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Therapeutic monitoring of serum digoxin for patients with heart failure using a rapid LC-MS/MS method

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Abstract

Objective: Here we develop a liquid chromatography tandem mass spectrometry (LC-MS/MS) method for the determination of digoxin in serum.

Design and methods: The serum samples were extracted with methyl tert-butyl ether using an isotope-labeled digoxin-d3 as internal standard. The analyte was separated on a reverse phase Capcell C18 column and detected in positive electrospray ionization multiple reaction monitoring mass spectrometry.

Results: The chromatographic analysis was carried out within 3 min, but the complete analysis took longer because of the liquid–liquid extraction. The lower limit of quantification was 0.1 ng/mL for digoxin. The intra- and inter-batch precisions were less than 12%, and the bias ranged from −9.1% to 10.7%. The external quality assessment (EQA) results obtained with the LC-MS/MS method were comparable to target values. Subsequently, this method has been applied to the therapeutic monitoring of digoxin in a clinical setting.

Conclusion: In this study, we have developed a rapid and reliable LC-MS/MS method for the therapeutic monitoring of digoxin in human serum.
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Keywords: Digoxin; LC-MS/MS; Liquid–liquid extraction; Therapeutic drug monitoring; Heart failure; Human serum

Introduction

Digoxin is commonly prescribed for the treatment of heart failure (HF) in clinical practice. Data from the Digitalis Investigation Group (DIG) trial, a randomized double-blinded placebo-controlled study, demonstrated that digoxin reduced hospitalizations among patients with HF and decreased the risk of death attributed to worsening HF [1–3]. The role of serum digoxin concentration (SDC) is well established, as many studies have suggested that the effectiveness of digoxin therapy in patients with HF should be optimized in the range of 0.5–0.9 ng/mL. A SDC above 1.2 ng/mL may be harmful [4–6] and the traditional range of 0.8–2.0 ng/mL for SDC is now questioned, because this new lower therapeutic window is associated with improvement of clinical outcomes [7]. Therefore, a more intensive dosage refinement is proposed [8].

The measurement and assessment of digoxin concentration are often performed inappropriately and the quality of SDC monitoring is poor [9–11]. Hence, it is necessary to introduce therapeutic drug monitoring (TDM) of digoxin, in order to optimize therapeutic efficacy and avoid the incidence of toxicity. In most cases, immunoassay techniques are the primary method used for monitoring of digoxin in clinical practice.

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Nevertheless, cross-reactivity with endogenous digoxin-like substances [12] and interference from other drugs, including a number of herbal medicines [13,14], may be the main obstacle to accurate determination of digoxin in real clinical samples. This makes LC-MS/MS, a technique with significant advantages of specificity and sensitivity, the most appropriate method for digoxin monitoring, as it is free of interferences from endogenous and exogenous compounds. Recently, many LC-MS or LC-MS/MS methods for digoxin have been reported for the purposes of drug monitoring, pharmacokinetic studies, or drug interaction investigations [15–21]. Some of these methods use gradient elution program for chromatographic separation. As a result, a long turnaround is required (14 min [15], 17 min [16], 10 min [21]) to restore the column to its original starting conditions. Some reported methods use lengthy sample preparation [15,18,20,21], or 96-well plate [16,17], which further increase both turnaround time and cost. Consequently, there is still a critical need for a method that addresses both rapid throughput and economy for the TDM of digoxin in routine clinical practice.

The aim of this paper is to build a rapid and reliable method for the TDM of serum digoxin concentration for patients with heart failure. Our protocol is based on a technique of stable isotope dilution liquid chromatography-positive electrospray ionization tandem mass spectrometry. A simple and economical liquid–liquid extraction with methyl tert-butyl ether is adopted using 0.2 mL of sample volume. The total turnaround per analysis is only 3 min and this greatly improves the assay throughput. The validated method has been subsequently applied to national EQA and clinical drug monitoring of digoxin in patients with heart failure.

