-blocker eluted S-form first[6,13]. This result was confirmed with S-isomer of atenolol and metoprolol.

3.1.2 Selection of chromatographic condition

The wavelength for the detection of atenolol and metoprolol was selected by using photodiode-array detection (DAD). The maximum number and the height of the peak could be obtained and the baseline of chromatogram was stable at 225 nm. Therefore, 225 nm was chosen as detection wavelength. The peak purity of two compounds in the sample was 99.9% obtained from spectrum overlaying graphs of three-point purity detection. The optimization of the chromatographic conditions was performed by using different compositions of mobile phases (1) acetonitrile: Ammonium acetate, (2) acetonitrile– Triethylamine formic; with different ratio of solvent in isocratic mode and different pH. The result showed that the resolution and peak shape was poor with system (2). Good resolution, baseline, sharp and symmetrical peaks were obtained by using system (1). The mobile phase was acetonitrile– Ammonium acetate in the ratio of 50:50 (v/v) was chosen for next investigation. As the pH of mobile phase increased from 4.5 to 7.0, the resolution factor and asymmetry (As) was changed depending on β-blockers-GITC behavior. At pH 5.0 for atenolol and pH 6.0 for metoprolol, best resolution and asymmetry were achieved. Hence, the mobile phase consisted of acetonitrile and ammonium acetate (pH=6) with ratio of 55:45 for separation of metoprolol isomers and methanol - ammonium acetate (pH=5) with ratio of 33:67 for separation of atenolol isomers. The representative chromatogram of the sample and standard were shown in Figure 4**.**

3.2. Method validation

3.2.1 System suitability

System suitability was tested by performing six replicate injections and determining theoretical plate number (N), resolution (Rs), and symmetry factor (As) and repeatability (RSD retention time and area) for the analyte of interest. As summarized in Table 1, the %RSD values of area and retention time were less than 1% indicating the precise analysis of atenolol and metoprolol by this system. All the results showed that the proposed method met the requirement.

3.2.2 Specificity

The selectivity was tested by applying the UPLC method to analyze standard, commercial product and spike standard to sampe. It was evaluated by comparing the retention time of each standard reference compound with that of the peaks obtained by analyzing real sample. There was no interference with the peaks of atenolol and metoprolol in sample (Figure 5).

3.2.3 Linearity, limits of detection and quantification

The result for regression equation, and correlation coefficients (r^2) are summarized in Table 2.

The LOD and LOQ of atenolol isomer were 0.18 μg/ml and 0.35 μg/ml, respectively. The LOD and LOQ of metoprolol isomer were 0.06 μg/ml and 0.2 μg/ml, respectively

3.2.4 Precision

For intra-day variability test, two β-blockers in six sample solutions were determined within one day, while for inter-day variability tests, two concentrations of β-blockers were examined in twice a day on 3 consecutive

