Determination of Digoxin in Human Serum Using Stable Isotope Dilution Liquid Chromatography/Electrospray Ionization-Tandem Mass Spectrometry

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A method for the determination of digoxin in human serum using a liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS) technique is reported. Digoxin and the internal standard, [21,21,22-$^2H_3$]digoxin, were extracted from 250 μl of human serum using a solid phase extraction cartridge (Oasis HLB) 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 0.20—3.20 ng/ml. The concentrations of digoxin in the serum samples obtained from digitalized patients (n=19) were in the range of 0.25—2.84 ng/ml, which were compared to those obtained by radioimmunoassay.

Key words digoxin; serum; liquid chromatography/tandem mass spectrometry; digitalized patient; correlation; radioimmunoassay

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

Dg has been more commonly used than digitoxin and its therapeutic range in serum is 0.8—2.0 ng/ml.[1] Immunoassay is routinely used for therapeutic drug monitoring (TDM) of Dg. However, the commercially available antibodies show a high cross-reactivity with Dg metabolites, such as digoxigenin bisdigitoxoside (Bis-Dggenin), digoxigenin monodigitoxoside (Mono-Dggenin) or digoxigenin (Dggenin) (Fig. 1), which were formed by the successive cleavage of the sugar moiety, as well as with endogenous digitalis-like immunoreactive substances (DLIS).[2] These cause a false-elevation or -lowering of the serum Dg concentration due to the inappropriate prescriptions of Dg dosages by physician. To overcome this problem, Ikeda *et al.* designed a new hapten of Dg and developed a radioimmunoassay (RIA) for Dg in human serum, which can clearly discriminate Dg from the Dg metabolites (Bis-Dggenin, Mono-Dggenin and Dggenin), but not DLIS.[3] Liquid chromatography/mass spectrometry (LC/MS) also seems to be suitable for this purpose because of its high sensitivity, specificity and without interfering from DLIS. Recently, the determination of digitoxin or Dg using LC/MS has been reported,[4—8] but its application to real samples obtained from digitalized patients followed by comparison with the data obtained from immunoassay has only been done in our report for determining digitoxin.[4]

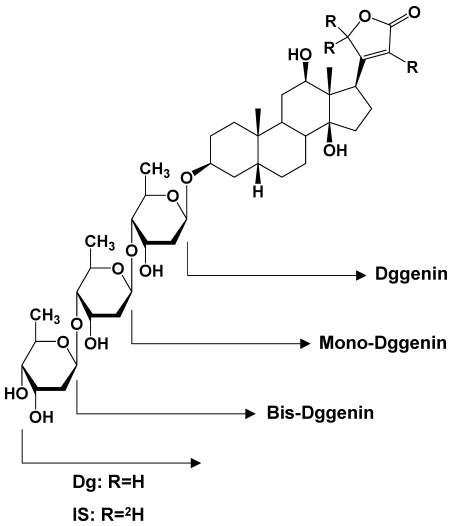

Fig. 1. Structures of Dg and Its Metabolites

Dggenin: digoxigenin, Mono-Dggenin: digoxigenin monodigitoxoside, Bis-Dggenin: digoxigenin bisdigitoxoside, Dg: digoxin.

In this study, we developed a determination method for Dg in human serum using stable isotope dilution LC/electrospray ionization (ESI)-tandem mass spectrometry (MS/MS) and applied this method to real samples. The data obtained in this study were compared to those obtained by using RIA with the anti-serum clearly discriminating Dg from its metabolites.[3]

MATERIALS AND METHODS

Materials and Reagents Dg was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). [21,21,22-$^2H_3$]Dg was synthesized in our laboratories by a known method.[7] LC/ESI-MS: m/z 801.3 $[M+NH_4]^+$ (100.0), m/z 802.6 (60.6), m/z 803.5 (11.7), m/z 804.4 (4.0), m/z 800.5 (1.7). The content of $[^2H_0]$Dg (m/z 798.3 $[M+NH_4]^+$) was less than 0.1%, and no $H/^2H$ exchange was observed during the ESI process. A stock solution of Dg in EtOH was prepared at 100 μg/ml and then further diluted with EtOH to 5, 10, 20, 40 and 80 ng/ml. An internal standard (IS: [21,21,22-$^2H_3$]Dg) solution in EtOH was also prepared at 40 ng/ml. Bis-Dggenin, Mono-

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Dggenin and Dggenin were prepared in our laboratories by a known method.[9]

The Oasis HLB cartridges (60 mg, 3 ml) were purchased from Waters Co. (Milford, MA, U.S.A.), 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 the Kinjo Hospital (Kanazawa, Japan).[3] Drug-free human serum samples were obtained from healthy volunteers with informed consent. Fresh frozen plasma (FFP; the Japan Red Cross Service; Tokyo) (250 μl), which was used as a blank sample, was spiked with each working solution at 0.20, 0.40, 0.80, 1.60 and 3.20 ng/ml, respectively, 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 (250 μl) at 0.20, 0.80 and 3.20 ng/ml.