Materials and methods

Chemicals and reagents

Digoxin (98.0% purity) was purchased from National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China. [$^2H_3$] digoxin (digoxin-d3) was purchased from Toronto Research Chemicals Inc., North York, Ontario, Canada. HPLC-grade acetonitrile, methanol, ammonium acetate, formic acid, and methyl tert-butyl ether were purchased from Tedia Company Inc., Fairfield, USA. All other reagents were of analytical grade. Double distilled water was used throughout the study.

Liquid chromatography

HPLC analysis was performed on a Shimadzu system (Kyoto, Japan) equipped with two LC-20AD pumps, a SIL-HTC autosampler, and an online DGU-20A3 vacuum degasser. Chromatographic separation was achieved on a Capcell C18 MG III analytical column (100 mm×2.0 mm I.D. 5 μm, Shiseido, Japan) coupled with a C18 guard column (4.0 mm×3.0 mm I.D. 5 μm, Phenomenex, USA) at a flow rate of 0.3 mL/min. The column and autosampler were kept at room temperature. The mobile phase consisted of 10 mM NH$_4$Ac/0.1% formic acid in water and 0.1% formic acid in acetonitrile and was run at an isocratic elution (60:40, v:v). The sample injection volume was 20 μL and the total run time was 3 min per injection.

Mass spectrometry

A 3200 QTRAP tandem mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, Canada) equipped with Turbo Ionspray source was used for quantitative analysis. The instrument was operated in positive ionization mode with an ion spray voltage at 5.5 kV and the source temperature at 400 °C. Multiple reaction monitoring (MRM) was used to detect digoxin and digoxin-d3, with precursor to product ion transitions of m/z 798.6/651.5 and m/z 801.6/654.5, respectively. The collision-activated dissociation (CAD) was set at medium. High purity nitrogen was used as the collision gas. The curtain gas, gas 1, and gas 2 were set at 20, 50, and 50, respectively. Dwell time of 200 ms was selected. Analyst 1.4.2 software was used for instrument control and data acquisition.

Standard solutions

Standard stock solutions of digoxin and digoxin-d3 (internal standard) were separately prepared at 0.1 mg/mL in methanol and stored at 4 °C. Dilutions were made to prepare calibration standards, at serial concentrations of 0.1, 0.4, 1, 4, and 10 ng/mL, by spiking appropriate amount of digoxin stock solution into blank serum. Quality control (QC) samples were prepared in the same way, at concentrations of 0.3, 1.5 and 8 ng/mL. All of the spiked standards were stored at −20 °C. A working solution of internal standard was prepared from digoxin-d3 stock at 10 ng/mL in 40% methanol and stored at 4 °C.

Liquid–liquid extraction

A 200 μL serum sample was mixed with 20 μL isotope-labeled internal standard working solution (10 ng/mL) and then extracted with 1 mL methyl tert-butyl ether by vortexing for 5 min. The mixture was subsequently centrifuged at 12 000 rpm for 5 min. The upper layer was transferred to a clean polypropylene tube and dried with a stream of nitrogen gas at 45 °C. The residue was reconstituted in 100 μL 40% methanol and 20 μL was injected onto the LC column for LC/MS/MS analysis.

Method validation

The method was evaluated by validation of the extraction recovery, matrix effect, linearity, precision and accuracy, and stability in three independent runs. The validation procedure was performed in respect to the guideline for the bioanalytical method validation recommended by U.S. Food and Drug Administration [22].

The linearity of the method was evaluated by a calibration curve prepared in duplicate over a range of 0.1–10 ng/mL digoxin in serum. A linear regression using $1/x$ weighting was

constructed based on the measured peak area ratio of digoxin to the internal standard, versus the nominal concentration. The linearity was considered acceptable when the correlation coefficient (r) was higher than 0.99.

Precision (expressed by RSD for replicate measurements) and accuracy (expressed by the percentage of bias between nominal and calculated concentrations) were evaluated by analysis of six replicates of QCs at four concentration levels (0.1, 0.3, 1.5 and 8 ng/mL) for three randomized batches.

The recovery and matrix effect were assessed by comparing the peak areas of digoxin from blank serum, neat QC chemical standards, and standards spiked before and after extraction, in six different lots of pooled sera at three concentration levels. Stock solution stability, three cycles of freeze–thaw stability,

bench-top stability, and post-processing stability were all checked as part of method validation.

