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Validation and application of a 96-well format solid-phase extraction and liquid chromatography–tandem mass spectrometry method for the quantitation of digoxin in human plasma

Yoshitaka Hashimoto*, Kimio Shibakawa, Susumu Nakade, Yasuyuki Miyata

Pharmacokinetic Research Laboratories, Research Headquarters, Ono Pharmaceutical Co. Ltd., 17-2 Wadai Tsukuba-shi, Ibaraki 300-4247, Japan

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ABSTRACT

To evaluate the pharmacokinetics of digoxin in humans, a sensitive and specific LC/MS/MS method was developed and validated for the determination of digoxin concentrations in human plasma. The method was shown to be more sensitive, specific, accurate, and reproducible than common techniques such as RIA. For detection, a LC/MS/MS system with electro spray ionization tandem mass spectrometry in the positive ion-multiple reaction-monitoring (MRM) mode was used to monitor precursor to product ions of *m*/*z* 798.5–51.5 for digoxin and *m*/*z* 782.5–35.5 for the internal standard, digitoxin. The method was validated over a concentration range of 0.02–5 ng/mL and was found to have acceptable accuracy, precision, linearity, and selectivity. The mean extraction recovery from spiked plasma samples was above 80%. Imidafenacin, coadministered in a drug–drug interaction study, had no detectable influence on the determination of digoxin in human plasma. The novel method was applied to a drug–drug interaction study of digoxin and imidafenacin and the characterization of steady-state pharmacokinetics of digoxin in humans after oral administration at a dose of 0.25 mg on days 1 and 2 followed by 0.125 mg daily doses on days 3 through 8.

1. Introduction

Digoxin (Fig. 1), a medication commonly prescribed to treat arrhythmias and heart failure, has a narrow therapeutic index (recommended therapeutic plasma concentrations of 0.8–2.0 ng/mL). Thus, small alterations in digoxin pharmacokinetics could lead to decreased therapeutic effect or potentially serious toxicity. Digoxin, a *p*-glycoprotein substrate that is primarily cleared by kidneys, has been shown to clinically interact with substrates or inducers of *p*-glycoprotein [1–4]. Therefore, drug–drug interaction studies of digoxin with coadministered drugs have been performed in humans. Almost all digoxin interaction studies have been carried out at the maintenance dose of 0.25 mg. The mean C_{max} in these studies ranged from 1.16 to 3.70 ng/mL (Table 1) [4–10]. Depending on the situation, side effects may be caused by exceeding the upper limit of recommended therapeutic plasma concentration. Therefore, we want to carry out an interaction study of digoxin at doses lower than 0.25 mg to evaluate the pharmacokinetics of digoxin. The pharmacokinetics are generally preferred to be evaluated with the lower limit of quantitation of 5–10% of C_{max}. For example, when a clinical study was carried out with a maintenance dose of 0.125 mg, C_{max} may become the half of 1.16 ng/mL (about 0.6 ng/mL), which requires the lower limit of quantitation of 0.03–0.06 ng/mL. However, the lower limit of quantitation of immunoassays which are commonly used method for digoxin assays (0.1–0.4 ng/mL, see Table 1) are not sensitive enough to evaluate the pharmacokinetics of digoxin administered at less than 0.25 mg such as 0.125 mg. It has been reported that immunoassays are relatively nonspecific and cross-react with digoxin metabolites and endogenous digoxin-like substances [11–16]. Recently, LC/MS/MS, which is highly sensitive and specific, has become accepted as the leading technique for the quantitation of exogenous substances in biological samples. One previous report described LC/MS/MS for the quantitation of digoxin in rat plasma [17]. In the report [17], the lower limit of quantitation of digoxin in rat plasma is 0.1 ng/mL. It is not sensitive enough to achieve our desires. Moreover, this report employed the liquid–liquid extraction for sample preparation, which has the high cleanup effect but a rather low throughput rate. Furthermore to our knowledge, no reports have described using LC/MS/MS to detect digoxin in human plasma. In this study, we reported the development and validation of 96-well format solid-phase extraction method and a more sensitive and specific method using LC/MS/MS for the quantitation of digoxin in human plasma. Using this method, we have performed a pharmacokinetic study of digoxin at a dosage of 0.125 mg.

* Corresponding author. Tel.: +81 29 877 4556; fax: +81 29 877 4545.
E-mail address: yos.hashimoto@ono.co.jp (Y. Hashimoto).