Instruments An LC/MS system, which consisted of an LC-10AT chromatograph (Shimadzu, Kyoto, Japan) coupled with an API 2000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, U.S.A.), was operated with ESI in the positive-ion mode. A semi-micro column, Develosil ODS-HG-5 (5 μm, 150×2.0 mm i.d.) (Nomura Chemical, Seto, Japan), was used at the flow rate of 0.2 ml/min at 40 °C, and MeOH–5 mM HCO_2NH_4 (3 : 2, 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, 20 V; focusing potential, 350 V; entrance potential, 12 V; curtain gas, 20 psi. For the selected reaction monitoring (SRM) detection [precursor ions, *m/z* 798.3 (Dg) and 801.3 (IS); product ions (monitoring ions), *m/z* 651.3 (Dg) and 654.3 (IS)], both Q1 and Q3 were operated under unit mass resolution (0.7 amu at full width half-maximum). Nitrogen gas was used as the collision gas at 6, and the collision energy and collision cell exit potentials were 23 V and 16 V, respectively.

Pretreatment of Serum Samples After the addition of the IS solution (400 pg/10 μl), the serum samples (250 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 nitrogen 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 40% MeOH (2 ml), Dg and IS were eluted with MeOH (1.5 ml). The eluate was evaporated in nitrogen gas stream, and then the residue was dissolved with MeOH–5 mM HCO_2NH_4 (3 : 2, v/v) (30 μl) and an aliquot of which was subjected to the LC/ESI-MS/MS analysis.

Absolute Recovery The first set of FFP (250 μl) spiked with the Dg solution (50 or 800 pg/10 μl) or IS solution (400 pg/10 μl) was subjected to the above pretreatment and then the same amount of IS (400 pg/10 μl) or Dg (800 pg/10 μl) was added (A). The second set of FFP (250 μl) was subjected to the pretreatment, and then Dg (50 or 800 pg/10 μl) and IS (400 pg/10 μl) were added (B). The absolute recoveries of Dg and IS were calculated by dividing the peak area ratios (Dg/IS and IS/Dg, 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 concentrations of 0.20, 0.80 and 3.20 ng/ml on the same day. The inter-assay precision and accuracy including the frozen and thaw stability were evaluated by analyzing these QC samples on day 5, which were frozen at −20 °C and thawed at room temperature.

RESULTS AND DISCUSSION

LC-MS Analysis The LC/MS behavior of the authentic Dg was investigated using ESI (positive ion mode) as reported for the digitoxin analysis.[4] The ESI-MS detected only the adduct ion, $[M+NH_4]^+$ at *m/z* 798.3, and the product ion spectra of this ion showed fragment ions at *m/z* 651.3 [Bis-Dggenin+H]$^+$ as the base peak, 521.3 [Mono-Dggenin+H]$^+$ and 391.2 [Dggenin+H]$^+$ (Fig. 2). The limit of detection of Dg determined by SRM (precursor ion, *m/z* 798.3; monitoring ion, *m/z* 651.3) was 2.5 pg (signal/noise=5.00). Using a Develosil ODS-HG-5 column and MeOH–5 mM HCO_2NH_4 (3 : 2, v/v) as the mobile phase, Dg was eluted at 4.9 min and then completely separated from the Dg metabolites (Bis-Dggenin, Mono-Dggenin, Dggenin) which were eluted within 4.5 min.

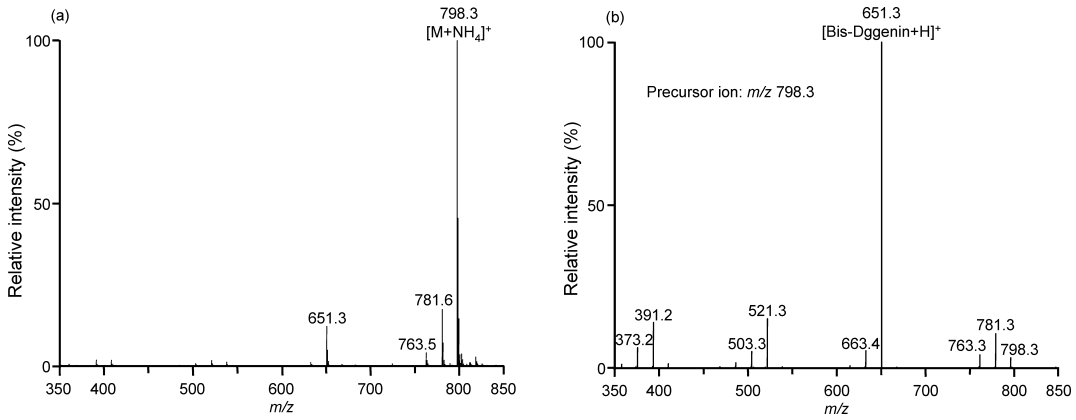

Fig. 2. Mass Spectrum (a) and Product Ion Spectrum (b) of Dg Analyzed by LC/ESI-MS(MS)