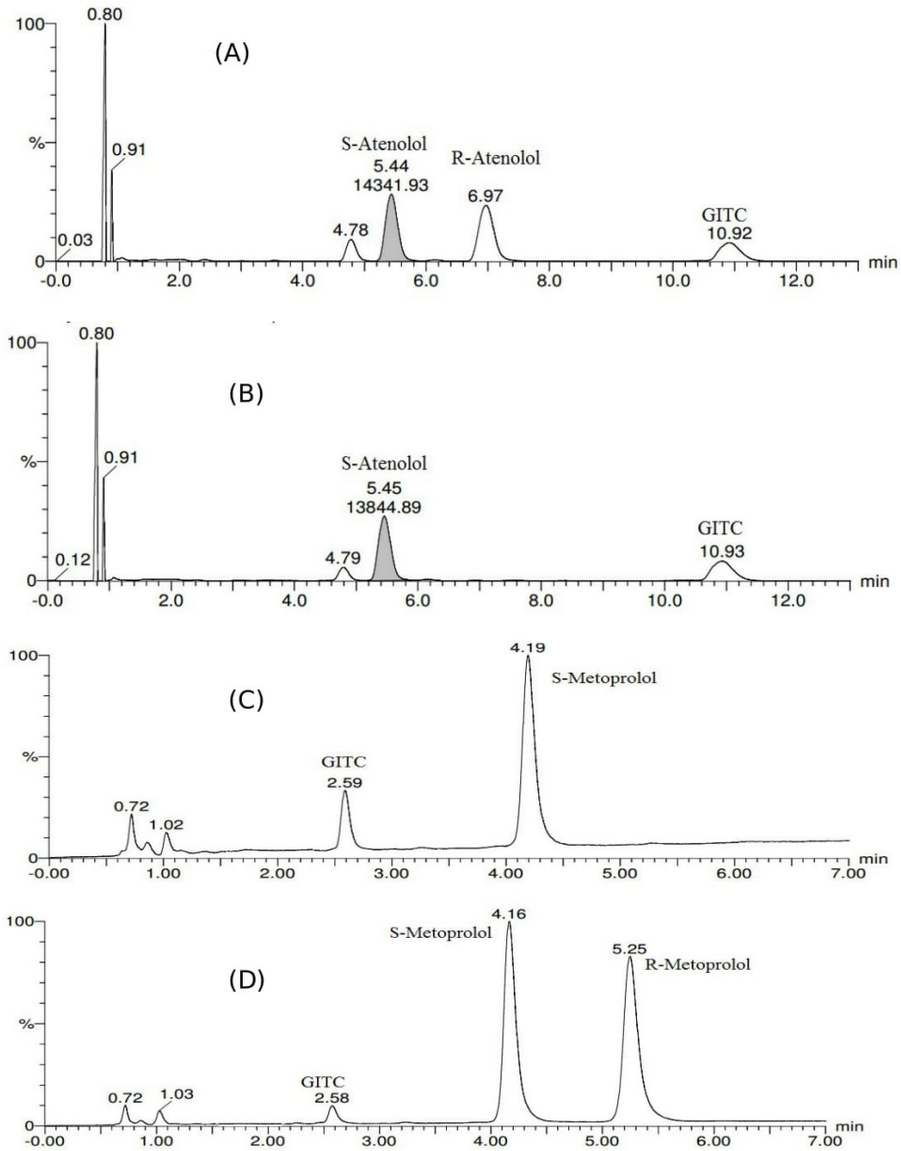

Figure 4. Chromatograms of the diastereomers obtained from β-blockers racemic (A and D) and S-isomer (B and C) after derivatization with GITC; detailed chromatographic conditions see method section.

Table 1. System suitability parameters

	t_R (min) (n=6)		peak area (mAu.s)		R_S	A_S
	average	RSD%	average	RSD%	average	average
S-atenolol	5.48	0.66	13748	0.60	2.21	1.45
R-atenolol	7.08	0.75	14499	1.49	3.19	1.26
S-metoprolol	4.01	0.90	9003	1.12	4.25	1.45
R-metoprolol	5.03	0.97	8736	1.25	2.95	1.43

Rs: resolution, As: asymmetry factor

days. All the results were expressed as relative standard deviations (RSD). The RSDs of intra-day and inter-day were 0.74-1.32% and 1.20-1.80% for atenolol isomers and metoprolol isomers, respectively (data were shown in Table 2).

Table 2. Linear regression data, LOD and LOQ, precision of the HPLC method for determination of atenolol and metoprolol isomers

Parameters	S-atenolol	R-atenolol	S-metoprolol	R-metoprolol
Regression equation	y = 398.8x + 11.72	y = 411.7 + 43.02	y = 96.554x – 159.6	y = 91.754x – 15.44
Linearity range (µg/ml)	0.35 - 70	0.35 - 70	0.2 - 200	0.2 - 200
r	0.9998	0.9999	0.9980	0.9996
LOD (µg/ml)	0.18	0.18	0.06	0.06
LOQ (µg/ml)	0.35	0.35	0.2	0.2
Precision (intra-day, % RSD)	0.74	1.04	1.20	1.65
Precision (inter-day, % RSD)	1.28	1.32	1.57	1.80

Regression curve data for six calibration points is y = ax + b, where y is peak area of analytes, x is concentration, a is slope, b is intercept, and r is correlation of determination.

