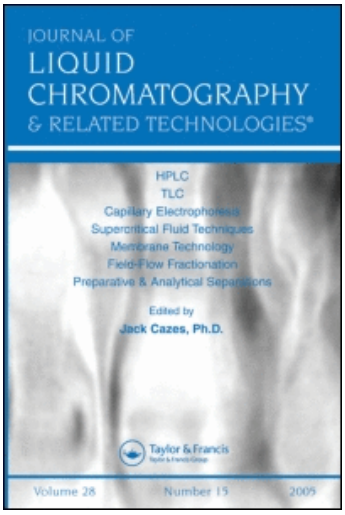

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Abstract: A method for the determination of digitoxin in human serum using a liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS) technique is reported. Digitoxin and the internal standard, [21,21,22-$^{2}H_3$]digitoxin, were extracted from 200 μL of human serum using a solid phase extraction cartridge and analyzed by LC/ESI-MS/MS in the selected reaction monitoring mode. The intra- and inter-assay reproducibility and accuracy were satisfactory within the quantification range of 5–100 ng/mL, which was sensitive enough to measure the digitoxin in real samples. The concentrations of digitoxin in serum samples obtained from digitalized patients (n = 19) were in the range of 5.3–24.1 ng/mL, and these correlated well with those obtained by radioimmunoassay using a very specific antiserum.

Keywords: Digitoxin, Digitalized patient, Liquid chromatography/mass spectrometry, Electrospray ionization, Radioimmunoassay

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INTRODUCTION

Digitalis glycosides, represented by digitoxin (Dt) and digoxin, have been commonly used for the treatment of congestive heart failure and other cardiac diseases. In addition to the narrow therapeutic range of serum digitalis glycosides, their bioavailability is easily affected by kidney or liver failure and the dosing of other medicines. Therefore, it is important to measure digitalis glycosides in sera from digitalized patients.

Dt is mainly biotransformed in the liver, whereas digoxin is predominantly eliminated by the kidney.[1] Dt has been prescribed in the presence of deteriorating renal function and its therapeutic range in serum is 15–25 ng/mL. Immunoassay is routinely used for therapeutic drug monitoring (TDM) of Dt. However, the commercially available antibodies show a high cross-reactivity with Dt metabolites such as digitoxigenin bisdigitoxoside (Bis-Dtgenin), digitoxigenin monodigitoxoside (Mono-Dtgenin), or digitoxigenin (Dtgenin) (Fig. 1), which were formed by the successive cleavage of the sugar moiety, as well as with endogenous digitalis-like immunoreactive substances (DLIS) in the sera of pregnant women, newborn infants, or patients with renal, diabetic, or hepatic disease conditions.[2] These cause a false-elevation or -lowering of the serum Dt concentration. To overcome this problem, one of the authors designed a new hapten of Dt and developed a very specific radioimmunoassay (RIA) for Dt in human serum.[3] Liquid chromatography/mass spectrometry (LC/MS) also seems to be suitable for this purpose due to its high sensitivity and specificity.

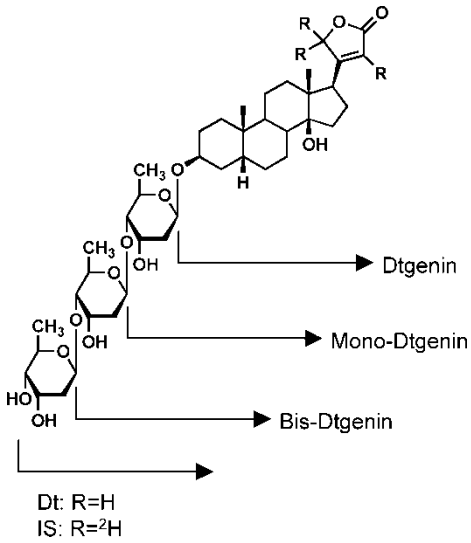

Figure 1. Structures of Dt and its metabolites.

Recently, the determination of Dt or digoxin using LC/tandem mass spectrometry (MS/MS) has been reported,[4–6] but its application using real samples obtained from digitalized patients has not been achieved.

In this study we developed a determination method for Dt in human serum using stable isotope diluted LC/electrospray ionization (ESI)-MS/MS and applied this method to a real sample. The obtained data was compared to those obtained by the RIA as described above.