External quality assessment (EQA) and therapeutic drug monitoring (TDM)

After being validated, the LC-MS/MS method was evaluated by participating national external quality assessment (EQA) program (2008–2009) offered by National Center for Clinical Laboratory, Ministry of Health of China. The program is offered two times a year, with five blind serum samples each time.

The method was applied to therapeutic drug monitoring of digoxin in patients with heart failure in a clinical setting. Patients who had been clinically diagnosed with heart failure
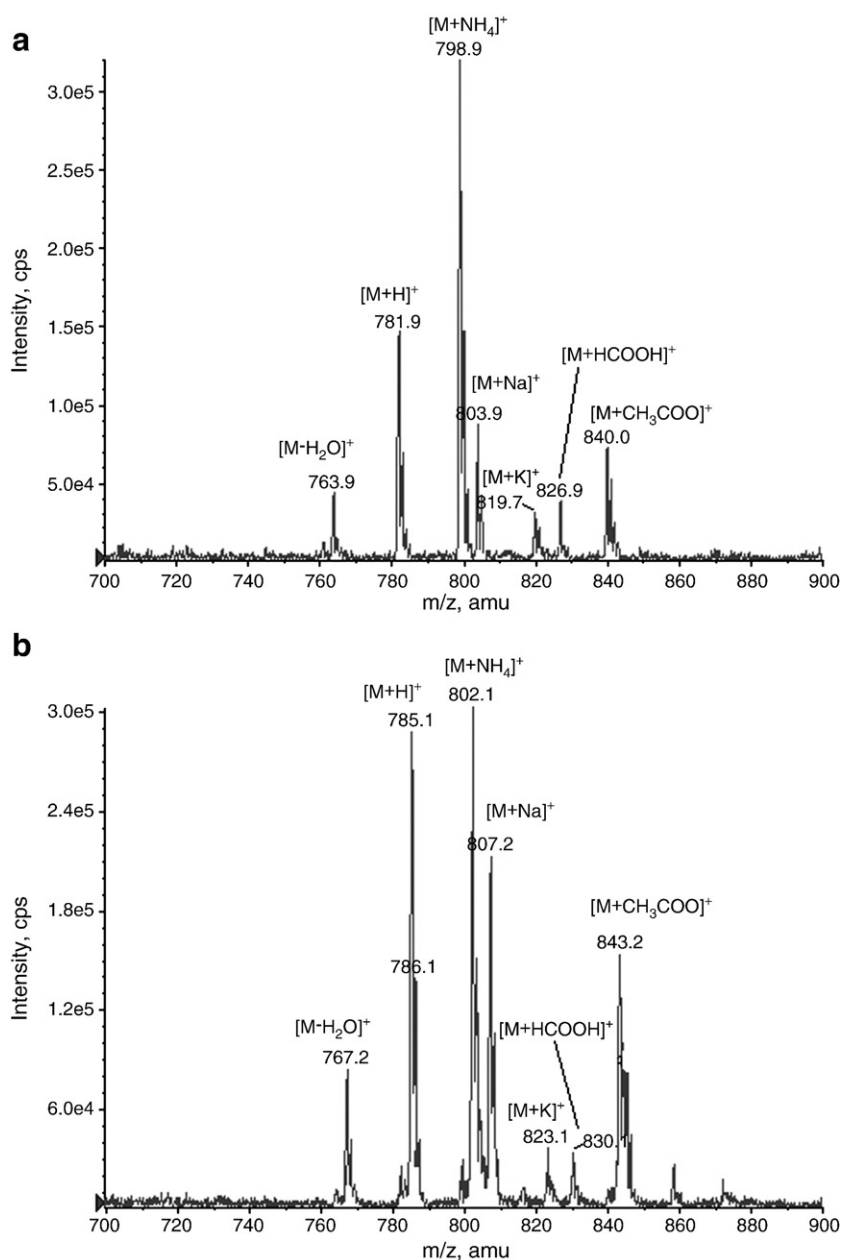

Fig. 1. Q1 full scan spectra of digoxin (a) and internal standard digoxin-d3 (b), the ion adducts are annotated.

were orally administrated digoxin at 0.125 mg/day. Blood samples were collected into heparinized tubes after digoxin had reached a stable state concentration. The time of blood drawing was at least 6 h following digoxin intake. Samples were centrifuged at 3000 rpm for 10 min and the resulting serum was stored at −20 °C until analysis.

