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Determination of celiprolol in human plasma using high performance liquid chromatography with fluorescence detection for clinical application

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ABSTRACT

A new method of analysis has been developed and validated for the determination of plasma celiprolol concentration. Plasma samples (1 ml) were pre-purified by solid-phase extraction with Bond Elut® C18. The separation was achieved with XBridge™ C18 column (150 mm × 3.0 mm i.d., 3.5 μm) at 35 °C using a mixture of acetonitrile and 10 mM ammonium acetate buffer (pH 10.5) (34:66, v/v) under isocratic conditions at a flow rate of 0.4 ml/min. The peak was detected using a fluorescence detector at excitation 250 nm and emission 482 nm. Retention times for the internal standard (acebutolol) and celiprolol were 4.2 min and 6.3 min, respectively. Calibration curves were linear over the range of 1.0–1000 ng/ml ($r > 0.999$), with a limit of quantification at 1.0 ng/ml. Intra- and inter-assay precision (relative standard deviation) were less than 13.3% and the accuracy (relative error) was −5.1% to 11.5% at four different concentrations. This proposed method was successfully applied to a study of pharmacokinetic interactions between celiprolol and apple juice in humans.

1. Introduction

Celiprolol, a β_1-selective adrenergic receptor antagonist with a weak vasodilating property, has been used for the treatment of hypertension, angina pectoris, and arrhythmia [1]. It is a weak base with a pK_a-value of 9.7 and has intermediate lipophilic properties in comparison with other β-adrenergic receptor antagonists [2]. The mean elimination half-life is about 5 h, and it is eliminated mostly in an unchanged form in both bile and urine after oral administration [1].

Recently, it has been shown that membrane transporters, such as P-glycoprotein (P-gp) and organic anion transporting polypeptides (OATPs), play a significant role in the drug disposition [3]. Celiprolol is one of the best-characterized P-gp substrates among the β-blockers [2]. In humans, itraconazole, a P-gp inhibitor, was reported to increase plasma celiprolol concentration without affecting the elimination half-life, indicating that the absorption of celiprolol was increased by inhibition of P-gp in the intestine [4]. On the other hand, fruit juices such as grapefruit juice and orange juice significantly reduce the bioavailability of celiprolol by 87% and 83%, respectively [4,5]. The remarkable reduction in the bioavailability of celiprolol by fruit juices can be considered to be clinically important. Regarding the mechanism of the interaction, previous studies have revealed that celiprolol is a substrate of uptake transporters such as OATP1A2 [6] and OATP2B1 [7]. Both of them are expressed in the intestine, and the transport function is inhibited by fruit juices including grapefruit, orange and apple juice [8,9]. These findings suggest that a reduced absorption of celiprolol in the intestine is the most likely mechanism for the fruit juice and celiprolol interaction [4,5]. However, clinical evidence for the fruit juice–drug interaction mediated by uptake transporters is limited. Celiprolol can be one of the model drugs for further in vivo studies to elucidate the contribution of uptake transporters to drug interactions. A simple and sensitive analytical method for celiprolol in plasma would facilitate such interaction studies conducted in vivo.

Several HPLC methods, using UV detection [10–12], fluorescence detection [13,14], and diode array detection [15,16], have been developed for the determination of celiprolol in plasma. However, the sensitivity of these methods (quantification limits: >5 ng/ml) may be insufficient for interaction studies in humans since an induced drug metabolism and/or a reduced absorption by coadministered drug/food decreases drug levels in plasma. A higher sensitivity to detect lower plasma concentrations is necessary for further interaction studies. In addition, reported methods require a traditional liquid–liquid extraction procedure with a considerable amount of toxic and flammable organic solvents for sample preparation [10–15].

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Although, the LC–MS/MS method for determination of celiprolol in plasma has been reported [17], it requires expensive instruments and highly skilled technical experts. A more convenient method, using HPLC, would be helpful for measuring plasma celiprolol concentrations for laboratories where LC–MS/MS is not available.

The aim of the present study was to develop a simple and sensitive HPLC method for the determination of celiprolol in human plasma. The time–plasma concentration profile of celiprolol determined by this method was also assessed.