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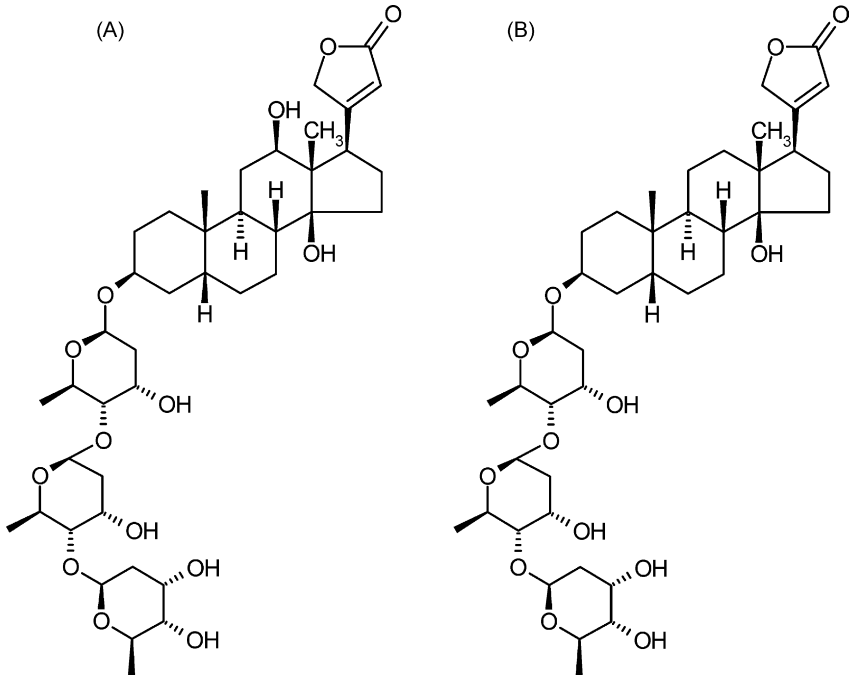

Fig. 1. Chemical structures of digoxin (A) and digitoxin (B, the internal standard).

2. Experimental

2.1. Chemicals and reagents

Digoxin (purity, 99.1%) and an internal standard (IS), digitoxin (purity, >97%), were purchased from Sigma–Aldrich Japan (Tokyo, Japan). Imidafenacin was synthesized at Ono Pharmaceutical Company (Osaka, Japan). Methanol (HPLC grade), ammonium acetate, and ammonium hydrogen carbonate were purchased from Kokusan Chemical (Tokyo, Japan). Ammonium (1 mol/L) was purchased from Nacalai Tesque (Kyoto, Japan). Heparinized human plasma was supplied by Octo (Hicksville, NY). Water was purified by a Milli-Q-System from Yamato Scientific Corp. (Tokyo, Japan). OASIS HLB 96-well plates (10 mg) were obtained from Waters (Milford, MA, USA).

2.2. Instrumentation

The LC/MS/MS system consisted of Waters 2690 Alliance systems (each including an HPLC pump and an autosampler) and a Thermo Electron TSQ 7000 triple quadrupole mass spectrometer. The Xcalibur software (Thermo Electron Co., USA) was used to control the LC/MS/MS system. The LCquan software (Thermo Electron Co., USA) was used to perform sample data analysis.

2.3. Liquid chromatography

The HPLC column (20 mm × 2.0 mm) was a 5-μm CAPCELLPAK C18 MG analytical column from Shiseido (Tokyo, Japan). Column temperature was held constant at 50 °C. The mobile phase consisted of 10 mmol/L ammonium hydrogen carbonate/methanol (9:1, v/v; solvent A) and 10 mmol/L ammonium hydrogen carbonate/methanol (1:9, v/v; solvent B). Chromatography was performed using the gradient conditions shown in Table 2. The total run time was 17 min for each injection.

2.4. Mass spectrometry

The ESI interface was used in positive ion mode. Quantification was performed in selected reaction monitoring (SRM) mode. The capillary temperature was 200 °C, the ionization voltage was 4500 V, collision gas was at 2 mTorr, and collision energy was −15 V for both digoxin and the IS. The digoxin was monitored at a transition of *m*/*z* 798.5–651.5 and the IS at *m*/*z* 782.5–635.5. The precursor and product ion mass spectra for digoxin are shown in Fig. 2.

Table 1
Comparison of the analytical method used by the clinical study and of the dose and C_{max} of the clinical study

Reference No.	Method	LLOQ (ng/mL)	Maintenance dose (mg)	C_{max} (ng/mL)	
				(a)	(b)
[4]	EIA	0.10	0.25	3.70	3.00
[5]	EIA	0.40	0.25	1.50	1.24
[6]	EIA	0.20	0.25	2.10	1.80
[7]	RIA	0.15	0.25	2.10	2.00
[8]	RIA	0.15	0.25	1.15	1.40
[9]	RIA	0.10	0.25	1.25	1.20
[10]	RIA	0.15	0.25	1.20	1.16
Our method	LC/MS/MS	0.04	0.125	0.97	0.85

(a) Administered digoxin with concomitant drug; (b) administered digoxin alone.