3.2.5 The accuracy

The accuracy of the method was investigated by recovery studies; in particular, samples solutions were spiked with 1.0 ml aliquot of a solution containing standard compounds in sample, at three concentration levels and three samples for each level. The spiked samples were then derivatization reaction processed, and quantified in accordance with the methods mentioned above. Table 3 showed a summary of sample. The developed method had good accuracy with overall recovery was 99.82% for S-atenolol, 99.67% for R-atenolol, 99.58% for S-metoprolol and 100.6% for R-metoprolol with RSD less than 2% for the analytes. Considering the results of the recovery test, the method was deemed to be accurate.

Table 3. Recoveries for the assay of the investigated *atenolol and metoprolol isomers* in tablets

Analytes	Sample	Concentration (µg/ml)		Recovery	Mean recovery n=9	RSD (%)
		Added	Found			
S-Atenolol	S_1^a	40	39.88	99.71		0.28
	S_2^b	50	49.21	98.43	98.82	0.93
	S_3^c	60	59.00	98.33		0.44
R-atenolol	S_1^a	40	40.09	100.23		0.44
	S_2^b	50	49.56	99.11	99.67	0.70
	S_3^c	60	59.80	99.67		0.29
S-metorolol	S_1^a	240	238.66	99.44		1.31
	S_2^b	300	298.90	99.63	99.58	1.36
	S_3^c	360	358.76	99.66		1.37
R-metoprolol	S_1^a	240	239.51	99.80		0.59
	S_2^b	300	303.02	101.01	100.60	1.16
	S_3^c	360	363.58	100.99		0.95

Recovery (%) = (found/added) ×100.
a The samples added known amounts of standards at low level (80% of the known amounts).
b The samples added known amounts of standards at medium level (same as the known amounts).
c The samples added known amounts of standards at high level (120% of the known amounts)

3.3. Method application

The proposed method is applied to determine content of atenolol and metoprolol isomers in commercial tablets (Result in Table 4). A typical chromatography of commercial tablet is shown in Figure 6. The content of atenolol and metoprolol isomers in sample is calculated using calibration curve method. The samples are prepared in a manner similar to that described for the repeatability test. The contents based on the average of three replicate measurements are found.

The enantiomeric ratios R-AT/S-AT and R-MT/S-MT in pharmaceuticals are important because of their relationship to drug potency. With racemic tablets (sample A and C) results show nearly equal proportions (50.45/49.55 for atenolol and 50.47/49.53 for metoprolol). The U.S. Pharmacopeia[14] gives limits of between 45 and 55% for the diastereomeric content of each isomer, but no information is provided for enantiomeric ratios. With single isomer (sample B and D), the amounts of R-isomer of atenolol and metoprolol in commercial tablets in sample B about 1.17 % and in sample D about 1.04 % of R-isomer was found. For determination of chiral drugs, one isomer is active compound, otherwise minor isomer was considered as impurity. According to ICH guideline, trace enantiomer impurities do not exceed 0.2%[5]. The results of the assay indicate that this method is sensitive, selective for the analysis of atenolol and metoprolol isomer without interference from the excipients used to formulate and produce these tablets.

4. CONCLUSION

To sum up, the main advantage of this method is a simple sample preparation permits fast and efficient application of the proposed method to the quantitation of atenolol and metoprolol enantiomers with precision and accuracy.

5. Acknowledgments

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Table 4. Content of atenolol and metoprolol isomers in commercial tablets

Sample[a]	Content (%) in tablet[b]	
	S-isomer	R-isomer
A (atenolol racemic)	50.45±0.88	49.55±1.85
B (S-atenolol)	95.83±1.85	1.17±0.37
C (metoprolol racemic)	50.47±1.02	49.53±1.10
D (S-metoprolol)	92.53±0.75	1.04±0.56

a The sample solutions were prepared as described for the precision test.

b Each value is the mean response of 3 determinations: AT at 100 µg/ml; MT at 300.0 µg/ml, mean ±SD (n=3)

