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Labeling of alprenolol with fluorescent aryl iodide as a reagent based on Mizoroki–Heck coupling reaction

Naoya Kishikawa, Mizuho Ohkuma, Mitsuhiro Wada, Kaname Ohyama, Rie Ikeda, Kenichiro Nakashima, Naotaka Kuroda*

Graduate School of Biomedical Sciences, Course of Pharmaceutical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan

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ABSTRACT

A novel fluorescent labeling method for alprenolol was developed based on Mizoroki–Heck coupling reaction. We designed and synthesized fluorescent aryl iodide, 4-(4,5-diphenyl-1*H*-imidazol-2-yl)iodobenzene (DIBI) as a labeling reagent. DIBI has a lophine skeleton carrying an iodide atom acting as fluorophore and reactive center, respectively. In order to evaluate the usefulness of DIBI, a high-performance liquid chromatography (HPLC) with fluorescence detection method was developed for the determination of alprenolol as a model compound of terminal double bond. The fluorescent labeling of alprenolol with DIBI was achieved in the presence of palladium acetate as a catalyst, and the labeled alprenolol was detected fluorometrically. In addition, it was found that the fluorescence of DIBI derivative increased and red shifted when compared with that of DIBI. Furthermore, the proposed method could be applied to determine the alprenolol concentration in rat plasma after administration of alprenolol without interferences from biological components. The detection limit (S/N = 3) for alprenolol in rat plasma was 0.74 ng/mL (30 fmol on column).

1. Introduction

The fluorescent labeling reagent is useful for sensitive fluorescence analysis of non- or weakly fluorescent analytes in combination with appropriate separation techniques such as high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE). Many fluorescent labeling reagents have been developed for a various functional groups such as amine, phenol, thiol, aldehyde and carbonyl groups [1–4]. Although the fluorescent labeling reagents are frequently applied to the drug analysis in biological fluids, there are drawbacks as follows: (1) fluorescent labeling technique cannot be applied to compounds which do not possess derivatizable functional groups, (2) fluorescent labeling reagent reacts with co-existing biological substances and the resultant products can interfere with the detection of target analyte. Recently, we developed a novel fluorescent labeling technique based on Suzuki coupling reaction which is a palladium-catalyzed cross-coupling reaction between aryl halides and aryl boronic acids [5]. We designed and synthesized fluorescent aryl boronic acid, 4-(4,5-diphenyl-1*H*-imidazol-2-yl)phenylboronic acid (DPA, a lophine derivative) as a fluorescent labeling reagent for aryl halides [5,6]. The Suzuki coupling reaction with DPA introduces fluorophore to the target analytes having an aryl halide in their structures, even in the absence of the derivatizable functional groups by a conventional fluorescent labeling reagent. Furthermore, DPA-labeled compounds can be detected clearly without interference on the chromatogram because DPA does not react with most of biological compounds. The practicability of the labeling procedure by DPA was confirmed in the successful analysis of the aryl halide drugs including clofibrate [5], haloperidol [7] and hydroxyzine [8] in biological fluids.

In addition to Suzuki coupling reaction, several types of palladium coupling reaction have been developed. Among them, the Mizoroki–Heck coupling reaction, one of such coupling reaction, is a palladium-catalyzed carbon–carbon bond formation between aryl halides and terminal double bonds [9–11]. We considered that Mizoroki–Heck reaction would become a new type of labeling reaction against terminal double bond by using fluorescent aryl halide as a labeling reagent. The aim of the present work is the establishment of a novel fluorescent labeling technique for terminal double bond based on Mizoroki–Heck coupling reaction in order to expand the application range of palladium coupling reaction in the field of analytical chemistry. For this purpose, we synthesized fluorescent aryl iodide, 4-(4,5-diphenyl-1*H*-imidazol-2-yl)iodobenzene (DIBI) as a specific fluorescent labeling reagent for terminal double bond (Fig. 1). DIBI has a lophine skeleton carrying an iodide atom acting as fluorophore and reactive center, respectively. In this study, we applied DIBI to the development

* Corresponding author. Tel.: +81 95 819 2894; fax: +81 95 819 2444.
E-mail address: n-kuro@nagasaki-u.ac.jp (N. Kuroda).