Table 2
Method gradient profile

Time (min)	Percentage of mobile phase in elute		Flow rate (mL/min)
	A	B	
0	70	30	0.6
13	35	65	0.6
13.1	0	100	1.5
16	0	100	1.5
16.1	70	30	1.5
17	70	30	1.5

The percentages drawn from each component of the tertiary gradient system over the 17 min run time are presented. (A) 10 mmol/L ammonium hydrogen carbonate and methanol at a ratio of 9:1 (v/v); (B) methanol and 10 mmol/L ammonium hydrogen carbonate at a ratio of 9:1 (v/v).

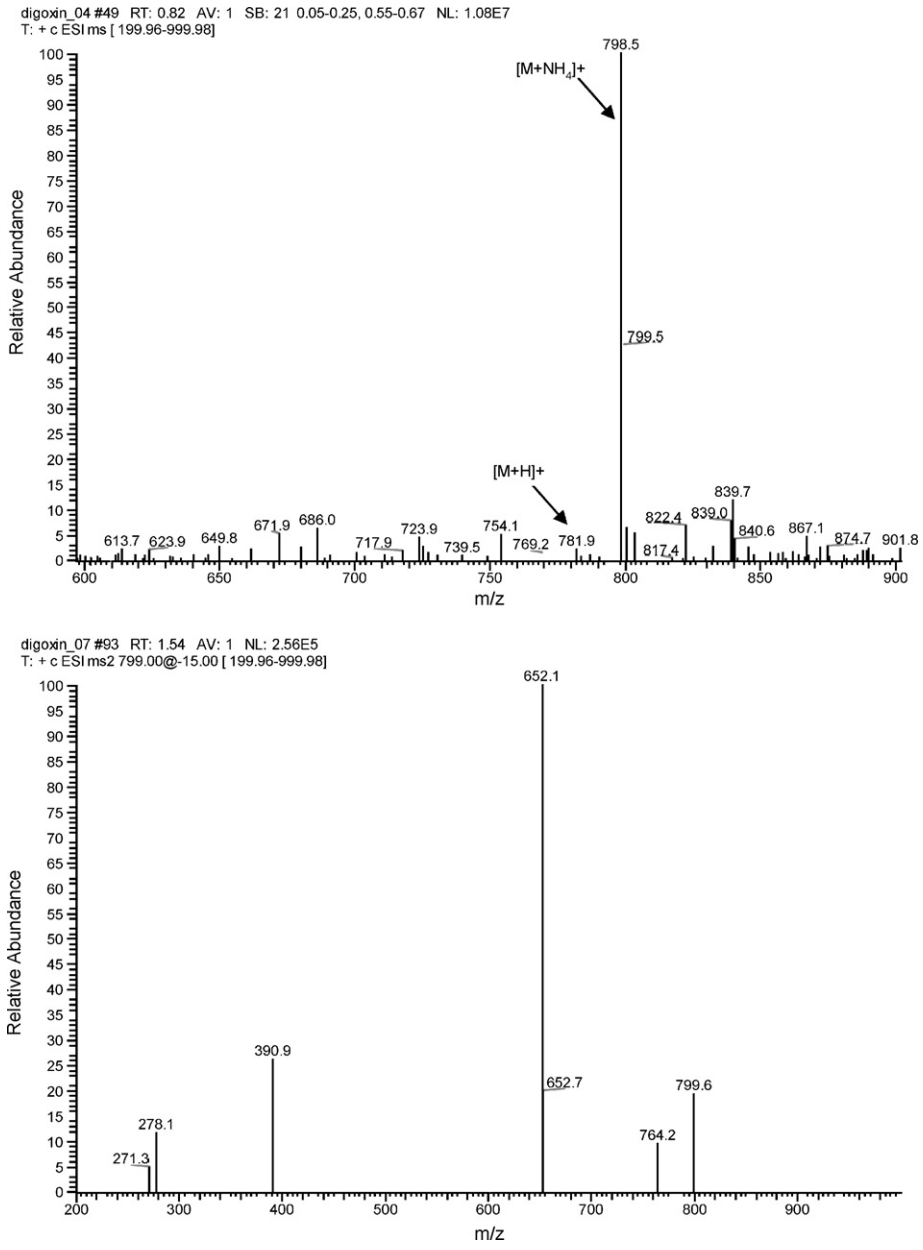

Fig. 2. Precursor ion and product ion (ammonium cluster of digoxin) mass spectra of digoxin.