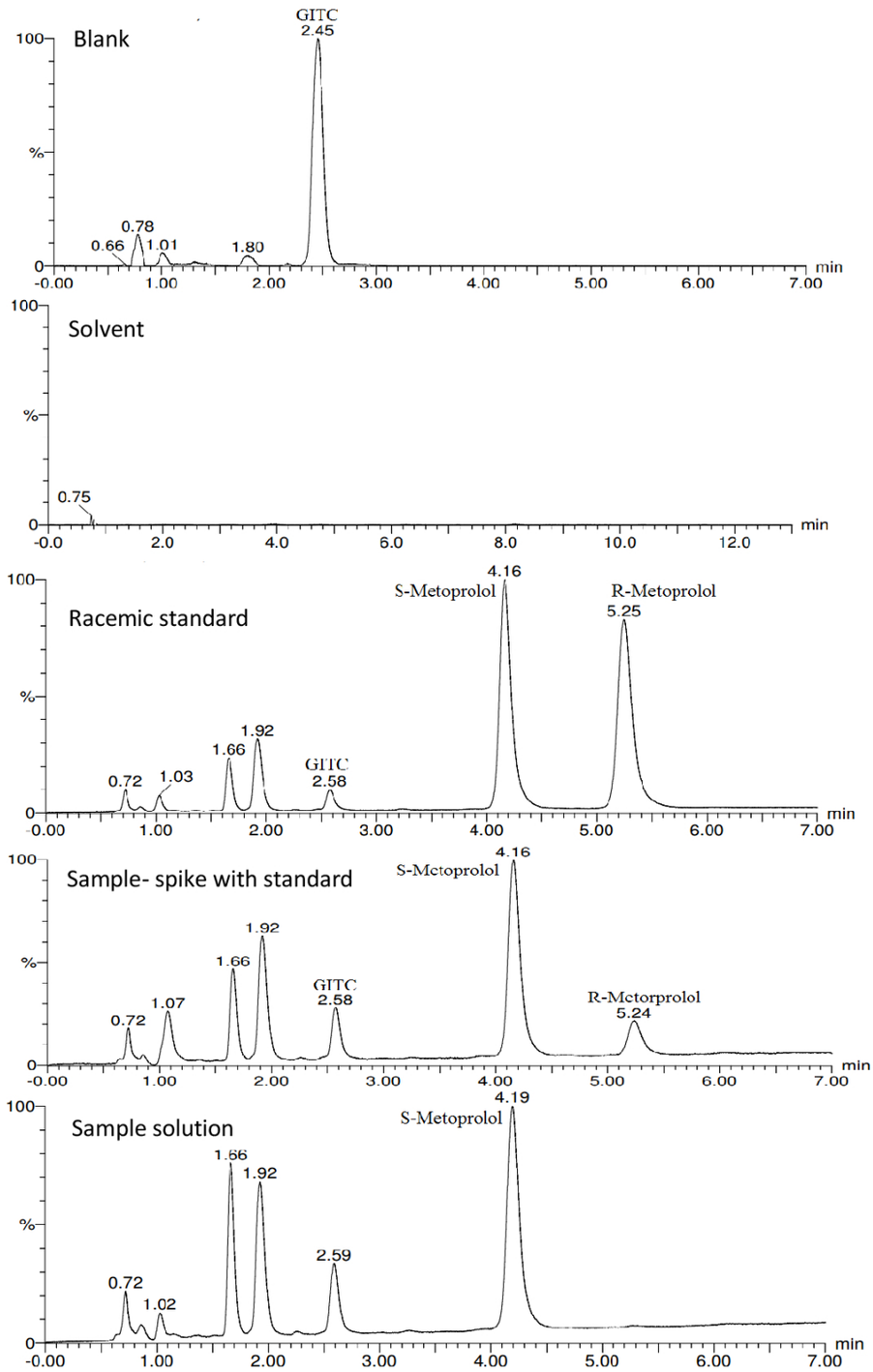

Figure 5. Representative UPLC chromatograms of mixed standards and the commercial product at 225 nm, detailed chromatographic conditions see (method section)