EXPERIMENTAL

Materials and Reagents

Dt was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). [21,21,22-$^2H_3$]Dt was synthesized in our laboratories by a known method.[6] The content of [$^2H_0$]Dt was less than 0.1%, and no H/^{2}H exchange was observed during the ESI process. A stock solution of Dt in EtOH was prepared at 100 μg/mL and was further diluted with EtOH to 0.05, 0.1, 0.25, 0.5, and 1 μg/mL. An internal standard (IS) solution in EtOH was also prepared at 1 μg/mL. Bis-Dtgenin, Mono-Dtgenin, and Dtgenin were prepared in our laboratories by a known method.[7]

Oasis HLB cartridges (60 mg, 3 mL) were purchased from Waters Co. (Milford, MA, USA), and successively conditioned with MeOH (2 mL) and H_2O (2 mL) prior to use. All the other reagents were of analytical grade and commercially available.

Serum and Blank Sample

Serum samples from digitalized patients were donated from Kinjo Hospital (Kanazawa, Japan).[3] Drug-free human serum samples were obtained from healthy volunteers. Large quantities of control human serum was not available, fresh frozen plasma (FFP; the Japan Red Cross Service; Tokyo) (200 μL), which was used as a blank sample, was spiked with each working solution (1, 2.5, 5, 10, 20 ng/20 μL) and kept at room temperature for 10 min, and then used for obtaining the calibration curve. Quality controlled (QC) samples used for the precision and accuracy evaluations were prepared by spiking the working solution with FFP at 5, 25, 50 ng/mL.

Instruments

An LC/MS system, which consisted of an LC-10AT chromatograph (Shimadzu, Kyoto, Japan) coupled with an API 2000 triple stage quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA), was operated with ESI or atmospheric pressure chemical ionization (APCI) in the positive-ion mode. For

the ESI, a semi-micro column, Develosil ODS-HG-5 (5 μm, 150 × 2.0 mm i. d.) (Nomura Chemical, Seto, Japan), was used at a flow rate of 0.2 mL/min at 40°C, and MeOH–5 mM HCO_2NH_4 (7 : 3, v/v) was used as the mobile phase. The ionization conditions were as follows: ion spray voltage, 5 kV; heated nebulizer temperature, 500°C; ion source gas 1 (nebulizer gas), 70 psi, ion source gas 2 (turbo gas), 80 psi; declustering potential, 11 V; focusing potential, 370 V; entrance potential, 7 V; curtain gas, 20 psi. For selected reaction monitoring (SRM) detection [precursor ions, m/z 782.2 (Dt) and 785.2 (IS); product ions (monitoring ions), m/z 635.2 (Dt) and 638.2 (IS)], N_2 was used as the collision gas at 6, and the collision energy and collision cell exit potentials were 21 V and 16 V, respectively. For the APCI, a J'sphere ODS-H80 (4 μm, 150 × 4.6 mm i. d.) (YMC, Kyoto, Japan) was used at a flow rate of 1.0 mL/min at 40°C, and MeOH–H_2O (7 : 3, v/v) was used as the mobile phase. The ionization conditions were as follows: ion spray voltage, 5 kV; heated nebulizer temperature, 450°C; ion source gas 1 (auxiliary gas), 80 psi, ion source gas 2 (nebulizer gas), 80 psi; declustering potential, 11 V; focusing potential, 370 V; entrance potential, 7 V; curtain gas, 20 psi; detection, selected ion monitoring mode (SIM), m/z 635.3 (Dt).

Pretreatment of Serum Samples

After addition of the IS solution (1 μg/mL, 20 μL), the serum samples (200 μL) were deproteinized with MeCN (500 μL) and centrifuged at 1500 *g* for 10 min. The supernatant was collected and the precipitate was re-suspended with MeCN (500 μL), followed by centrifugation. The collected supernatant was combined with the above one and evaporated in a N_2 gas stream. The residue was dissolved with H_2O (2 mL) and applied to a solid-phase extraction cartridge, Oasis HLB. After washing with H_2O (2 mL) and 30% MeOH (2 mL), Dt and IS were eluted with AcOEt (1.5 mL). The eluate was evaporated in a N_2 gas stream, and then the residue was dissolved with MeOH–5 mM HCO_2NH_4 (7 : 3, v/v) (30 μL) and an aliquot of which was subjected to the LC/ESI-MS/MS analysis.