2.5. Stock solutions and working solutions

Digoxin was dissolved in methanol to give stock solutions containing 1 mg/mL digoxin. Separate stock solutions were made for the standard and QC samples. The stock solutions for the standard samples were serially diluted in methanol to obtain working solutions of 0.08, 0.2, 0.4, 0.8, 2, 4, 8, and 20 ng/mL. The working solutions for the QC samples (5, 50, and 500 ng/mL), the IS samples (10 ng/mL), and the imidafenacin samples (5 ng/mL) were obtained in the same manner. All working solutions were stored at −20 °C until use.

2.6. Preparation of standards and QC samples

In a glass tube, 200 μL of human plasma was added to 50 μL of each working standard solution to obtain calibration standard samples ranging from 0.02 to 5 ng/mL. For blanks and double blanks, 50 and 100 μL of ethanol was added in place of the standard and IS working solutions, respectively. In a polypropylene tube, 10 mL of human plasma was added to 80 or 100 μL of each working QC solution to obtain QC samples containing 0.02, 0.05, 0.4, and 4 ng/mL. Ten milliliter of human plasma was added to the QC working solution and the imidafenacin working solution to obtain QC samples (digoxin, 0.05 and 4 ng/mL; imidafenacin, 2.5 ng/mL), which was used to study the influence of imidafenacin on the LC/MS/MS. Calibration standards were freshly prepared, and QC samples were stored at −20 °C until use.

2.7. Sample preparation

In a glass tube, 200 μL of QC sample were transferred and added to 50 μL of methanol. Then, 0.1 mol/L ammonium acetate buffer (500 μL, pH 9.5) and IS working solution (50 μL) were added to the standards and QC samples. Each sample was applied to an Oasis

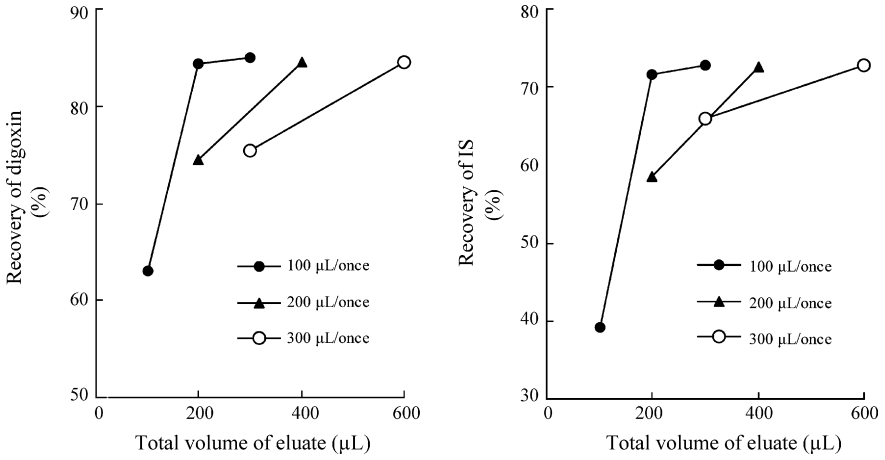

Fig. 3. Recovery of digoxin and IS under various elute conditions (optimization of eluting condition).