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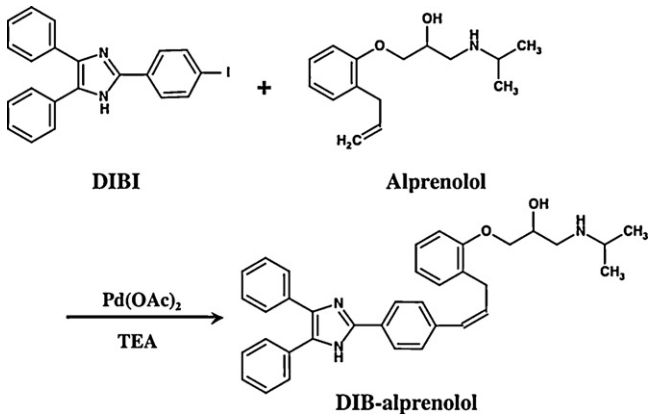

Fig. 1. Fluorescent labeling reaction of alprenolol with DIBI based on Mizoroki–Heck coupling reaction.

of determination method for alprenolol as a model compound of double bond. Alprenolol is a β-blocker that has a terminal double bond moiety and is used for treatment of cardiovascular diseases. Since the serial side effects of alprenolol, such as heart failure were reported, the sensitive and selective determination method for alprenolol should be useful to guide dosing of alprenolol [12,13]. We confirmed that alprenolol could be sensitively determined after its derivatization with DIBI (Fig. 1). Furthermore, the developed method was successfully applied to the determination of alprenolol in rat plasma without any interference with plasma components.

2. Experimental

2.1. Materials and reagents

Alprenolol was obtained from Sigma (St. Louis, MO, USA). Palladium (II) acetate ($Pd(OAc)_2$), acetic acid, *N*,*N*-dimethylacetamide (DMAc) and chloroform were purchased from Nacalai Tesque (Osaka, Japan). Acetonitrile (HPLC grade) was from Kanto Chemical (Tokyo, Japan). Triethylamine (TEA), ammonium acetate, iodobenzene and sodium hydroxide (NaOH) were from Wako (Tokyo, Japan). Benzil, *p*-iodobenzaldehyde and dimethyl sulfate were purchased from Tokyo Chemical Industries (Tokyo, Japan). DIBI, DIB–alprenolol and *N*,*O*-dimethylated alprenolol as an internal standard (IS) were synthesized as described in later sections. Water was distilled and passed through a Pure Line WL21P system (Yamato, Tokyo, Japan). All other chemicals were the highest purity and quality available. Stock solutions of alprenolol, IS and DIBI were prepared in DMAc and stored at 4 °C. $Pd(OAc)_2$ and TEA were dissolved in DMAc and water, respectively, just before use.

2.2. Equipment

The HPLC system consisted of a Shimadzu LC-9A pump (Kyoto, Japan), a Shimadzu RF-10AxL fluorescence detector, a Rheodyne 7125 injector with a 20-μL loop (Cotati, CA, USA), and a Tosoh FBR-1 recorder (Tokyo, Japan). Fluorescence spectra were measured with a Hitachi 650-10S fluorescence spectrophotometer (Tokyo, Japan). ^{1}H NMR spectral data were obtained on a Varian Unity plus spectrometer (Palo Alto, CA, USA). Mass spectral data were obtained on a JEOL JMS-700 N spectrometer (Tokyo, Japan). Elemental analyses were performed on a Perkin Elmer 2400II (Norwalk, CT, USA). Melting points were measured with a Yanagimoto MP-53 melting point apparatus (Tokyo, Japan).

