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A sensitive method for digoxin determination using formate-adduct ion based on the effect of ionization enhancement in liquid chromatograph–mass spectrometer

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ABSTRACT

A sensitive and rapid method based on formate-adduct ion detection was developed and fully validated for digoxin determination in rat plasma. For LC/MS/MS detection with formate-adducts as precursor ions, transitions of m/z 825.5 → 779.9 for digoxin and m/z 809.5 → 763.4 for the internal standard (digitoxin) were monitored in negative mode. To investigate the impact of formic acid on the mass response and method sensitivity, a formic acid concentration range of 0–0.1% (0, 0.0005%, 0.002%, 0.01%, 0.1%, v/v) was evaluated. A concentration of 0.002% gave the highest sensitivity, which was 16- to 18-fold higher than deprotonated ions, and was designated as the contribution giving the strongest ionization enhancement and adduction. A number of parameters were then varied in order to optimize the method, and a limit of quantitation (LOQ) at 0.2 ng/mL was reached with an injection volume of 5 μL, a total run time of 3 min, and 0.1 mL of rat plasma. A calibration curve was plotted over the range 0.2–50 ng/mL (R^2 = 0.9998), and the method was successfully applied to study pharmacokinetics in rat following a single oral administration of digoxin (0.05 mg/kg). Four additional steroid saponins (digitoxin, deslanoside, ginsenoside Rg1 and Rb1) were investigated to assess the impact of formic acid on the mass response of steroid saponins. Compounds with a conjugated lactonic ring in their structures such as digoxin, digitoxin and deslanoside tended to form stable formate-adduct ions more easily. The LC/MS/MS method developed here is therefore well suited for the quantification of steroid saponins that are difficult to deprotonate using other MS approaches.

1. Introduction

Digoxin is widely used in the clinic for the treatment of congestive heart failure and arrhythmia. The extremely narrow therapeutic window of 0.5–2 ng/mL in human plasma increases the complexity of using digoxin, and therapeutic drug monitoring (TDM) is required to ensure the appropriate dose is administered [1]. In addition, digoxin is a substrate for permeability-glycoprotein (P-gp). Many other substrate-binding sites are also present on P-gp, and co-administered drugs transported by P-gp may interact with each other, which can influence the final concentrations in the blood and ultimately affect toxicity and therapeutic effectiveness [2]. In this case, digoxin is commonly used as a probe compound to study drug–drug interaction (DDI). Therefore, methods to more accurately determine digoxin will therefore benefit both clinical TDM and studies in DDI.

Two rapid and sensitive methods widely used in practical digoxin monitoring are radio-immunoassay (RIA) and LC/MS/MS [3,4]. RIA is highly sensitive but suffers from low accuracy due to high cross-reactivity in many immunoreactions. LC/MS/MS is a highly efficient analytical tool for biological and nonbiological analysis. Some LC/MS/MS methods for quantifying digoxin have been reported, but in this study we developed a novel rapid and sensitive approach using formate-adduct ions $[M+HCOO]^-$, found in Q1 scanning exhibited higher response than any other fragment ions [2,5–13]. Formate-adducts afforded a limit of quantitation (LOQ) of 0.2 ng/mL and an amount on column (AOC) of 0.001 ng, which are both lower than most previously reported methods (Table 1). Despite advantages in LOQ and AOC, one previous method involved a basic mobile phase consisting of 0.05% ammonium carbonate (pH 9.0) may degrade the silica in some columns [2]. Similarly, a large injection volume of 80 μL reported in another method (using a column of 50 mm × 2.1 mm, 5 μm) is considerably bigger than

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Table 1
Comparison of recently used method for digoxin determination.

Reference	Year	Matrix	Extraction method	Detected ion	Additives in mobile phase	Gradient	Total run time (min)	Sample volume before/after processed (mL/mL)	Injection Volume (μL)	LOQ (ng/mL)	AOC[a] (ng)
Present	2014	Rat plasma	μ-Elution (SPE)	$[M+HCOO]^-$	0.002% formic a acid	No	3	0.1/0.1	5	0.2	0.0010
5	2013	Human plasma	PP	$[M-C_6H_{11}O_3+H]^+$	0.1% formic acid	Yes	13.5	0.1/0.05	–[c]	1	–[c]
6	2012	Whole blood	SPE	$[M+NH_4]^+$	5 mM ammonium formate	No	1.5	1/0.1	10	0.28	0.0280
2[b]	2011	Human and rat blood	LLE	$[M-H]^-$	0.05% ammonium carbonate (pH 9.0)	Yes	4.5	0.03/0.2	25	0.1	0.0004
7	2011	Rat blood	LLE	$[M+NH_4]^+$	5 mM ammonium formate and 0.1% formic acid	No	3	0.08/0.12	10	0.5	0.0033
8	2010	Human serum	LLE	$[M+NH_4]^+$	10 mM NH_4Ac and 0.1% formic acid	No	3	0.2/0.1	20	0.1	0.0040
9	2009	Human plasma	PP	$[M+NH_4]^+$	0.1% formic acid in 10 μM sodium acetate	No	2.3	0.2/0.3	20	0.5	0.0067
10	2008	Human plasma	LLE	$[M+Na]^+$	0.25 mM sodium acetate	Yes	14	1/0.1	15	0.05	0.0075
11	2008	Human plasma	SPE	$[M+NH_4]^+$	10 mM ammonium hydrogen carbonate	Yes	17	0.2/0.3	80	0.02	0.0011
12	2007	Human serum and urine	PP and SPE	$[M+NH_4]^+$	5 mM ammonium formate	No	7	0.25/0.03	30	0.2	0.0500
13	2003	Rat blood	PP and SPE	$[M+NH_4]^+$	5 mM ammonium formate (pH 3.4)	No	4	0.1/0.05	10	0.1	0.0020