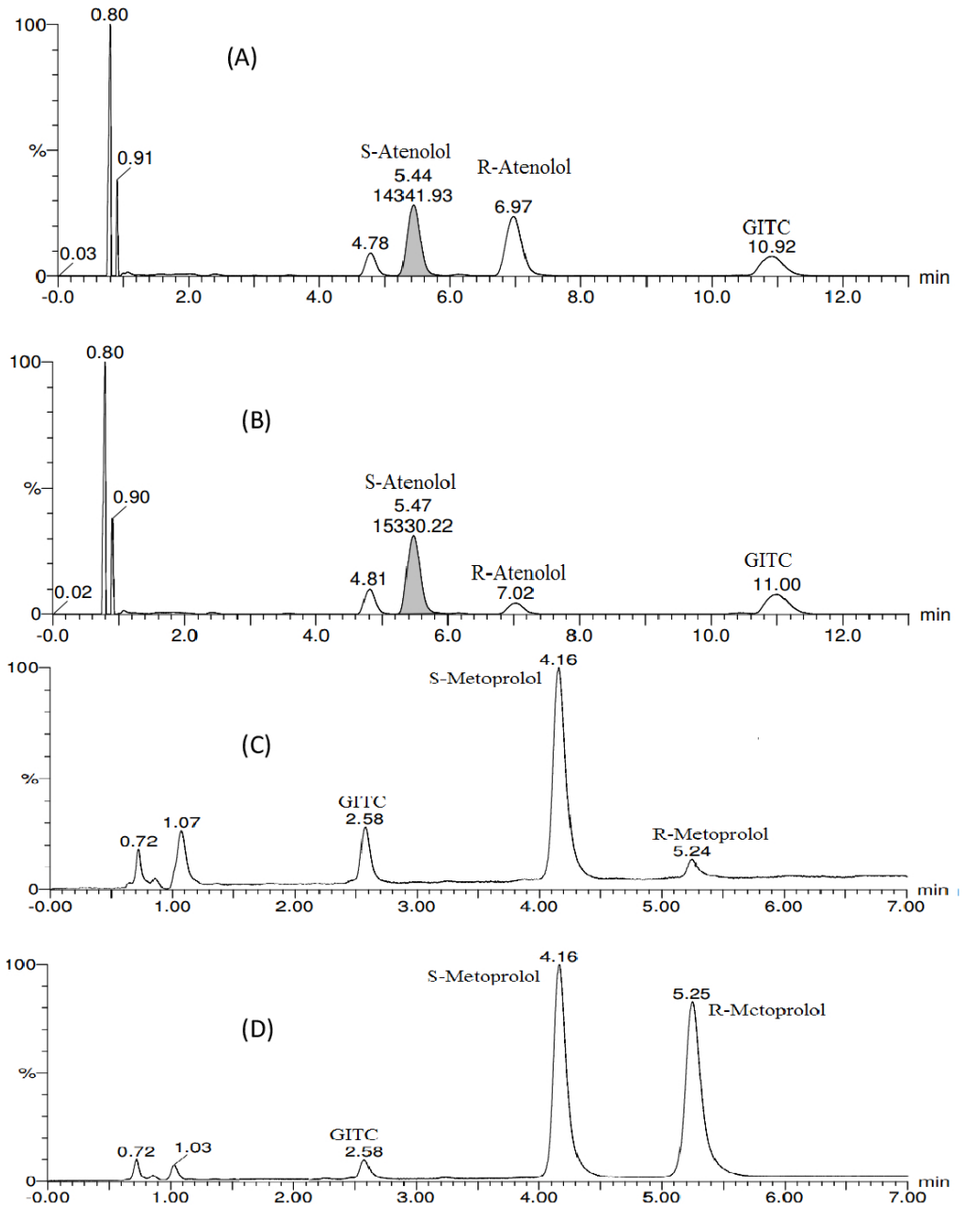

Figure 6. A typical chromatography of commercial tablets: sample A and B (atenolol tablets); sample C and D (metoprolol tablets)

REFERENCES

1. AU.S. Food and Drug Administration. Guideline for Submitting Supporting Documentation in Drug Applications for the Manufacture of Drug Substances [document on the Internet]; 1987 [updated 2018 Mar 22; cited 2018 Mar 27]. Available from: https://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm149499.htm
2. Nguyen LA, He H, Pham-Huy C. Chiral Drugs: An Overview. Int J Biomed Sci. 2006;2(2):85-100.
3. Nagori BP, Deora MS, Saraswat P. Chiral drug analysis and their application. Int. J. Pharm. Sci. 2011;6:106-13.