2.3. Synthesis of DIBI

DIBI was synthesized according to the previous papers [14,15]. *p*-Iodobenzaldehyde (110 mg, 0.5 mmol), ammonium acetate (97 mg, 1.25 mmol) and benzil (53 mg, 0.25 mmol) were dissolved in 2.5 mL of acetic acid. This mixture was heated at 100 °C for 8 h. After cooling to room temperature, the mixture was poured into cold water. The resultant precipitate was recrystallized from methanol to give colorless crystals; yield: 102 mg, 96%, mp: 260 °C. Elemental analysis; calculated for $C_{21}H_{15}N_2I$: C, 59.72%; H, 3.58%, N, 6.63%, found: C, 59.94%, H, 3.34%, N, 6.51%. High resolution EI-MS (*m*/*z*) found: 422.0291, calculated: 422.0279 [M^+]. ^{1}H NMR (500 MHz, DMSO-d_6): δ, ppm = 12.75 (s, 1H, NH), 7.89 (d, *J* = 8.5 Hz, 2H, 2-phenyl), 7.85 (d, *J* = 8.5 Hz, 2H, 2-phenyl), 7.53–7.23 (m, 10H, 4 and 5-phenyl).

2.4. Preparation of authentic DIB–alprenolol

Alprenolol (143 mg, 0.5 mmol) and DIBI (106 mg, 0.25 mmol) were dissolved in DMAc (7 mL). To this solution, $Pd(OAc)_2$ (1.7 mg, 7.5 μmol) and TEA (70 μL, 0.5 mmol) were added and then the solution was purged with N_2 (5.0 mL/min) for 5 min. After heating at 100 °C for 3 h, the solution was filtered and poured into cold water. To this solution, 1 M NaOH aqueous solution was added until precipitate was obtained. The resulting precipitate was filtered, and a portion of the crude product was purified by chromatography on silica gel using ethyl acetate containing 4% TEA to give DIB–alprenolol as yellow crystals; yield: 20 mg, 15%, mp: 87 °C. Elemental analysis: calculated for $C_{36}H_{37}N_3O_2{\cdot}1.2H_2O$, calculated: C, 76.49%; H, 7.02%; N, 7.43%, found: C, 76.23%; H, 7.12%; N, 7.48%. High resolution FAB-MS (*m*/*z*) found: 544.2971, calculated: 544.2964 [M+H]$^+$. ^{1}H NMR (500 MHz, CD_3OD): δ, ppm = 7.92–7.19 (m, 18H, phenyl), 6.84 (d, *J* = 16 Hz, 1H, –Ph–C*H*=CH–CH_2–), 6.42 (m, 1H, –Ph–CH=C*H*–CH_2–), 4.15 (m, 1H, –CH_2–C*H*(OH)–CH_2–), 4.02 (m, 2H, –O–CH_2–CH(OH)–), 3.60 (m, 2H, =CH–CH_2–Ph–), 2.98 (m, 2H, –CH(OH)–CH_2–NH–), 2.84 (m, 1H, –NH–C*H*(CH_3)$_2$), 1.12 (m, 6H, –CH(CH_3)$_2$).

2.5. Preparation of N,O-dimehtylated alprenolol

Alprenolol (206 mg, 0.72 mmol) and dimethyl sulfate (288 μL, 3.04 mmol) were dissolved in 2 M NaOH aqueous solution (0.9 mL). After the reaction at 4 °C for 2 h, the mixture was purified by preparative HPLC using Tosoh TSK-GEL ODS-80TM (300 mm × 7.8 mm i.d., 10 μm) eluted with a mixture of acetonitrile and water (=50:50, v/v) containing 0.1% trifluoroacetic acid at a flow rate of 1.0 mL/min. *N*,*O*-Dimethylated alprenolol was extracted from the eluted fraction with dichloromethane, and then the organic phase was evaporated to give *N*,*O*-dimethylated alprenolol as a colorless oil; yield: 135 mg, 67%. Elemental analysis: calculated for $C_{17}H_{27}N_1O_2{\cdot}0.85CH_2Cl_2$, calculated: C, 61.33%; H, 8.27%; N, 4.01%, found: C, 61.39%; H, 8.09%; N, 3.63%. FAB-MS (*m*/*z*) found: 263, calculated: 263 [$M-CH_3$]$^+$. ^{1}H NMR (500 MHz, CD_3OD): δ, ppm = 7.26–6.90 (m, 4H, phenyl), 5.94 (m, 1H, CH_2=C*H*–CH_2–Ph–), 4.90 (m, 2H, CH_2=CH–CH_2–Ph–), 4.76 (m, 1H, –CH_2–C*H*(OCH_3)–CH_2–), 4.02 (m, 2H, –O–CH_2–CH(OCH_3)–), 3.82 (m, 2H, CH_2=CH–CH_2–Ph–), 3.53 (m, 1H, –N(CH_3)–C*H*(CH_3)$_2$), 3.32 (m, 2H, –CH(OCH_3)–CH_2–N(CH_3)–), 3.27 (s, 3H, –CH_2–CH(OCH_3)–CH_2–), 3.24 (s, 3H, –CH_2–N(CH_3)–CH_2–), 1.43 (dd, *J* = 6.3, 16 Hz, 6H, –CH(CH_3)$_2$).