2. Materials and methods

2.1. Reagents

Celiprolol hydrochloride and acebutolol hydrochloride as an internal standard (IS) were purchased from Santa Cruz Biotechnology Inc. (CA, USA) and Sigma–Aldrich Japan, Inc. (Tokyo, Japan), respectively. Acetonitrile, methanol, 25% aqueous ammonia, ammonium acetate and sodium acetate were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Acetic acid and triethylamine were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). All chemicals and solvents were of analytical-reagent grade. Purified water was obtained by Milli-Q water purification system (Japan Millipore, Ltd., Tokyo, Japan).

2.2. Stock solutions and calibration standard

Stock solutions containing 100 μg/ml celiprolol and acebutolol (internal standard, IS) were prepared in methanol. Working solutions were prepared by serial dilution of the stock solution with 10 mM ammonium acetic acid buffer (pH 10.5) and stored at 4 °C. For preparation of calibration standards and quality control samples, appropriate aliquots of the stock solutions were added to drug-free plasma. The final concentrations were 1, 10, 50, 100, 500 and 1000 ng/ml for the calibration standards and 1, 50, 500, 1000 ng/ml for quality control (QC) samples. These plasma samples were stored at −30 °C.

2.3. Chromatography

Chromatography was performed using an Agilent 1220 infinity LC® (Agilent Technologies, Inc., Santa Clara, CA, USA). EZChrom Elite Compact Software (Agilent) was used for data acquisition and processing. The fluorometric detector was set at 250 nm excitation and 482 nm emission wavelengths. Chromatographic separations were performed on an XBridge™ C18 (3.5 μm, 150 mm × 3.0 mm i.d., Waters, Milford, MA, USA) operated at 35 °C. The mobile phase consisted of 10 mM ammonium acetate buffer, adjusted to pH 10.5 with 25% ammonium solution, and acetonitrile (66:34, v/v). The flow rate was 0.4 ml/min.

2.4. Sample preparation

Bond Elut C18® columns (3 ml, 500 mg packing, Varian, CA, USA) were used for sample preparation. To 1 ml of plasma, 25 μl of the internal standard working solution (1 μg/ml) and 975 μl of 0.2 M sodium acetate buffer (pH 4.0) were added. The mixtures were loaded to the columns that were conditioned with 2 ml of methanol and 2 ml of water in order, and then acidified with 1.5 ml of 0.2 M sodium acetate buffer (pH 4.0). Following sample loading, the columns were washed with 2 ml of water, 2 ml of 50% methanol/water, and 1 ml of 70% methanol/water. Columns were dried by suction and the analytes were eluted in duplicate with 1 ml of 50 mM triethylamine in methanol (TEA solution). Eluates were dried at 40 °C in a vacuum. The residues were reconstituted with 100 μl of mobile phase, vortexed for 30 s, and filtrated with millex LG® (0.2 μm, Millipore, MA, USA). The samples were transferred to micro-vials and 15 μl was injected into the HPLC system.

2.5. Method validation

The method validation assays were carried out according to the US Food and Drug Administration (FDA) bioanalytical method validation guidance [18] on selectivity, linearity, precision, accuracy, recovery and stability.

2.5.1. Selectivity

Selectivity of the assay method was assessed by evaluating potential interference from endogenous compounds. Six randomly selected blank plasma samples were analyzed. Each blank sample was tested for interference using the proposed extraction procedure and chromatographic conditions and was compared with those obtained with working standard solution of celiprolol.

2.5.2. Linearity of calibration curves

Calibration curves for celiprolol were constructed from the working standard solutions over a range 1–1000 ng/ml (i.e., 1, 10, 50, 100, 500, 1000 ng/ml). The linearity of each calibration curve was determined by the peak area ratio (y) of celiprolol to IS versus nominal concentration (x) of celiprolol. The calibration curves were constructed by weighted ($1/x$) least squares linear regression. Calibration curves were constructed for five consecutive days to evaluate the linearity of calibration curves. The lower limit of quantification (LLOQ) is defined as the lowest concentration on the calibration curve. The deviations of back-calculated concentrations of calibration standards from their nominal values should be within ±20% for LLOQ and ±15% for all other calibration levels.