Results

LC-MS/MS optimization

A variety of molecular ions for digoxin, including $[M -H_2O]^+$, $[M +H]^+$, $[M +NH_4]^+$, $[M +Na]^+$, $[M +K]^+$, $[M +HCOOH]^+$ and $[M +CH_3COOH]^+$, were observed in Q1 positive full-scan with respective m/z at 763.9, 781.9, 798.9, 803.9, 819.7, 826.9, and 840.0, respectively (Fig. 1a). A similar ion addition pattern was observed for the digoxin-d3 internal standard by adding a mass of 3 to each of these ions (Fig. 1b). A higher abundance was found for the ammonium addition of $[M +NH_4]^+$, which was used for further fragmentation in product ion scan. The product ions were obtained by fragmentation of the ammonium adduct precursor ion in a collision cell. Products with m/z at 651.8, 521.6 and 391.4 were produced by the losses of glycosides from digoxin one by one. The product ion mass spectra of digoxin are presented in Fig. 2. Multiple reaction monitoring (MRM) mode was used for quantitative detection, with sensitive ion transitions of m/z 798.6/651.5 and 802.6/654.5 for digoxin and its internal standard, respectively. Fig. 3 presents typical chromatograms of blank human serum, blank serum spiked with digoxin at the LLOQ level, and serum from a patient who had been orally administered a 0.125-mg digoxin tablet. As shown in the chromatograms, the baseline was flat and no endogenous interference was observed at the retention time of digoxin or

its IS, indicating a good selectivity for the method. The retention time was about 1.6 min, allowing a turnaround of 3 min per injection, but the complete analysis took longer because of the liquid–liquid extraction. The signal to noise (S/N) at the LLOQ was more than 10.

Method validation

Compared with an equal amount of digoxin chemically spiked to post-extracted blank serum, the extraction recovery was 83.9–87.1% by using liquid–liquid extraction with methyl tert-butyl ether as extraction solvent. Table 1 shows the results of extraction recovery and the matrix effect for digoxin and its IS, by comparing the mean peak areas obtained from six different lots of pooled sera after extraction with methyl tert-butyl ether. Although the matrix effect of 64.9–68.6% seems insufficient for LC-MS/MS analysis, good reproducibility and consistency were obtained by using an isotope-labeled internal standard.

The linearity was evaluated by analyzing three batches of standard curves over the concentration range of 0.1–10 ng/mL in human serum. Table 2 shows the linearity results of digoxin in human serum. Good linearity was observed over the quantification range when a linear regression was used with $1/x$ weighting. The correlation coefficients (r) were greater than 0.9961 for all analytical batches, with a bias within ±12%.

The intra- and inter-batch precision and accuracy are summarized in Table 3. These were obtained by spiking blank human serum at the LLOQ (0.1 ng/mL), and low (0.3 ng/mL), medium (1.5 ng/mL) and high (8.0 ng/mL) QC levels, then analyzing these in six replicates each batch, for three randomized analytical batches. The intra- and inter- batch precisions were less than 10% and 12%, respectively, and the bias ranged
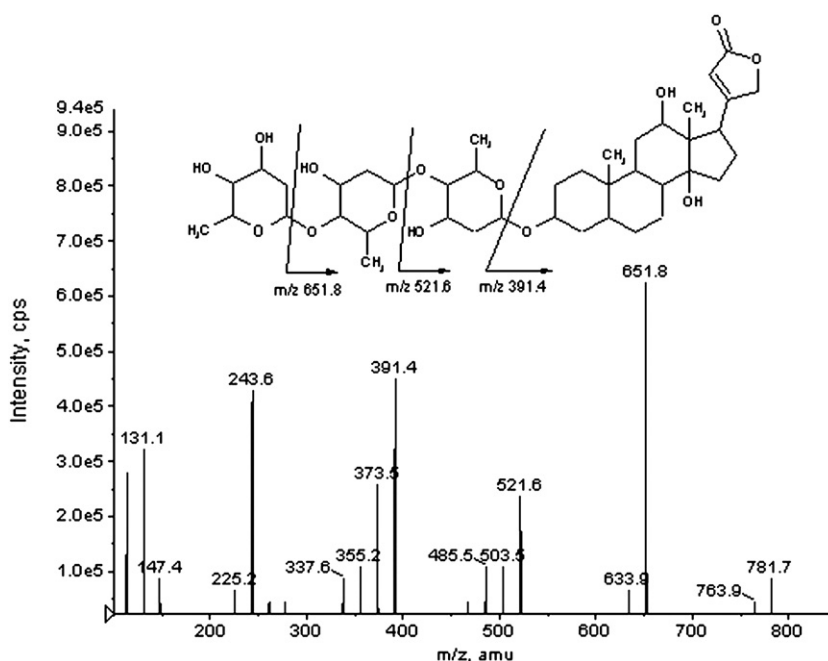