2.6. Fluorescent labeling procedure and HPLC conditions

To the DMAc solution (50 μL) including 4.00–600 ng/mL alprenolol and 150 ng/mL IS, 7 mM DIBI in DMAc (50 μL), 20 μM $Pd(OAc)_2$ in DMAc (50 μL) and 50 mM TEA in water (50 μL)

were successively added and mixed well. After purging with N_2 (5.0 mL/s) for 15 s, the reaction mixture was heated at 100 °C for 15 min. After filtration through a membrane filter (0.45 μm, HLC-DISK 3, Kanto chemical), a 20 μL of aliquot was injected into HPLC system.

Chromatographic separation was performed on a Merck Lichrosorb Si60 (250 mm × 4 mm i.d., 5 μm) by using an isocratic elution with a mixture of acetonitrile and water (=90:10, v/v) containing 0.02% TEA and 0.02% acetic acid at a flow rate of 1.0 mL/min. The excitation and emission wavelengths were set at 330 nm and 420 nm, respectively.

2.7. Assay procedure for alprenolol in rat plasma

Wistar male rat (274 g, Tagawa experimental animals, Nagasaki, Japan) was used for experiment. Rat was anesthetized with ethyl carbamate (1.5 μg/kg, i.p.). Alprenolol was administered (i.a.) via arteria femoralis at a dose of 5 mg/kg. Blood samples were collected through indwelling arterial catheters, transferred to EDTA tubes and centrifuged at 5000 × *g* for 10 min, then the plasma was separated and stored at −80 °C in the dark until analysis. The experiment was performed with an approval of the Nagasaki University Animal Care and Use Committee.

One-hundred microliters of plasma was spiked with 10 μL of 3 μg/mL IS in DMAc and then 400 μL of 0.1 M NaOH aqueous solution was added. To this solution, 2 mL of chloroform was added and vortexed for 60 s followed by centrifugation at 5000 × *g* for 10 min. This extraction was repeated again. The organic layer was transferred to a screw-capped amber-colored reaction vial and evaporated in a centrifugal evaporator for 10 min. The residue was reconstituted in 50 μL of DMAc and then introduced into the labeling reaction as previously described.

3. Results and discussion

3.1. Fluorescence characteristics of DIBI and DIB–alprenolol

We selected aryl iodide as a reactive group for labeling because it was known that the reactivity order of aryl halides in Mizoroki–Heck reactions is usually aryl iodide > aryl bromide > aryl chloride [16]. DIBI could be obtained by the relatively simple reaction as described in Section 2.2. Fig. 2 shows the fluorescence spectra of the DIBI and authentic DIB–alprenolol. The fluorescence intensity of DIB–alprenolol was approximately 4 times higher than that of DIBI. The increase in fluorescence might be attributed to the elimination of iodine group during the reaction, which acts as a quencher of fluorescence. Additionally, the maximum emission wavelength of DIB–alprenolol (415 nm) was significantly longer than that of DIBI (390 nm). The extension of the conjugation structure might cause the red shift of the fluorescence emission wavelength. The changes of fluorescence characteristic of DIBI along with labeling reaction should be advantageous because the reagent blank peaks derived from excess DIBI can be reduced on the chromatogram.

3.2. Optimization of labeling reaction conditions

Fig. 3(A) and (B) shows the typical chromatograms obtained from reagent blank and the reaction mixture of alprenolol and IS with DIBI, respectively. The alprenolol and IS were successfully labeled with DIBI, and were detected at 17 and 25 min on the chromatogram, respectively.