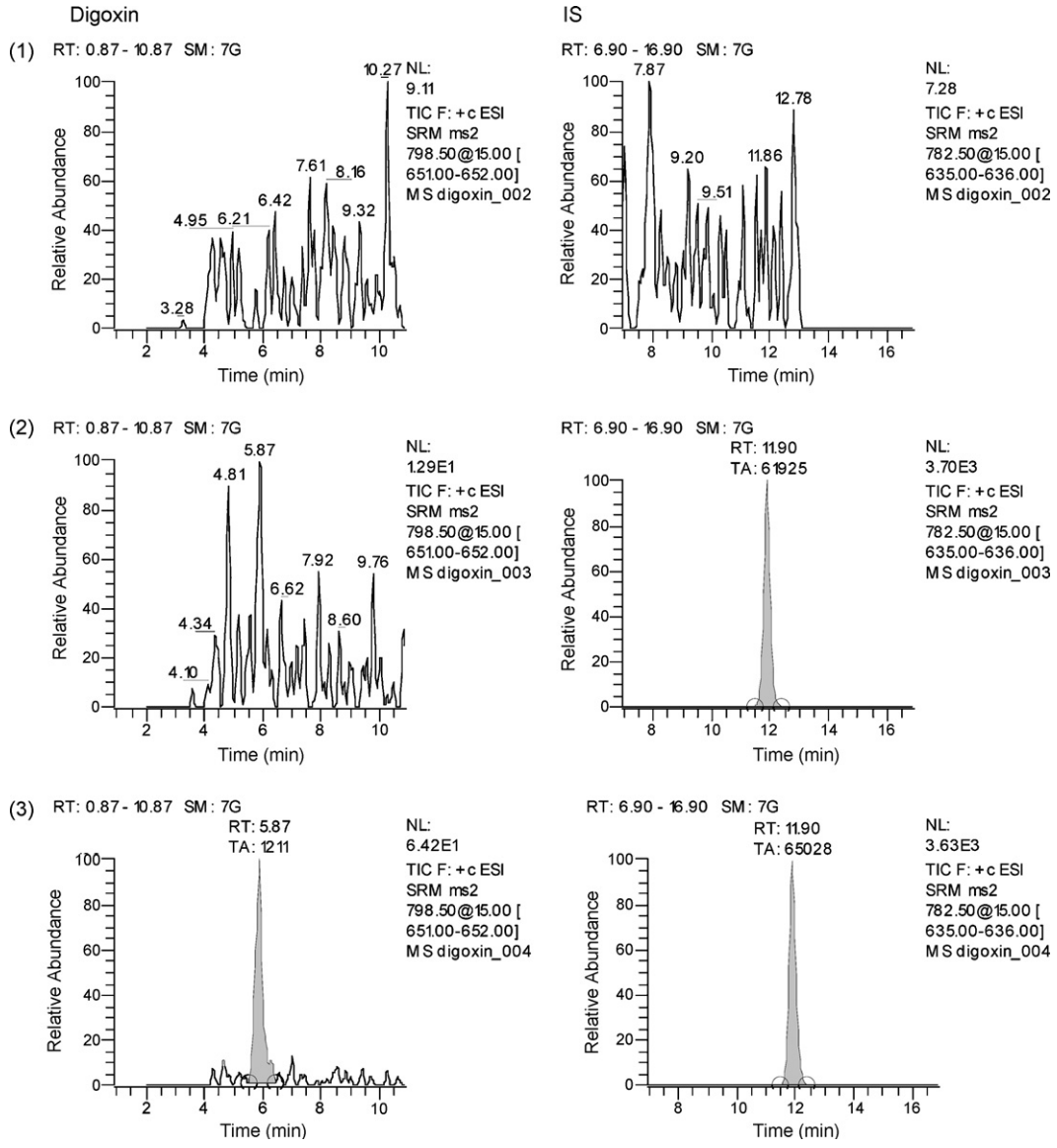

Fig. 4. Representative mass chromatograms of (1) blank human plasma; (2) human plasma spiked with the IS; and (3) human plasma spiked with digoxin (0.02 ng/mL) and the IS.

Table 3
Accuracy and precision of the digoxin assay in human plasma

Nominal concentration (ng/mL)	Batch	*n*	Measured concentration (ng/mL)	Intra-day		*n*	Measured concentration (ng/mL)	Inter-day	
			Mean ± S.D.	RE (%)	CV (%)		Mean ± S.D.	RE (%)	CV (%)
0.02	1	5	0.0211 ± 0.0021	5.4	9.8	15	0.0212 ± 0.0017	6.0	8.2
	2	5	0.0226 ± 0.0007	13.2	2.9				
	3	5	0.0199 ± 0.0011	−0.5	5.5				
0.05	1	5	0.0499 ± 0.0052	−0.3	10.4	15	0.0503 ± 0.0040	0.5	8.0
	2	5	0.0528 ± 0.0031	5.5	5.8				
	3	5	0.0481 ± 0.0026	−3.7	5.5				
0.4	1	5	0.369 ± 0.013	−7.8	3.5	15	0.376 ± 0.014	−6.1	3.8
	2	5	0.375 ± 0.012	−6.4	3.1				
	3	5	0.383 ± 0.017	−4.3	4.4				
4	1	5	4.09 ± 0.12	2.3	3.0	15	4.05 ± 0.16	1.4	3.9
	2	5	4.02 ± 0.21	0.5	5.3				
	3	5	4.05 ± 0.15	1.3	3.6				

HLB 96-well extraction plate (30 mg) pre-conditioned with 1 mL of methanol and 1 mL of water. Following sample loading, the plate was washed sequentially with 250 μL of 0.1 mmol/L ammonium acetate buffer (pH 9.5) and 250 μL of methanol/water (1:1, v/v), and then eluted twice with 100 μL of methanol. We added 200 μL of water to the eluate and then injected 80 μL into the LC/MS/MS.