2.5.3. Precision and accuracy

The precision and accuracy of the method were evaluated by assaying the QC samples at three different concentrations. The intra-day precision and accuracy were performed by assaying five spiked samples on a single day. The inter-day precision and accuracy were performed by assaying each QC sample (in duplicate) on five different days. Intra-assay and inter-assay precision were expressed as the relative standard deviation (RSD, %) of measured concentrations of the QC samples. Accuracy was expressed as the relative error (RE%) for each QC level by comparing the nominal concentration and the estimated concentration determined after extraction. The precision criterion for the acceptability of data was that the variation for each concentration level should not exceed 15% except for the LLOQ, for which it should not exceed 20%. Similarly, for accuracy, the mean value should not deviate by ±15% from the nominal concentration except for LLOQ, for which the limit was ±20%.

2.5.4. Extraction recovery

The extraction recovery for celiprolol for four QC samples was assessed by comparing the peak areas for extracted spiked plasma samples with the peak areas for pure compounds of the same concentrations in solvent. The recovery of the IS was evaluated at the concentration used in sample analysis.

2.5.5. Stability

The stability of QC samples was evaluated by duplicate assay at two concentration levels (1 ng/ml and 50 ng/ml), including (a) stability of plasma samples at −30 °C for one month, (b) stability after two freeze–thaw cycles, (c) stability of the extracted samples at room temperature (kept in the autosampler) for 24 h and 48 h. Samples were analyzed and the concentrations obtained were compared with those of the respective freshly prepared QC samples. For

all these experiments, stability was acceptable when ≥85% of the analyte was recovered.

2.6. Application

The proposed method was applied to a pharmacokinetic drug–apple juice interaction study in healthy volunteers. The clinical study protocol was approved by the Institutional Review Board of Oita University Hospital and a written informed consent was obtained from all participants. After physical examinations and clinical laboratories tests, 15 healthy volunteers enrolled in the randomized crossover study. Each subject was given 200 mg celiprolol (Selectol®, Nippon Shinyaku Co. Ltd., Kyoto, Japan) with 500 ml water (control) or apple juice (Welch's apple 100®, CALPIS Co. Ltd., Tokyo, Japan). Blood samples were collected into heparinized tubes at time points as 0, 1, 2, 3, 4, 5, 6, 8, 12 and 24 h after oral celiprolol administration. Samples were centrifuged and plasma was separated and stored at −30 °C until analyzed. We applied the above described analytical method to this human study. In this report, plasma celiprolol concentrations and the pharmacokinetic parameters in five subjects who were initially enrolled in the human study have been presented.

Individual subject pharmacokinetic (PK) parameters of celiprolol were derived using non-compartmental analysis method (WinNonlin® version 6.2, Pharsight Co., Mountain View, CA). The peak concentration (C_{max}) and concentration peak time (t_{max}) were obtained directly from the original data. The elimination half-life ($t_{1/2}$) was calculated using the equation 0.693/λ, where λ is the terminal slope of time versus log concentration. The area under the plasma concentration–time curve from time zero to 24 h (AUC_{24}) was calculated by the trapezoidal rule.

Statistical analyses were performed with SPSS for Mac (ver. 16.0JA, SPSS Inc., Tokyo, Japan). The log-transformed C_{max}, AUC_{24}, and $t_{1/2}$ were analyzed by the paired t test. Wilcoxon signed-rank test was used for t_{max}. A value of $P < 0.05$ was considered statistically significant.