In order to obtain higher reactivity, the labeling reaction conditions were optimized using a standard solution of alprenolol and IS. The effect of organic solvent for alprenolol and DIBI was examined with DMAc, dimethyl formamide, *N*-methylpyrrolidone, dioxane, methanol and acetonitrile. Among these solvents, the optimal result was obtained with DMAc and it was selected for subsequent work. The concentration of DIBI was investigated over a range of 0.5–10 mM, and the maximum peak height of alprenolol was obtained at a concentration of 7 mM (Fig. 4). Under the optimized condition, the molar ratio of DIBI to alprenolol was approximately 14,000. The effect of base was investigated using TEA, sodium carbonate, tripotassium phosphate, potassium fluoride and dipotassium carbonate. Among the examined bases, the optimal result was obtained with TEA. Then, the concentration of TEA was studied over a range of 20–60 mM and 50 mM of TEA

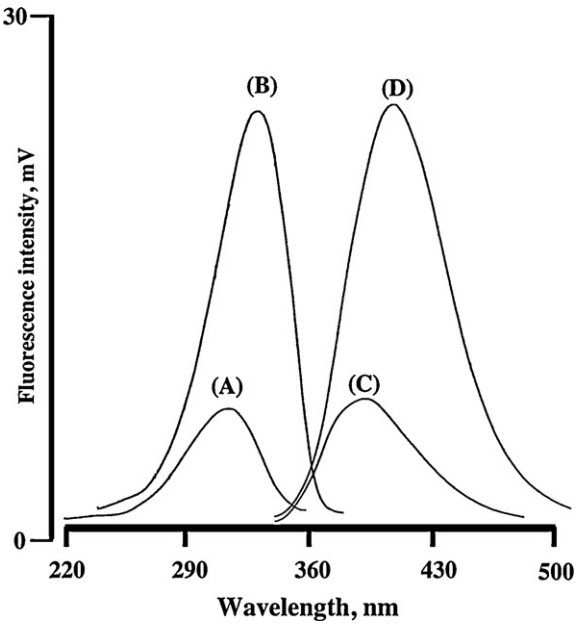

Fig. 2. Fluorescence spectra of 2.5 μM DIBI and 2.5 μM DIB–alprenolol. Excitation spectra of (A) DIBI with emission at 390 nm and (B) DIB–alprenolol with emission at 415 nm. Emission spectra of (C) DIBI with excitation at 315 nm and (D) DIB–alprenolol with excitation at 335 nm.

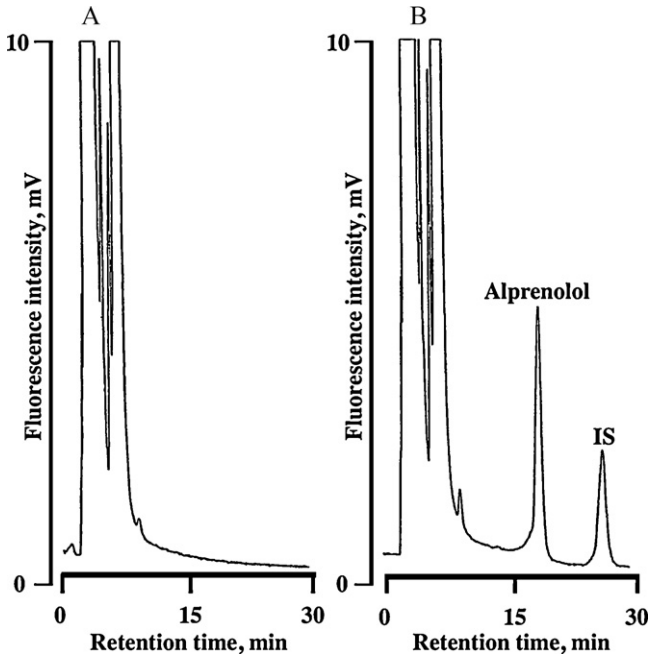

Fig. 3. Chromatograms of (A) reagent blank and (B) standard solution of alprenolol and IS. The concentrations of alprenolol and IS were 120 and 150 ng/mL, respectively.