2.8. Validation procedure

Validation of the analytical method was performed according to the FDA guidance [18]. The validation parameters included specificity, linearity, intra- and inter-assay precision and accuracy, extraction recovery, and stability. The specificity of the method was assessed by analyzing blank plasma, imidafenacin-spiked plasma, and digoxin-spiked plasma from six (three male and three female) individuals. Ion chromatograms were examined to determine the presence of any endogenous constituents or imidafenacin, which could potentially interfere with the analysis of digoxin and IS. Extraction recoveries were carried out in triplicate at concentrations of 0.05, 0.4, and 4 ng/mL for digoxin and 10 ng/mL for IS. The matrix effect on ionization was evaluated by comparing neat standards to the absolute peak areas of digoxin standard added to the plasma extracts. Linearity, as well as intra- and inter-assay precision (expressed as the percentage coefficient of variation, CV (%)) and accuracy (expressed as relative error from the nominal concentration, RE (%)), were determined by analyzing three separate analytical batches, including standards and QC samples. Each batch contained standards consisting of seven points (0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, and 5 ng/mL) and two blanks (blank and double blank), and four concentrations (0.02, 0.05, 0.4, and 4 ng/mL) of QC samples in five replicates for each concentration. The influence of imidafenacin on the determination of digoxin in human plasma was evaluated by determining the concentration of digoxin in imidafenacin-spiked QC samples (digoxin, 0.05 and 4 ng/mL; imidafenacin, 2.5 ng/mL). Dilution precision and accuracy were evaluated by determining the digoxin concentration in diluted QC samples (80 ng/mL) in three replicates. The dilution QC samples were diluted 100-fold in human plasma prior to analysis and processing. Calibration curves were established by linear least-squares regression ($1/x^2$ weighting) from peak area ratios (digoxin/IS) versus nominal concentrations. Intra- and inter-assay experiments, determination of the influence of imidafenacin, and determination of dilution accuracy and precision had ≤15% CV and RE, except for the lower limit of quantification (LLOQ), where ≤20% was acceptable for all parameters. The short-term stability at room temperature for 24 h, the stability following three freeze–thaw cycles (−20 °C to room temperature), and the long-term stability (3 months at −20 °C) were determined for spiked plasma samples. The stability of processed samples was also assessed. In each case, triplicate QC samples at 0.05 and 4 ng/mL were analyzed.

2.9. Clinical study

The study was conducted at the Kitasato Institute Bio-Iatric Center (Tokyo, Japan) and approved by the local institutional review board. Healthy male subjects participated in this study after submitting individual informed consent forms. This was a randomized, two-way crossover study. During both treatment periods, subjects received daily oral loading doses of digoxin (DIGOSIN®, Chugai) as 0.25 mg on days 1 and 2 followed by 0.125 mg daily doses on days 3 through 8. During either Period 1 or 2, twice-daily doses of imidafenacin 0.1 mg were coadministered with digoxin for 8 days, from days 1 through 8. On Trial day 8, imidafenacin and digoxin were coadministered after an overnight fast of at least 8 h. Blood samples (2 mL per point) were collected before administration on days 1 through 8, and on day 8 at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, and 24 h post-digoxin dose. Samples were collected into tubes containing sodium heparin and then centrifuged; plasma was harvested from samples and stored at −20 °C until use.

3. Results and discussion

3.1. Method development

The positive ion full-scan mass spectra (Q1) of digoxin and IS indicated the presence of the ammonium adduct ion $[M+NH_4]^+$ as the predominant ion for each compound, with *m*/*z* values of 798.5 and 792.5 for digoxin and the IS, respectively. The negative ion full-scan mass spectra (Q1) of digoxin and IS indicated the presence of the molecular-related ion $[M-H]^-$ as the predominant ion for each compound. The ammonium adduct ion in the positive mode showed very strong intensity compared to the molecular-related ion in the negative mode. Therefore, the product ion mass spectra were obtained by choosing each ammonium adduct ion $[M+NH_4]^+$ as a precursor ion (Fig. 2). The intensity of ammonia adduct ion was the strongest when the temperature of capillary was 200°, and the ion intensity became weaker as the temperature of capillary increased, and the ammonium adduct ion was not detected at 280°. As for the salt in the mobile phase, ammonium hydrogen carbonate exhibited higher ion intensity than the formic acid ammonium