Fig. 2. Product ion spectra from $[M +NH_4]^+$ m/z 798.6 and the fragmentation pattern of digoxin.
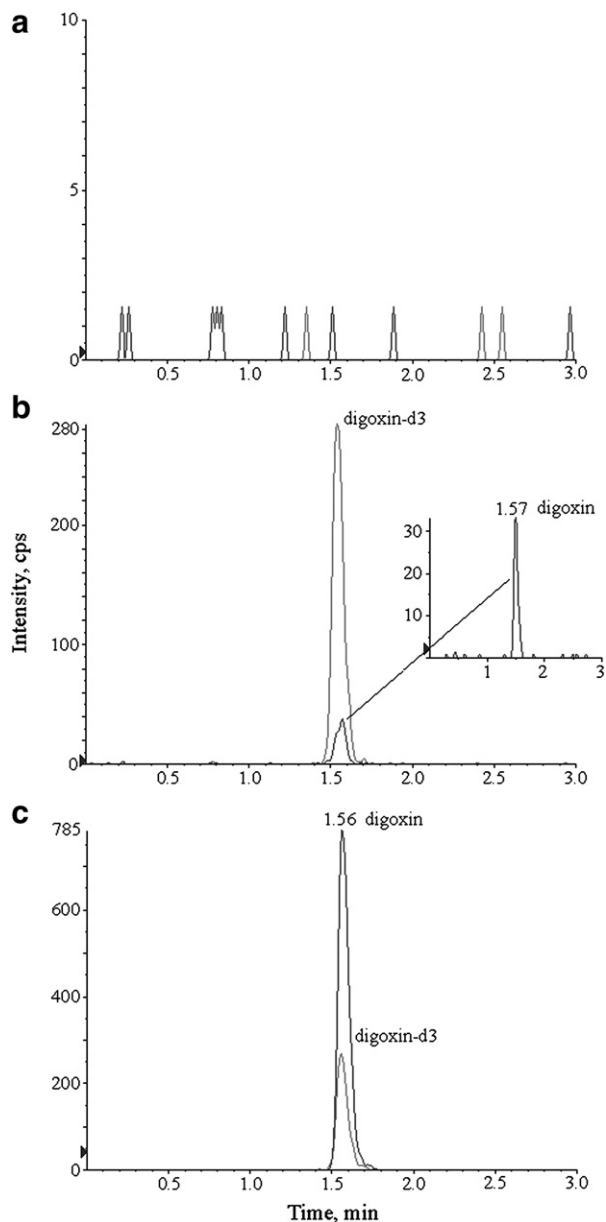
Fig. 3. Typical chromatograms of blank human serum (a), blank serum spiked with digoxin at the LLOQ (0.1 ng/mL digoxin) level (b), and serum collected from a patient who had been orally administered a 0. 125-mg digoxin tablet (c).

from −5.2% to 8.3% and −9.1% to10.7%, respectively. The values were within acceptable range and the method proved sufficiently precise and accurate.

Table 1
Extraction recovery and matrix effect of digoxin and its internal standard obtained from six different lots of pooled sera (RSD listed in bracket).