3. Result and discussion

3.1. Chromatography

Under the described chromatographic conditions, the retention times of celiprolol and acebutolol were 6.3 min and 4.2 min, respectively and well separated from endogenous interferences (Fig. 1). We have already developed HPLC methods for the analysis of basic compounds, fexofenadine [9] and domperidone [19], by using XBridge™ C18 (5 μm, 150 mm × 4.6 mm i.d., Waters, MA, USA). Initially, we used the same column for the determination of celiprolol with the mobile phase, a mixture of acetonitrile and 10 mM ammonium acetate buffer (pH 10.5) (36:64, v/v), at a flow rate of 1.0 ml/min. In previous studies, similar type of columns has been used to analyze plasma celiprolol [11,13–16]. However, the global shortage of acetonitrile continued from 2008 to 2009 [20]. Because of this issue, the column was switched from 4.6 mm i.d. to 3.0 mm i.d. with smaller particle size (3.5 μm) that allows us to reduce acetonitrile consumption.

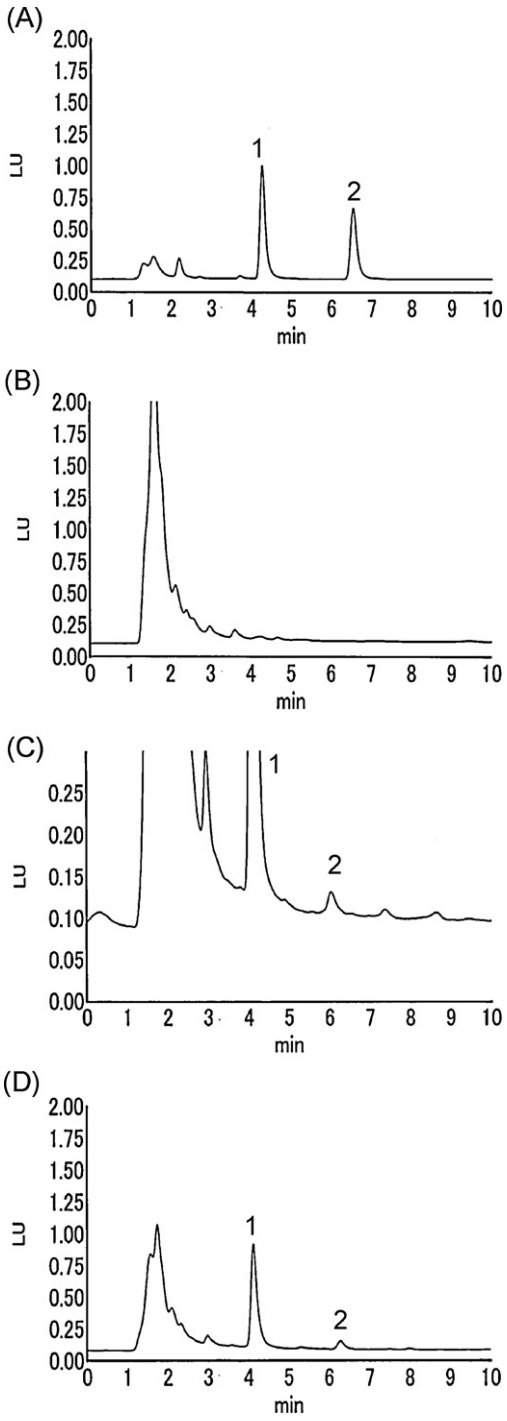

Fig. 1. Representative chromatogram of IS (1) and celiprolol (2): (A) standard mixture of celiprolol (500 ng/ml) and IS (250 ng/ml); (B) blank plasma sample; (C) blank plasma spiked with celiprolol at the LLOQ (1 ng/ml) concentration with IS; (D) plasma sample from a volunteer 2 h after a single oral administration of celiprolol 200 mg with apple juice.

3.2. Sample preparation

Solid phase extraction treatment was applied to remove the interferences of blank plasma. Firstly, the extraction method previously described by Imanaga et al. [9] was evaluated for sample pretreatment. The method involves three washing steps (2 ml of water, 2 ml of 50% methanol/water, and 1 ml of methanol) and an elution step (1 ml of 50 mM TEA solution). However, the recovery rates of celiprolol and IS were less than 60%. To obtain a sufficient recovery, a reduced volume of methanol (0.5 ml) as a third washing step and duplicated the elution step using 50 mM TEA solution (1 ml, 2 times) were applied to the solid phase extraction. Although this procedure improved the recovery (>85%), a small interference appeared on the celiprolol peak of chromatogram. One milliliter of 70% methanol/water was finally selected as the third washing solvent. More than 90% recoveries of celiprolol and IS were achieved without any interference peaks by using this washing procedure.