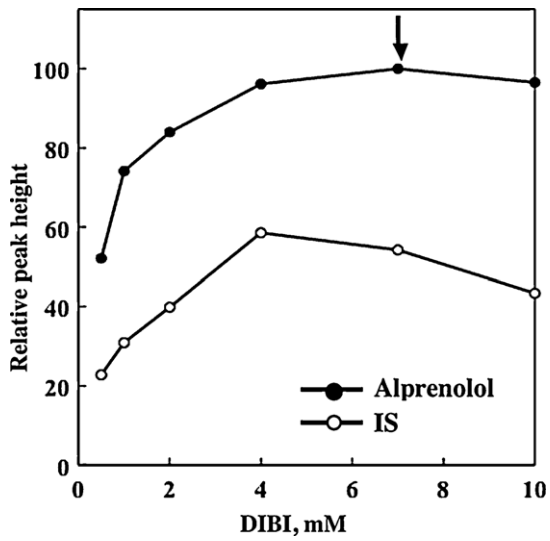

Fig. 4. Effect of DIBI concentration on the peak heights of alprenolol and IS. The concentrations of alprenolol and IS were 120 and 150 ng/mL, respectively.

was selected because it gave maximum peak heights. The effect of $Pd(OAc)_2$ concentration was investigated over a range of 5–40 μM, and the maximum peak height of alprenolol was observed at 20 μM; 20 μM of $Pd(OAc)_2$ was selected. The reaction temperature was selected at 100 °C because the peak heights increased with increasing the temperature. The effect of reaction time was investigated at 100 °C until 30 min. Since the maximum peak heights were achieved by heating for more than 10 min, 15 min was selected. Under the optimized conditions, the reaction yields for alprenolol was calculated by comparing the peak heights of the reaction products and authentic DIB–alprenolol. The reaction yields was 97%, suggesting that the labeling reaction proceeded in excellent yield.

3.3. Optimization of extraction conditions

The extraction conditions of alprenolol from rat plasma were investigated. For the effective extraction of alprenolol and IS from plasma, liquid–liquid extraction was investigated as a sample treatment by using 100-μL plasma spiked with alprenolol. To choose the extraction solvent, chloroform, *n*-hexane and diethyl ether were compared and the maximum recovery was obtained with chloroform; chloroform was employed. Both the volume of extraction solvent and the number of extraction were studied using 1.0–3.0 mL of chloroform with one or twice extraction. The optimal volume and number were 2.0 mL and twice extraction, respectively. NaOH was added to plasma in order to inhibit the dissociation of amino group of alprenolol. The effect of NaOH concentration was examined over a range of 0.01–0.5 M. Maximal and constant recoveries were obtained more than 0.05 M of NaOH; 0.1 M NaOH was selected. After the optimization of the extraction conditions, the recoveries of alprenolol and IS were 89 and 75%, respectively.

3.4. Calibration curve, detection limit and repeatability

A calibration curve was prepared by spiking plasma with known amount of alprenolol. A linear relationship ($r = 0.999$) was obtained between peak height ratio of alprenolol to IS and alprenolol concentration over the range from 2.00 to 300 ng/mL. The slope and intercept of regression equation (mean ± standard error, $n = 3$) were

Table 1
Within- and between-day accuracy and precision of the proposed method for determination of alprenolol in rat plasma.

Alprenolol (ng/mL)	Within-day ($n = 5$)		Between-day ($n = 5$)	
	Accuracy (%)	Precision (RSD[a])	Accuracy (%)	Precision (RSD)
2	98.8	4.4	90.2	9.5
15	103.9	4.2	105.0	5.3
60	95.1	2.7	99.6	3.8

[a] Relative standard deviation.