Concentration (ng/mL)	Extraction recovery (% RSD)		Matrix effect (% RSD)	
	Digoxin	IS	Digoxin	IS
0.3	85.2 (8.3)	82.4 (7.1)	68.6 (8.6)	68.6 (6.7)
1.5	83.9 (5.5)	78.8 (6.2)	64.9 (7.2)	67.6 (5.7)
8	87.1 (3.6)	81.7 (4.8)	66.7 (5.3)	67.3 (6.4)

Table 2
Mean inter-assay calibration curve results of digoxin in human serum ($n=2$ for three batches).

Nominal concentration (ng/mL)	RUN1	RUN2	RUN3	Mean	%RSD	%Bias
0.1	0.088	0.090	0.086	0.088	1.77	−12.00
0.4	0.396	0.417	0.455	0.423	7.17	5.65
1	1.089	1.067	1.034	1.063	2.60	6.33
4	4.333	4.013	4.511	4.285	5.89	7.13
10	9.595	9.914	9.458	9.656	2.42	−3.44
Intercept	0.0 405	0.0 262	0.0 279			
Slope	0.889	0.931	0.937			
R	0.9 967	0.9 986	0.9 961			

Digoxin was considerably stable after three freeze–thaw cycles, on bench top at room temperature for 8 h, in autosampler at room temperature for 24 h, and in storage at −20 °C for at least 1 month. The stability results are listed in Table 4. The stock solution of digoxin was stable in methanol for up to one year when kept at 4 °C. The working solution was found to be stable for a week at 4 °C.

External quality assessment results

The EQA results determined by our LC-MS/MS method are comparable to the target values, which were established by 73 participants in 2008 and 66 participants in 2009 in China. Linear regression analysis was performed to define the relationship between LC-MS/MS values (y) and EQA target values (x), the regression formula was $y=0.92x+0.034$ ($r=0.99$). Linear regression correlation between the values obtained with LC/MS/MS digoxin method and the EQA target values was presented in Fig. 4.

Application to therapeutic drug monitoring

The LC-MS/MS method was applied to determine the serum digoxin concentrations from heart failure patients receiving a dose of 0.125 mg/day of digoxin therapy. Among 48 collected serum samples, only 7 samples (14.6%) fell into the clinically recommended range of 0.5–0.9 ng/mL. The digoxin concentrations in 37 samples (77.1%) were found to be higher than the target range, while 4 samples (8.3%) were lower. Among the over-therapeutic-range samples, 13 samples (27.1%) had digoxin concentrations higher than 2 ng/mL.

Table 3
Accuracy and precision results of digoxin in human serum ($n=6$ for three batches).

Nominal concentration (ng/mL)	Intra-assay ($n=6$)		Inter-assay ($n=18$)	
	%RSD	%Bias	%RSD	%Bias
0.1	4.25	−5.18	11.79	−9.1
0.3	9.83	3.93	7.87	3.71
1.5	4.44	8.29	4.65	10.69
8.0	4.99	−4.81	5.82	1.23

Table 4
Stability of digoxin in human serum.

Nominal concentration (ng/mL)	Found concentration (ng/mL)	%RSD	%Bias
Freeze–thaw stability (three cycles)			
0.3	0.31	1.22	3.33
1.5	1.53	3.87	2.00
8.0	7.98	2.42	−0.25
Bench top stability (room temperature for 8 h)			
0.3	0.29	10.08	−3.33
1.5	1.59	5.78	6.00
8.0	7.66	5.20	−4.25
Auto-sampler stability (room temperature for 24 after processing)			
0.3	0.30	5.43	0.31
1.5	1.50	2.35	0.12
8.0	7.93	2.55	−0.88
Long-term storage stability (−20 °C for 3 months)			
0.3	0.30	4.71	0.03
1.5	1.59	2.21	6.00
8.0	7.94	1.28	−0.75

Discussion

Here we have described a rapid, economical, specific and reliable liquid chromatography electrospray ionization tandem mass spectrometry method for the quantification of serum digoxin. The analytical performance parameters including linearity, precision, accuracy, recovery, matrix effect, and stability were fully validated. The digoxin assay with LC-MS/MS method demonstrated high-throughput in terms of turnaround and cost-saving in terms of inexpensive reagents used for sample preparation.