Table 1
Intra-day and inter-day precision and accuracy for assay of celiprolol in human plasma.

Nominal conc. (ng/ml)	Intra-day ($n = 5$)			Inter-day ($n = 5$)		
	Measured conc. (mean ± SD)	RSD (%)	RE (%)	Measured conc. (mean ± SD)	RSD (%)	RE (%)
1	1.12 ± 0.07	6.1	11.5	0.95 ± 0.13	13.3	−5.1
50	54.1 ± 2.0	3.6	8.1	52.8 ± 4.3	8.1	5.5
500	485.0 ± 18.6	3.8	−3.0	502.2 ± 10.8	2.2	0.5
1000	997.3 ± 11.8	1.2	−0.3	1002.8 ± 9.5	1.0	0.3

The key advantages of the present method are the simplicity of pretreatment extraction process and a high sensitivity to determine plasma celiprolol concentration. Liquid–liquid extraction procedures with a considerable amount of toxic and flammable organic solvents, such as hexane, methyl-*tert*-butyl ether, or dichloromethane have been used in previously reported methods [10–15]. These methods require two-step extraction with a back extraction or a repeated extraction [10,11,13,15]. These procedures take more than 30 min for the extraction (shaking with organic solvent, centrifugation, and separation of lipophilic from hydrophilic phases). A more simple method would contribute to pharmacokinetic studies that require a large number of blood sample analyses. Therefore, we used the solid phase extraction that is characterized by its simplicity compared with liquid–liquid extraction. There is one previous report that used the solid phase extraction method for the analysis of plasma celiprolol [16]. Our method has a higher sensitivity than this report (the limit of quantification; 1 versus 10 ng/ml).

3.3. Method validation

3.3.1. Selectivity

Selectivity was assessed by comparing the chromatograms of six different blank human plasma with the corresponding spiked plasma. As shown in Fig. 1, there was no interference at the retention times of celiprolol and IS.

3.3.2. Linearity of calibration curves

The linear regression of the peak area ratios versus concentrations was fitted over the concentration range 1–1000 ng/ml for celiprolol in human plasma. All calibration standards were up to the acceptance criteria. A typical regression equation was $y = 0.0148x + 0.00316$ ($r = 0.999$), where y was the peak area ratio of celiprolol to IS and x was the concentration of celiprolol. The LLOQ was set at 1 ng/ml with RE ranging from −2% to 13% and an RSD value of 6.3%. The RE values at all other concentration levels were less than 15%.

3.3.3. Precision and accuracy

The intra- and inter-day precision and accuracy were found to be within the accepted limits, as given in Table 1. The results indicated that the present method was reliable and reproducible for the quantitative analyses of celiprolol in human plasma samples.

Table 2
Extraction recovery for celiprolol and acebutolol (IS) from spiked plasma ($n = 5$).

Samples	Nominal concentration (ng/ml)	Recovery (mean ± SD, %)	RSD (%)
Celiprolol	1	117.8 ± 11.4	9.7
	50	99.7 ± 1.5	1.5
	500	101.0 ± 7.2	7.1
	1000	90.7 ± 2.5	2.8
Acebutolol (IS)	250	97.8 ± 2.7	2.8

Table 3
Stability of celiprolol in human plasma and processed samples ($n = 2$).

Storage condition	Stability (% remained)	
	1 ng/ml	50 ng/ml
−30 °C for 1 month	99.0	108.0
Two freeze–thaw cycles	90.3	93.8
Room temperature for 24 h	95.3	102.8
Room temperature for 48 h	108.1	96.5

3.3.4. Extraction recovery

Table 2 summarizes the extraction recoveries for celiprolol and IS. In the present study, acebutolol was chosen as the IS because celiprolol and acebutolol are structural analogs, with similar chemical characteristics and properties. Acebutolol exhibits good fluorescence response at the excitation wavelength (250 nm) and emission wavelength (482 nm). It also showed an appropriate chromatographic retention with a sufficiently separated peak from that of celiprolol.