LC/ESI-MS(MS) conditions are written in Materials and Methods. Bis-Dggenin: digoxigenin bisdigitoxoside.

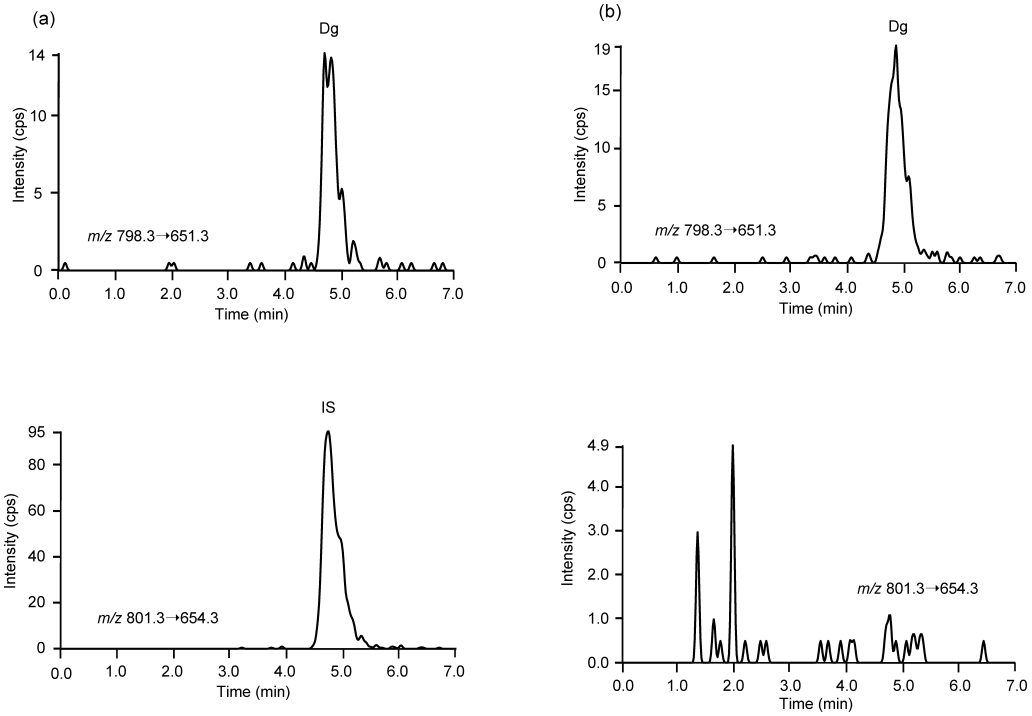

Fig. 3. Typical SRM Chromatograms of Dg (Upper Column) and IS (Lower Column) in QC Sample Spiked with Dg (0.20 ng/ml) and IS (1.60 ng/ml) (a), and Digitalized Patient Serum (b)

LC/ESI-MS(MS) conditions are written in Mateials and Methods. Dg: digoxin, IS: [21,21,22-$^2H_3$]Dg.

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 92.0% (Dg, 50 pg), 91.5% (Dg, 800 pg) and 96.3% (IS, 400 pg) (mean, $n=2$). The representative SRM chromatograms of the blank sample spiked with authentic Dg 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 Dg 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 Curves Calibration curves were constructed using the blank samples (FFP, 250 μl) spiked with graduated amounts of Dg (50, 100, 200, 400, 800 pg) and IS (400 pg), and the peak area ratios (Dg/IS) (y) were plotted *versus* the amounts of Dg (pg/tube) (x). The obtained regression line showed a satisfactory linearity with the regression coefficient values (r^2) of greater than 0.9996 within the range of 50—800 pg/tube (0.20—3.20 ng/ml). The slopes and the intercepts of 5 lines drawn for different days were reproducible (0.00248±0.00007 and 0.00928±0.00534, respectively) [mean±standard deviation (S.D.)]. The accuracies of the back calculated concentrations were within 95.4—108.5%, as shown in Table 1. Therefore, the quantification limit of this method is 50 pg/tube (0.20 ng/ml).