[a] AOC, amount on column, $=LOQ^*V_{injection}{}^*V_{before\ processed}/V_{after\ processed}$, counts in concentrating and diluting effects and indicates the real amount injected into LC/MS/MS.
[b] This method is really comparable with our supplied, but the latter is more convenient because of faster sample preparation, less additives in mobile phase (effectively shorting the whole process), no gradient, and shorter run time.
[c] Unknown.

the recommended maximum volume of 20 μL [11]. The method developed here has an injection volume of 5 μL (using a column of 100 mm × 2.1 mm, 5 μm), well within the suggested limit, which allows analysis of trace amounts of sample and improves column performance in the LC/MS/MS procedure. Additionally, unlike previously described methods that use gradient elution [2,5–13], which leads to a higher column pressure and compromises HPLC analysis, our method does not use gradient elution. Other advantages include a short analysis time, a simple mobile phase and sample extraction procedure, and the used of Oasis HLB μElution plates in the final optimized method, which can determine digoxin from only 100 μL of biological sample (rat plasma). The method was fully validated and successfully applied to study rat pharmacokinetics following a single oral administration. Four additional steroid saponins (digitoxin, deslanoside, ginsenoside Rg1 and Rb1) were also evaluated to assess the effect of formic acid on adduct formation and mass ionization in the LC/MS/MS procedure.

2. Materials and methods

2.1. Chemicals, reagents and instruments

All standards (digoxin, digitoxin, deslanoside, ginsenoside Rg1 and Rb1) were purchased from the National Institutes for Food and Drug Control (Beijing, China). Formic acid (HPLC-grade) was purchased from Dikma Technologies Inc. (Beijing, China). Ammonium formate (HPLC-grade) was purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Methanol (HPLC-grade) was purchased from J&K Scientific Ltd. Acetonitrile (HPLC-grade) was purchased from Merck KGaA. Deionized water was obtained from a Milli-Q Ultrapuresystem (Millipore, Shanghai, China) with the water outlet operating at 18.2 MΩ. An Agilent-1200 HPLC system was used for separation. An API4000 turbo ion spray ionization-triple quadrupole mass spectrometer (AB SCIEX) was used for detection. Oasis HLB μElution plates (Waters) were used for plasma purification.

2.2. Liquid chromatography conditions

A Venusil XBP Phenyl analytical column (2.1 mm × 100 mm, 5 μm) from Bonna-Agela Technologies (Tianjin, China) was used at a column temperature of 30 °C. The optimized mobile phase consisted of acetonitrile and 0.002% (v/v) formic acid (60:40, v/v) and a flow rate of 0.3 mL/min.

2.3. Mass spectrometry conditions

Turbo ion spray in negative ion mode and multiple reaction monitoring (MRM) were applied for mass spectrometric detection with the parameters shown in Table 2. Digoxin was monitored at two transitions of m/z 825.5 → 779.9 and m/z 779.4 → 649.4 and the internal standard (digitoxin) was at m/z 809.5 → 763.4. An m/z of 825.5 → 779.9 for digoxin and 809.5 → 763.4 for the internal standard were used to quantify digoxin. Since an m/z 779.4 → 649.4 in negative ion mode showed a high sensitivity in a previously

Table 2
Mass-spectrometric parameters.

Transitions (*m*/*z*)	DP (V)	CE (eV)	EP (V)	CXP (V)	IS (V)	TEM (°C)	GS1 (psi)	GS2 (psi)
825.5 → 779.9	−70	−47						
779.4 → 649.4	−80	−30	−10	−15	−4500	400	50	50
809.5 → 763.4	−66	−33						

reported method, we also tested this transition to allow a direct comparison of the methods.

2.4. Stock solutions

Stock solutions of digoxin (0.1 mg/mL) and IS (0.05 mg/mL) were prepared by dissolving the reference compounds in methanol, and solutions were stored at −4 °C until needed.