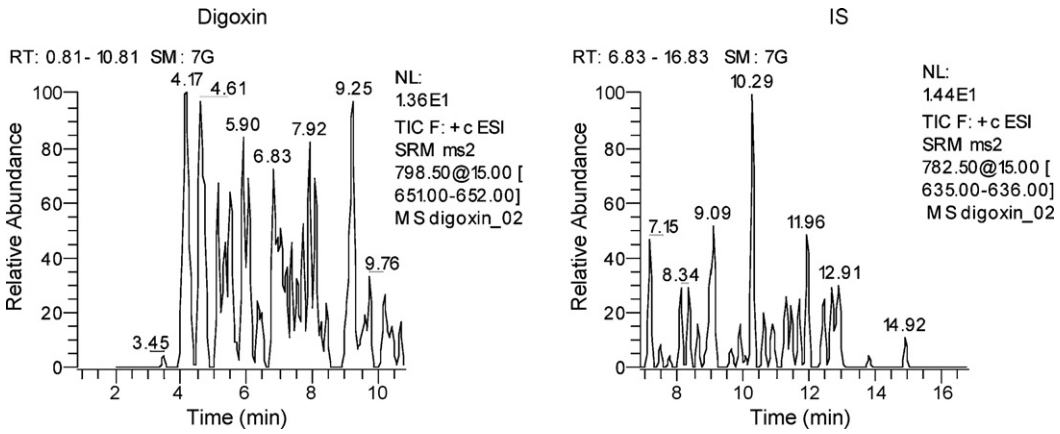

Fig. 5. Representative mass chromatograms of imidafenacin (2.5 ng/mL)-spiked human plasma.

(pH 3.4), the mobile phase for digoxin assay in rat plasma [17]. Therefore, 10 mmol/L ammonium hydrogen carbonate/methanol was selected as the mobile phase. In consideration of peak shape and retention time, the chromatography was performed under gradient conditions. For reduction in analysis time, the flow rate of the mobile phase was set to 0.6 mL/min until digoxin and IS were detected (0–13 min), then it was set to 1.5 mL/min in washing and equilibration process until 17 min. The system pressure at flow rate of 0.6–1.5 mL/min was less than 20 MPa. The 96-well format solid-phase extraction, which enables easy and high-throughput sample treatment, was employed to permit many samples, such as those obtained from drug–drug interaction studies, to be swiftly measured. In order to find the optimal washing condition, the effect of methanol composition in methanol/water on washing was investigated. Digoxin and IS were eluted with 60% methanol in a washing process, but those were not eluted with 50% methanol. Therefore, 50% methanol was used as washing solvent. Next, in order to find the optimal eluting condition, recoveries of digoxin and IS were tested under various elute conditions (1–3 times with 100 μL, once with 200 μL, and once with 300 μL of methanol). As shown in Fig. 3, it was found to be optimal to elute digoxin and IS twice with 100 μL of methanol.

Table 4
Influence of imidafenacin on the digoxin assay (accuracy and precision of digoxin assay in imidafenacin-spiked human plasma)

Nominal digoxin concentration (ng/mL)	Imidafenacin concentration (ng/mL)	*n*	Measured concentration (ng/mL) Mean ± S.D.	RE (%)	CV (%)
0.05	2.5	5	0.0508 ± 0.0038	1.6	7.5
4	2.5	5	3.97 ± 0.18	−0.8	4.5

3.2. Validation study

3.2.1. Specificity

Under optimized HPLC and MS conditions, digoxin and the IS were separated with retention times of 5.9 and 11.9 min, respectively (Fig. 4). Blank human plasma from six volunteers showed no significant interfering peaks at the retention times of digoxin or the IS (Fig. 4).

3.2.2. Linearity

The correlation coefficients (r^2) were not less than 0.995 in all cases, and the accuracy was 0.8–4.0% at LLOQ and −8.8% to 9.7% at other concentrations. It was assessed that calibration curves showed good linearity within the assay range of 0.02–5 ng/mL and consistent slope values when evaluated by weighted ($1/x^2$) linear regression.

3.2.3. Intra- and inter-day accuracy and precision

Table 3 shows the validation results for intra- and inter-day assay accuracy and precision. The intra-day accuracy and precision for digoxin were −0.5% to 13.2% and 2.9% to 9.8%, respectively, at the LLOQ, and −7.8% to 5.5% and 3.0% to 10.4%, respectively, at other concentrations. The inter-day accuracy and precision for digoxin were 6.0% and 8.2%, respectively, at the LLOQ, and −6.1% to 1.4% and 3.8% to 8.0%, respectively, at other concentrations. These results satisfied the acceptance criteria and the analytical method was judged to be reproducible in terms of intra- and inter-day accuracy and precision.

3.2.4. Influence of imidafenacin on the determination of digoxin in human plasma

Imidafenacin-spiked human plasma showed no significant interfering peaks near the retention times of digoxin and the IS

Table 5
Stability of digoxin in human plasma and processed samples.