Absolute Recovery of Dt and IS

The first set of FFP (200 μL), spiked with the Dt solution (1 or 10 ng/20 μL) or IS solution (20 ng/20 μL), was subjected to the above pretreatment and then the same amount of IS (20 ng/20 μL) or Dt (10 ng/20 μL) was added (A). The second set of FFP (200 μL) was subjected to the pretreatment, and then Dt (1 or 10 ng/20 μL) and IS (20 ng) were added (B). The absolute recoveries of Dt and IS were calculated by dividing the peak area ratios (Dt/IS and IS/Dt, respectively) obtained from A with B. Each sample was prepared in duplicate and the recovery rates are shown as the mean values.

Precision and Accuracy

The intra-assay precision and accuracy were evaluated by analyzing replicates ($n = 5$) of the QC samples at 5, 25, and 50 ng/mL on the same day. The inter-assay precision and accuracy were evaluated by analyzing these QC samples on 5 days, which were frozen at $-20^{\circ}C$ and thawed at room temperature.

RESULTS AND DISCUSSION

LC-MS Analysis

The LC/MS behavior of the authentic Dt was investigated using APCI and ESI (positive ion mode). The APCI-MS detected an ion at m/z 635.3 [Bis-Dtgenin + H]$^+$ as the base ion, together with the ions at m/z 765.3 [M + H]$^+$ (relative intensity, 40.7%) and 747.3 [M + H − H_2O]$^+$ (62.1%). Because the characteristic and abundant product ion was not detected by the MS/MS analysis, Dt was analyzed by SIM, and the limit of detection (LOD) was 50 pg [signal/noise (S/N) = 5.6]. On the other hand, the ESI-MS detected only the adduct ion, [M + NH_4]$^+$ at m/z 782.2, and the product ion mass spectra of this ion showed fragment ions at m/z 635.2 [Bis-Dtgenin + H]$^+$ as the base ion peak, 505.2 [Mono-Dtgenin + H]$^+$ and 375.2 [Dtgenin + H]$^+$ (Fig. 2). The LOD of Dt determined by SRM (precursor ion, m/z 782.2; monitoring ion, m/z 635.2) was 12 pg (S/N = 5.2), which showed that the ESI-MS was more sensitive than APCI-MS. Therefore, the determination of Dt in human serum was performed by LC/ESI-MS/MS. Using a Develosil ODS-HG-5 column and MeOH–5 mM HCO_2NH_4 (7 : 3, v/v) as the mobile phase, Dt was eluted at 6.8 min and then was completely separated from the Dt metabolites (Bis-Dtgenin, Mono-Dtgenin, Dtgenin), which were eluted within 5 min.

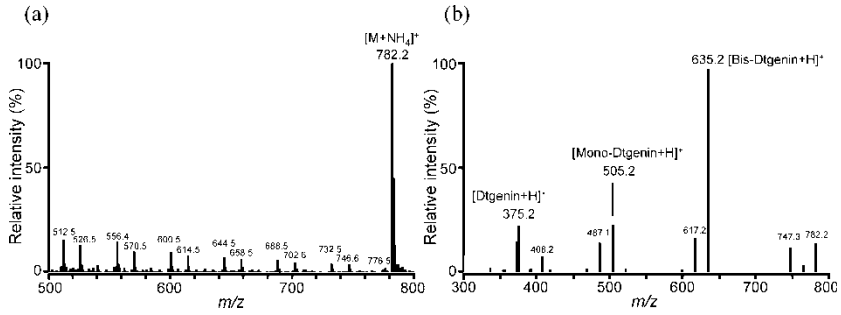

Figure 2. Mass spectrum (a) and product ion mass spectrum (b) of Dt analyzed by LC/ESI-MS/(MS).

Pretreatment of Serum Sample

The human serum was deproteinized with MeCN, purified by a solid-phase extraction cartridge containing a hydrophilic-lipophilic reversed-phase sorbent, an Oasis HLB cartridge, and then determined by LC/ESI-MS/MS. The absolute recoveries were 87.4% (Dt, 1 ng), 87.3% (Dt, 10 ng), and 95.4% (IS, 20 ng) (mean, n = 2). The representative SRM chromatograms of the blank sample spiked with authentic Dt and IS, and the serum obtained from a digitalized patient are shown in Figs. 3a and b, respectively. In the serum from the patient, peaks corresponding to Dt were observed and the structure was confirmed by comparison with an authentic sample based on its chromatographic behavior and mass spectral data. No interference peaks with IS were observed in the sera from a patient and healthy volunteer.