3.3.5. Stability

Stability data for celiprolol in spiked drug-free human plasma are summarized in Table 3. It was found to be stable under the following conditions: after two cycles of freeze/thaw, at −30 °C for one month, and at room temperature (in autosampler) for 24 h and 48 h post-extraction.

3.4. Application

The HPLC method was used for an interaction study of celiprolol in humans. Mean plasma concentration–time curves after single oral administration of celiprolol with water or apple juice are shown in Fig. 2. The pharmacokinetic parameters are given in Table 4. These parameters of control group were in agreement with those reported in the literature [21]. Apple juice

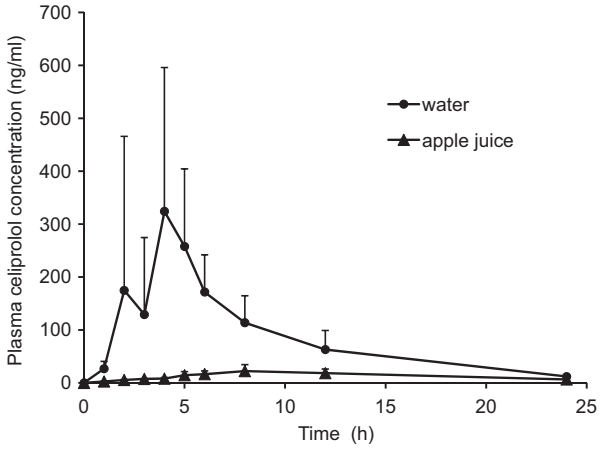

Fig. 2. Mean (SD) plasma concentrations of celiprolol in five healthy volunteers after a single oral administration of 200 mg celiprolol with water (circles) or apple juice (triangles).

Table 4
The pharmacokinetic parameters of celiprolol after ingestion of a single 200 mg dose of celiprolol with water or apple juice ($n = 5$).

Parameter	Water		Apple juice		Apple juice/water	*P* value
	Geometric mean	CV (%)	Geometric mean	CV (%)	Ratio (95% CI)	
t_{max} (h)	4 [2,6]		8 [6,12]			0.068
C_{max} (ng/ml)	397.0	50.5	23.0	39.3	0.058 (0.022, 0.15)	0.001
AUC_{24} (ng h/ml)	1829	40.1	284	35.0	0.16 (0.095, 0.25)	0.001
$t_{1/2}$ (h)[a]	5.3	22.5	9.0	15.7	1.7 (1.4, 2.1)	0.004

CV, coefficient of variance; CI, confidence interval; t_{max} value is given as median with range; C_{max}, peak plasma concentration; t_{max}, time to reach C_{max}; AUC_{24}, area under the plasma concentration–time curve from 0 to 24 h; $t_{1/2}$, half-life.

[a] $n = 4$. The $t_{1/2}$ of a volunteer could not be appropriately calculated because of a remarkable delay of t_{max} (12 h).

remarkably reduced plasma celiprolol concentrations. Celiprolol C_{max} and AUC_{24} were significantly decreased by 94% and 84%, respectively. A delay in t_{max} of celiprolol by apple juice was also observed. These results are consistent with previous studies regarding fruit juice and celiprolol interaction in humans [4,5].

Using the present HPLC method, the concentrations of celiprolol in plasma samples could be determined up to 24 h after administration, even when administered with apple juice that greatly reduced plasma celiprolol concentrations. The concentration range of plasma celiprolol in the absorption phase (from 0 to 4 h after administration) was less than 10 ng/ml (Fig. 2). It is unlikely that previously reported methods (the quantification limits: 5–10 ng/ml) are fully sensitive to detect this range of celiprolol concentrations [10–16].

4. Conclusion

A simple and sensitive HPLC-fluorescence detection method for the assay of celiprolol in human plasma was developed and validated. The proposed method was successfully applied to a pharmacokinetic interaction study of celiprolol in healthy volunteers.

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