Storage condition	*n*	Nominal concentration (ng/mL)	Measured concentration (ng/mL)	RE (%)
Room temperature for 24 h	3	0.05	0.477	−4.5
	3	4	3.85	−3.8
Three freeze–thaw cycles	3	0.05	0.0542	8.3
	3	4	3.97	−0.7
−20 °C for 3 months	3	0.05	0.0512	2.4
	3	4	4.15	3.8
Processed sample 4 °C for 48 h	3	0.05	0.0477	−4.6
	3	4	3.89	−2.8

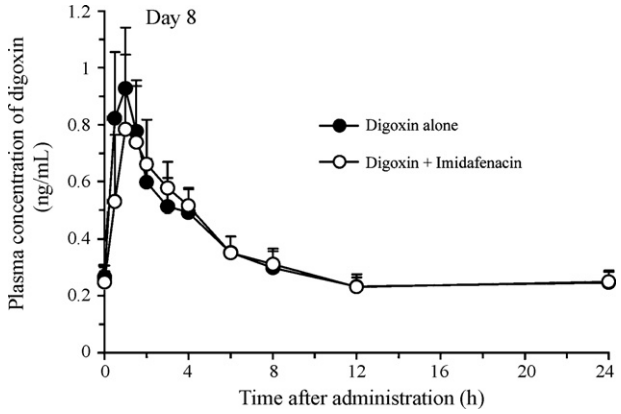

Fig. 6. Mean plasma concentration vs. time profiles of digoxin on day 8 after administration at a dose of 0.25 mg on days 1 and 2 followed by 0.125 mg daily dose on days 3 through 8 in healthy male subjects. Each value represents the mean ± S.D. of 12 subjects.

(Fig. 5). The accuracy and precision for digoxin in imidafenacin-spiked human plasma were −0.5% to 13.2% and 2.9% to 9.8%, respectively, at 0.05 and 4 ng/mL (Table 4). These results satisfied the acceptance criteria, and imidafenacin was judged to have no significant influence on the determination of digoxin in human plasma.

3.2.5. Dilution precision and accuracy

When dilution QC samples (80 ng/mL) were diluted 100-fold, the precision and accuracy were 1.1% and 3.3%, respectively. These results satisfied the criteria for dilution reproducibility. Therefore, it was assessed that when a plasma sample containing digoxin is diluted 100-fold in blank human plasma, reproducible measurements of digoxin concentrations in human plasma could be obtained by the current determination method.

3.2.6. Stability

The results of stability tests are summarized in Table 5. Digoxin was considered stable if the %RE was no greater than ±15%. Using these criteria, digoxin was determined to be stable in human plasma for up to 24 h at room temperature, through three freeze–thaw cycles, for up to 3 months at −20 °C, and in processed samples for up to 48 h at 4 °C.

3.2.7. Recovery and matrix effects

The recoveries of digoxin and the IS were consistent at 82.1–87.4% and 72.8% across the tested range, respectively. The effects of ion suppression on the peaks for digoxin and digitoxin were 8.7% and 13.6%, respectively. These results indicate good recovery and low ion suppression.

3.3. Application

The validated method of determination was successfully applied to determine the plasma concentrations of digoxin in subjects who participated in the drug–drug interaction pharmacokinetic study between digoxin and imidafenacin. The plasma concentration versus time profile and pharmacokinetic parameters are shown in Fig. 6 and Table 6, respectively. Imidafenacin had no effect on digoxin pharmacokinetics in healthy subjects. There were no peaks interfering with determination of digoxin on the chromatograms of actual blood sample. In this study, although measured in six batches (six 96-well plates), the linearity of the standard curve was good, being independent of the plate lots, and the QC samples ranged within 15% of the nominal concentration. This analytical method can be successfully applied to the analysis of plasma concentrations versus time profiles of digoxin in human after oral administration at a dose of 0.25 mg on days 1 and 2 followed by 0.125 mg daily on days 3 through 8.

Table 6
Pharmacokinetic parameters of digoxin in the absence and presence of imidafenacin

Parameter	Digoxin alone	Digoxin with imidafenacin
C_{max} (ng/mL)	0.971 ± 0.232	0.850 ± 0.196
t_{max} (h)	1.0 (0.50–1.5)	1.0 (0.50–2.0)
$AUC_{0-24\,h}$ (ng × h/mL)	7.98 ± 1.16	7.91 ± 0.90
$t_{1/2}$ (h)	15 ± 3	14 ± 3

Mean ± S.D. (n = 12); t_{max}: median (range).

4. Conclusions

An LC/MS/MS method to quantify plasma digoxin concentrations was developed and validated. LC/MS/MS was more sensitive, specific, accurate and reproducible than commonly used techniques, such as RIA. Imidafenacin, which was coadministered in the drug–drug interaction study, did not influence the determination of digoxin in human plasma. In addition, the use of the 96-well plate format greatly decreased the time required for sample preparation, allowing many samples to be analyzed daily. The new method was successfully applied to a drug-interaction study and the characterization of steady-state pharmacokinetics of digoxin in humans after oral administration at a dose of 0.25 mg on days 1 and 2 followed by 0.125 mg daily on days 3 through 8.

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