Calibration Curve

A calibration curve was constructed using the blank samples (200 μL) spiked with graduated amounts of Dt (1, 2.5, 5, 10, and 20 ng) and IS (20 ng), and the peak area ratios (Dt/IS) (y) were plotted versus the amounts of Dt (ng/tube) (x). The obtained regression line showed a satisfactory linearity with regression coefficient values (r^2) of greater than 0.99 within the range of

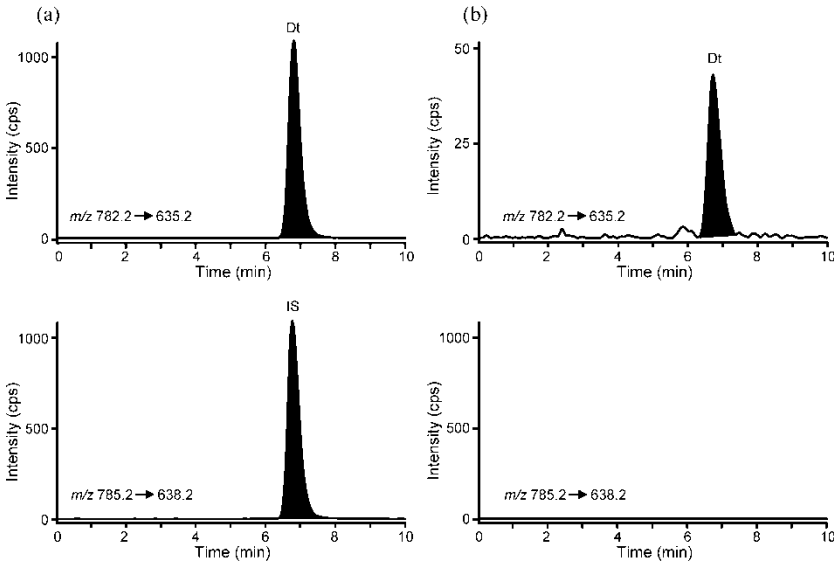

Figure 3. Typical SRM chromatograms of Dt (upper column) and IS (lower column) in QC sample spiked with Dt (20 ng/tube) and IS (20 ng/tube) (a), and digitalized patient serum (b).

Table 1. Accuracy of back calculated concentration

Nominal concentration (ng/tube)	Accuracy (%)					
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
1	114.6	108.4	106.0	108.6	93.6	107.8
2.5	108.1	105.0	102.3	102.6	95.5	101.8
5	106.0	103.9	101.0	100.6	96.2	99.8
10	104.9	104.0	100.4	99.6	96.5	98.8
20	104.3	103.1	100.1	99.1	96.6	98.3

1–20 ng/tube. The slopes and the intercepts of six lines drawn for different days were reproducible (0.0500 $\pm$ 0.0014 and 0.0033 $\pm$ 0.0026, respectively) [mean $\pm$ standard deviation (SD)]. The accuracies of the back calculated concentrations were within 93.6–114.6%, as shown in Table 1. These data showed that the lower limit of quantification is 1 ng/tube (5 ng/mL).

Validation of the Determination Method

The intra-assay precision and accuracy were determined by five replicates of QC samples at three different concentrations. Those of the inter-assay were determined by the same samples on five different days, and the samples were thawed and frozen before and after use each day. Satisfactory relative standard deviation (RSD) values of the intra- and inter-assays lower than 3.4% and 4.2% were obtained, respectively, as shown in Table 2. The accuracies of both assays were within 95.0–107.0% of the nominal concentration.

A large quantity of control human serum was not obtainable from the Japan Red Cross Service, all the validation experiments together with the construction of calibration curve, were performed using FFP as the blank

Table 2. Precision and accuracy for the determination of Dt

	Concentration (ng/mL)			
	Added	Found[a]	RSD (%)	Accuracy (%)
Intra-assay	5	5.35 $\pm$ 0.15	2.8	107.0
	25	25.50 $\pm$ 0.65	2.5	102.0
	50	51.05 $\pm$ 1.75	3.4	102.1
Inter-assay	5	5.29 $\pm$ 0.22	4.2	105.8
	25	24.41 $\pm$ 0.97	4.0	97.6
	50	47.49 $\pm$ 1.07	2.3	95.0

[a]Mean $\pm$ SD (n = 5).

Table 3. Analytical recoveries of Dt in sera

Concentration (ng/mL)				
	Found		Analytical recovery (%)	
Added	Serum 1	Serum 2	Serum 1	Serum 2
5	4.34	5.29	86.8	105.8
25	26.29	26.59	105.2	106.4
50	52.00	50.66	104.0	101.3

sample while the real sample was human serum. In order to examine the matrix-effect of the serum components for the ionization (ESI), the analytical recoveries were determined using two different human sera, which were not digitalized and spiked with three levels of authentic Dt. As shown in Table 3, the recovery rates using the blank sample calibration curve were within 86.8–106.4% in both sera, and the matrix-effect was not observed.