After being scanned with flow injection analysis at a continuous flow of standard solution, digoxin produced the most intense molecular ion of ammonium addition $[M + NH_4]^+$ at m/z 798.9. The most intense product ion at m/z 651.8 was produced by loss of a glycoside from the molecular ion. The ion transition of 798/651 was subsequently optimized, which was also employed by other reports [16,18,20], for the digoxin MS/MS monitoring. Furthermore, we found that ion source temperature affects the stability of ammonium adduct ion. Therefore, we
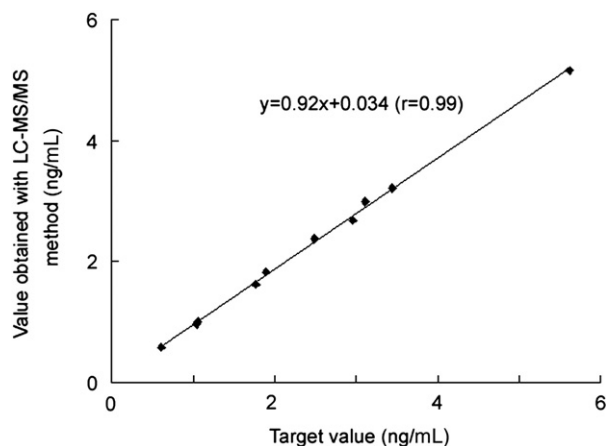
studied the impacts of different source temperatures on the intensity of the digoxin response. The best sensitivity was obtained when the source temperature was set at 400 °C.

In our method, we used methyl tert-butyl ether, a commonly-used and inexpensive solvent, for the liquid–liquid extraction procedure. During sample preparation, an isotope-labeled digoxin-d3 was used as the internal standard. As a result of it, good reproducibility and consistency were obtained during method validation. This effectively eliminated systematic errors during the process of sample preparation, chromatographic separation, and ionization in MS. Isotope dilution mass spectrometry (IDMS) provided data with reliable accuracy and precision [23–25].

The accuracy of the results with the proposed LC-MS/MS method was demonstrated by participating in external quality assessment (EQA) program (2008–2009) offered by National Center of Clinical Laboratory, Ministry of Health, China. Our LC-MS/MS digoxin method was capable of giving results close to the target value.

After being validated, our method was applied to the therapeutic monitoring of digoxin in a clinical setting. Among the collected serum samples from heart failure patients receiving a dose of 0.125 mg/day of digoxin therapy, only 14.6% fell into the recently recommended therapeutic window (0.5–0.9 ng/mL). These types of sub- or over-therapeutic concentrations of digoxin may bring potential risks of digoxin toxicity or inefficiency during clinical therapy. Therefore, therapeutic drug monitoring of digoxin is essential for dosage adjustment regimens in order to obtain desirable therapy outcome in clinical practice. With regard to the timing of the blood drawing for the digoxin TDM, digoxin will reach maximum serum concentration within 1–2 h following drug intake. Then its serum concentration will rapidly reduce within 5 h and maintain to a stable state 6–7 h after the drug intake. Therefore it should be reminded the importance to wait at least 6–7 h after the drug intake before performing a blood drawing for digoxin determination.

In conclusion, a LC-MS/MS protocol was developed and validated for the analysis of digoxin in human serum extracted with methyl tert-butyl ether. The method used isotope-labeled digoxin-d3 as an internal standard. After separation by reverse phase liquid chromatography, digoxin was detected with electrospray ionization tandem mass spectrometry. The method allowed a rapid chromatographic separation, with a total run time of 3 min for sample analysis, and a sensitive detection with a LLOQ of 0.1 ng/mL. The validated method was demonstrated to be acceptable in the EQA program and subsequently applied to therapeutic drug monitoring of digoxin in patients with heart failure who were receiving digoxin therapy in routine clinical practice.

Fig. 4. Linear regression comparing the values obtained with LC/MS/MS digoxin method vs. the national EQA target values.

y=0.92x+0.034 (r=0.99)

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