4. Sekhon BS. Exploiting the Power of Stereochemistry in Drugs: An Overview of Racemic and Enantiopure Drugs. J Mod Med Chem. 2013;1:10-36.
5. Lanchote VL, Bonato PS, Cerqueira PM, Pereira VA, Cesarino EJ. Enantioselective analysis of metoprolol in plasma using highperformance liquid chromatographic direct and indirect separations: applications in pharmacokinetics. J Chromatogr B Biomed Sci Appl. 2000;28;738(1):27-37.
6. Cheng B, Li L, Zhou R, Nie G, Zhang H. Enantioseparation and determination of atenolol enantiomers in tablet on a β-cyclodextrin-based chiral stationary phase by high performance liquid chromatography. Se Pu. 2014;32(11):1219-24.
7. Baranowska I, Adolf W, Magiera S. Enantioselective determination of metoprolol and its metabolites in human urine high performance liquid chromatography with fluorescence detection (HPLC-FLD) and tandem mass spectrometry (MS/MS). J Chromatogr B Analyt Technol Biomed Life Sci. 2015;1004:79-84.
8. Alwera S, Bhushan R. Liquid chromatographic enantioseparation of three beta-adrenolytics using new derivatizing reagents synthesized from (S)-ketoprofen and confirmation of configuration of diastereomers. Biomed Chromatogr. 2016;30(11):1772-81
9. Bhushan R, Tanwar S. RP-LC resolution of (R,S)-atenolol via diastereomerization with Marfey's reagent and its structural variants under conventional and microwave heating. Chromatographia. 2008;68(9-10): 849-53.
10. Kim KH, Choi PW, Hong SP, Kim HJ. Chiral separation of β-blockers after derivatization with (-)-menthyl chloroformate by reversed-phase high performance liquid chromatography. Arch Pharm Res. 1999;22(6):608-13.
11. Ko MY, Shin DH, Oh JW, Asegahegn WS, Kim KH. Chiral Separation of β-blockers after derivatization with a new chiral derivatization agent, GATC. Arch Pharm Res. 2006;29(11):1061-5.
12. Li X, Yao TW, Zeng S. Reversed-phase high-performance liquid chromatographic analysis of atenolol enantiomers in rat hepatic microsome after chiral derivatization with 2,3,4,6-tetra-*O*-acetyl-β-D-glycopyranosyl isothiocyanate. J Chromatogr B Biomed Sci Appl. 2000;742(2):433-9.
13. Péter M, Gyéresi A, Fülöp F. Liquid chromatographic enantioseparation of beta-blocking agents with (1R,2R)-1,3-diacetoxy-1-(4-nitrophenyl)-2-propyl isothiocyanate as chiral derivatizing agent. J Chromatogr A. 2001;910(2):247-53.
14. Kim KH, Choi PW, Hong SP, Kim HJ. Chiral purity test of metoprolol enantiomer after derivatization with (-)-menthyl chloroformate by reversed - phase high performance Liquid chromatogrophy. Arch Pharm Res. 1999;22(6):614-8.
15. U.S. Pharmacopeia (2015) 39th Ed., U.S. Pharmacopeial Convention, Inc., Rockville, MD, electronic version.
16. ICH, Q2(R1) Validation of Analytical Procedures: Text and Methodology, ICH Harmonized Tripartite Guideline, 2005
17. ICH, Q3(R2) Impurities in New Drug Products: Registration of Pharmaceuticals for Human Use, ICH Harmonized Tripartite Guideline, 2006