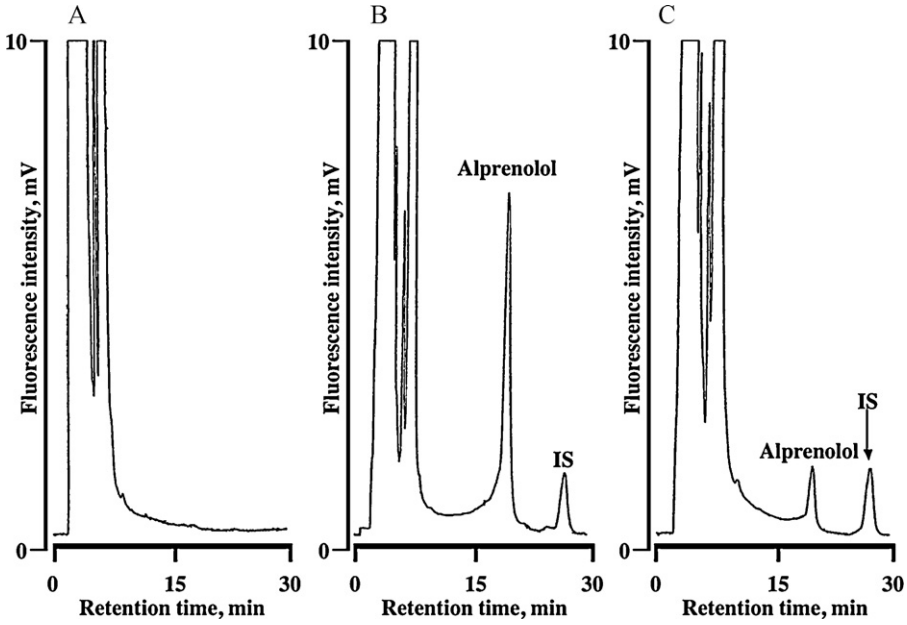

Fig. 5. Chromatograms of (A) blank rat plasma and rat plasma after (B) 60 min and (C) 420 min of the administration of alprenolol.

0.031 ± 0.006 and -0.059 ± 0.017, respectively. The detection limit of alprenolol in rat plasma was 0.74 ng/mL (30 fmol on column) at a signal-to-noise ratio (S/N) of 3. The sensitivity of the proposed method was approximately 600, 60 and 14 times higher than that of HPLC with amperometric detection [17], ELISA [18] and HPLC with atmospheric pressure chemical ionization mass spectrometry [19]. Although the sensitivity of the proposed method was comparable with that of HPLC with tandem mass spectrometry [20], the proposed method does not require expensive and elaborate instruments. Accuracy and precision of the proposed method were examined using three levels (2.0, 15 and 60 ng/mL) of alprenolol in rat plasma (Table 1). As shown in Table 1, the within- and between-day accuracy of alprenolol ranged from 90.2% to 105.0% with precision values less than 9.5%. These results indicated that the proposed method showed sufficient accuracy and precision.

3.5. Determination of alprenolol in rat plasma after administration

The alprenolol concentration in rat plasma after a single dose of alprenolol was determined by the proposed method. Typical chromatograms of rat plasma in the absence of alprenolol and IS, rat plasma collected after 60 and 420 min after the i.a. administration of alprenolol (5 mg/kg) are shown in Fig. 5(A–C), respectively. The peak of alprenolol could be detected clearly and there were no interfering peaks derived from plasma components because DIBI could react specifically with terminal double bond and did not react with most of biological compounds. As shown in Fig. 5(B) and (C), the plasma concentration of alprenolol decreased with time after administration. This decrease could be attributed to the disappearance of alprenolol from the blood by metabolism and excretion. Therefore, the proposed method should be useful to investigate the pharmacokinetic parameters of terminal double bond drugs. Also, it has been reported that (*S*)-enantiomer of the β-blocker showed stronger pharmacological activity than (*R*)-enantiomer [21]. Therefore, selective determination of individual enantiomers of alprenolol should be useful to properly assess their own pharmacological effects. The application of the proposed labeling reaction to the separation by HPLC equipped with a chiral stationary phase column should allow highly sensitive and enantioselective determination of alprenolol enantiomers.

4. Conclusion

In this study, we employed fluorescent aryl iodide, DIBI as a specific fluorescent labeling reagent for alprenolol. DIBI reacted with terminal double bond of alprenolol based on Mizoroki–Heck coupling reaction. By using DIBI, we developed a sensitive and selective HPLC determination method for alprenolol. The fluorescent labeled alprenolol was clearly detected on the chromatogram even in the presence of biological components. The proposed method could be applied to the determination of alprenolol concentration in rat plasma after administration of alprenolol. Since the proposed labeling technique is specific for terminal double bond and allows sensitive detection, DIBI should be useful for real sample analysis of several terminal double bond chemicals such as oxprenolol, levallorphan and cholecalciferol.

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