Although the presented method was validated using 200 μL of serum, the effect of the serum amounts was examined, and the result is shown in Table 4. Using the same calibration curve, satisfactory accuracies were obtained with 50–200 μL of the QC samples. Based on these results, the present method is highly reproducible and accurate in the quantification range of 5–100 ng/mL.

Determination of Dt in the Sera Obtained from Digitalized Patients

The proposed method was used for the determination of Dt in human serum samples obtained from digitalized patients ($n = 19$). As shown in Fig. 4, the obtained results were comparable to those of RIA,[3] which were determined using a very specific antiserum, and indicated a good correlation ($r = 0.8442$, $p < 0.0001$).

Table 4. Effect of amounts of serum

Concentration (ng/mL)	Serum volume (μL)	Observed concentration[a] (ng/mL)	Accuracy[a] (%)
10	100	10.78	107.8
	200	10.59	105.9
20	50	19.86	99.3
	100	19.02	95.1
	200	19.81	99.1

[a]Mean ($n = 2$).

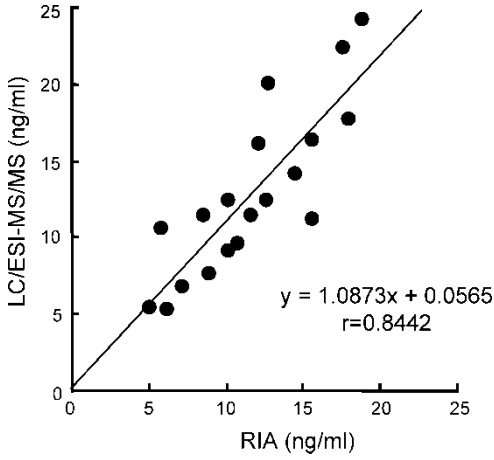

Figure 4. Correlation of the concentrations of Dt in digitalized patient sera determined by LC/ESI-MS/MS and those determined by RIA.

CONCLUSION

The reported LC/ESI-MS/MS method for the determination of Dt is concise, reproducible, accurate, and sensitive enough to measure the Dt concentration in the serum samples obtained from the digitalized patients. The method is not expected to be interfered by the Dt metabolites and endogenous DLIS, therefore, it is suitable for not only TDM, but also pharmacokinetic studies.

REFERENCES

1. Smith, T.W. Pharmacokinetics, bioavailability and serum levels of cardiac glycosides. J. Am. Coll. Cardiol. **1985**, *5* (5 Suppl A), 43A–50A.
2. Wu, S.L.; Li, W.; Wells, A.; Dasgupta, A. Digoxin-like and digitoxin-like immunoreactive substances in elderly people. Impact on therapeutic drug monitoring of digoxin and digitoxin concentartion. Am. J. Clin. Pathol. **2001**, *115* (4), 600–604.
3. Ikeda, Y.; Fujii, M.; Yamazaki, M.; Fujii, Y. Measurement of serum digitoxin in patients by radioimmunoassay using specific antiserum. Clin. Chim. Acta **2001**, *314* (1–2), 245–247.
4. Guan, F.; Ishii, A.; Seno, H.; Watanabe-Suzuki, K.; Kumazawa, T.; Suzuki, O. Identification and quantification of cardiac glycosides in blood and urine samples by HPLC/MS/MS. Anal. Chem. **1999**, *71* (18), 4034–4043.
5. Yao, M.; Zhang, H.; Chong, S.; Zhu, M.; Morrison, R.A. A rapid and sensitive LC/MS/MS assay for quantitative determination of digitoxin in rat plasma. J. Pharm. Biomed. Anal. **2003**, *32* (6), 1189–1197.

6. Kaiser, P.; Kramer, U.; Meißner, D.; Kress, M.; Wood, W.G.; Reinauer, H. Determination of the cardiac glycosides digoxin and digitoxin by liquid chromatography combined with isotope-dilution mass spectrometry (LC-IDMS)—a candidate reference measurement procedure. Clin. Lab. **2003**, *49* (7–8), 329–343.
7. Satoh, D.; Aoyama, K. Studies on digitalis glycosides. XXXI. Stepwise degradation of polydigitoxosides of cardenolides. Chem. Pharm. Bull. **1970**, *18* (1), 94–99.

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