Validation of Determination Method The intra-assay precision and accuracy were determined by analyzing five replicates of the 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 2.0% and 6.5% were obtained, respectively, as shown in Table 2. The accuracies of both assays were within 97.2—101.5% of the nominal concentration. The data also showed the freeze-thaw stability of digoxin in the QC sam-

Table 1. Accuracy of Back Calculated Concentration

Nominal concentration (ng/ml)[a)]	Accuracy (%)				
	Day 1	Day 2	Day 3	Day 4	Day 5
0.20	104.1	108.5	104.4	97.2	97.6
0.40	104.8	102.5	101.9	96.7	107.3
0.80	101.4	99.4	95.4	99.2	98.5
1.60	100.8	99.8	97.9	100.5	97.3
3.20	102.3	101.4	98.7	98.8	100.0

a) Used 250 μl (FFP).

Table 2. Precision and Accuracy for Determination of Dg

	Concentration (ng/ml)[a)]		RSD (%)	Accuracy (%)
	Added	Found[b)]		
Intra-assay	0.20	0.197±0.004	2.0	98.5
	0.80	0.799±0.004	0.5	99.9
	3.20	3.248±0.018	0.6	101.5
Inter-assay[c)]	0.20	0.201±0.013	6.5	100.5
	0.80	0.799±0.030	3.8	99.9
	3.20	3.110±0.063	2.0	97.2

a) Used 250 μl (FFP). *b*) Mean±S.D. ($n=5$). *c*) Samples were thawed and frozen before and after use each day.

ples.

Matrix-effect As a large quantity of the human serum control was not obtainable from the Japan Red Cross Service, all the validation experiments together with the construction of the calibration curves were performed using FFP as the blank 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 five different human sera which were not digitalized and spiked with three levels of authentic Dg. As shown in Table 3, the recovery rates using the blank sample calibration curve were within 97.0—112.3% in these sera, and no matrix-effect was observed.

Determination of Dg in Sera Obtained from Digitalized Patients The proposed method was used for the determination of Dg in the human serum samples obtained from digitalized patients ($n=19$). As shown in Table 4, the obtained results were not comparable to those obtained by using the RIA with the anti-serum clearly discriminating Dg from its metabolites.[3] Five of these samples (patient No. 15—19) showed more than 2.5 times higher values than those obtained by LC/ESI-MS/MS. Without these five data, an almost good relationship ($y=1.16x-0.3266$, $r^2=0.9217$) was observed for the data obtained from RIA (y; ng/ml) and LC/ESI-MS/MS (x; ng/ml). The used anti-serum clearly discriminated Dg from its metabolites,[3] so the observed discrepancy could come from DLIS. DLIS has been reported in the sera of pregnant women,[10] newborn infants,[11] patients with renal[12] or hepatic disease.[13] The further experiments considering the patients' background are necessary to recognize the effect of DLIS with immunoassay, which can clarify the scope and limitation of immunoassay for the determination of Dg or digitoxin concentration in human fluids.

On another hand, the reported LC/ESI-MS/MS method for the determination of Dg is concise, reproducible, accurate, and sensitive enough to measure the Dg concentration in the serum samples obtained from the digitalized patients. The method is not expected to undergo interference by the Dg metabolites and endogenous DLIS. The automatic solid phase extraction[8] followed by LC/MS analysis might be suitable for not only TDM, but also pharmacokinetic studies.

Table 3. Analytical Recoveries of Dg to Evaluate Matrix-effect

Added[a,b]	Analytical recovery (%)				
	Serum 1	Serum 2	Serum 3	Serum 4	Serum 5
0.20	99.0 (0.198)[b]	111.0 (0.222)	99.0 (0.198)	103.5 (0.207)	97.0 (0.194)
0.80	98.8 (0.790)	112.3 (0.898)	99.6 (0.797)	105.9 (0.847)	104.5 (0.836)
3.20	97.3 (3.114)	101.5 (3.248)	97.6 (3.123)	110.4 (3.533)	106.4 (3.405)

a) Used 250 μl (serum). *b*) ng/ml.

Table 4. Concentrations of Dg in Digitalized Patients Sera Determined by LC/ESI-MS/MS and RIA

Patients	Dg concentration (ng/ml)	
	LC/ESI-MS(MS)	RIA
1	2.11	1.94
2	0.78	0.47
3	2.84	3.48
4	1.25	0.89
5	1.13	0.90
6	1.17	0.92
7	0.53	0.31
8	0.59	0.40
9	1.80	1.34
10	0.49	0.33
11	1.18	0.92
12	0.57	0.67
13	0.91	0.83
14	0.42	0.32
15	0.46	2.44
16	0.25	0.66
17	0.47	2.38
18	0.33	2.05
19	0.91	2.98

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