2.5. Standards and quality control (QC) samples

A series of digoxin working standard solutions were prepared by diluting with methanol to give concentrations of 2, 5, 10, 20, 50, 100, 200, and 500 ng/mL and each solution was spiked with IS at 100 ng/mL. Then, 10 μL of standard solution was added to 90 μL of blank rat plasma, vortexed for 30 s and centrifuged at 5000 rpm for 10 min to provide working standard plasma solutions at 0.2, 0.5, 1, 2, 5, 10, 20 and 50 ng/mL for digoxin and 10 ng/mL for IS. For QC samples, spiked plasma solutions of 50, 5 and 0.5 ng/mL were prepared in the same way.

2.6. Sample preparation

An Oasis HLB μElution plate (30 μm) was used to process plasma samples. Sample preparation was performed by conditioning each cartridge twice with 0.2 mL of methanol (0.4 mL in total), equilibrating twice with 0.2 mL of water, loading 0.1 mL of plasma sample in two separate 0.05 mL portions, washing with 0.2 mL of water, and eluting with 0.1 mL of 60:40 (v/v) acetonitrile:water (without formic acid) in two separate portions. 5 μL of eluate was injected into the LC/MS/MS system.

2.7. Method validation

2.7.1. Specificity

The specificity was evaluated by analyzing blank plasma, IS-spiked (10 ng/mL) plasma and IS- and digoxin-spiked (10 ng/mL) plasma from six rats.

2.7.2. Calibration curve, linearity and limit of quantitation (LOQ)

A calibration curve ranging from 0.2 to 50 ng/mL digoxin in rat plasma was constructed by plotting the peak area ratio of the digoxin *m*/*z* 825.5 → 779.9 to IS *m*/*z* 809.5 → 763.4 (*Y*-axis) versus digoxin concentration (*X*-axis). The regression parameters of the slope, intercept and correlation coefficient were calculated by weighting ($1/x^2$) linear regression in Analyst 1.6 software. The correlation coefficient (r^2) was used to evaluate linearity. Accuracy was evaluated by relative error (RE). The concentration with a signal-to-noise of 10 was defined as the LOQ.

2.7.3. Intra- and inter-assay precision and accuracy

Three analytical batches were processed on three separate days to evaluate the intra- and inter-assay precision and accuracy. Each batch consisted of eight concentrations (0.2, 0.5, 1, 2, 5, 10, 20 and 50 ng/mL) for the calibration curve, a blank, and three typical concentrations (0.5, 5 and 50 ng/mL) of QC samples, with five replicates for each concentration. Intra- and inter-assay accuracy was evaluated by relative error (RE). Assay precision was evaluated by percent relative standard deviation (RSD). Both accuracy and precision should ideally be less than 15%, except for the LOQ, where less than 20% is also acceptable in LC/MS/MS procedures.

2.7.4. Matrix effect and recovery

Three sets of solutions were prepared to assess the matrix effect and recovery: Set 1 = pre-spiked standards (QC samples); Set 2 = post-spiked standards (prepared as described in Section 2.6 followed by spiking of the eluate with working standard solutions); Set 3 = neat standards (prepared by diluting working standard solutions with 60:40 (v/v) acetonitrile:water). Each set consisted of digoxin-spiked samples at three typical concentrations (0.5, 5 and 50 ng/mL) and five replicates for each concentration. The matrix effect (ME) and recovery (RE) were calculated using the following formulae:

$$\text{Recovery}\ \ (\%) = \frac{\text{absolute peak area of Set 1}}{\text{absolute peak area of Set 2}} \times 100\%$$

$$\text{Matrix effect}\,(\%) = \frac{\text{absolute peak area of Set 2}}{\text{absolute peak area of Set 3}} \times 100\%$$

2.7.5. Stability

Freeze–thaw, short- and long-term stability was evaluated at concentrations of 1 ng/mL and 40 ng/mL. For freeze–thaw stability, three freeze–thaws were performed and samples were frozen for 24 h at −20 °C each time. For short-term stability, experimental samples were kept at room temperature for 24 h. For long-term stability, experimental samples were kept at −20 °C for at least five weeks. Comparison of the concentration of experimental samples and freshly prepared samples was performed to evaluate stability.

2.7.6. Application

Using the above procedure, profiles of digoxin plasma concentration versus time were determined for three rats (weight ranging from 204 to 208 g) following an oral administration of a single 0.05 mg/kg dose. A piece of tablet containing 0.25 mg of digoxin was ground into powder and dissolved in 25 mL of water to prepare the suspension for administration. Plasma was drawn 24 h after administration, centrifuged at 5000 rpm for 10 min and stored at −20 °C until needed for analysis.

3. Results and discussion

3.1. Method development

3.1.1. Influence of adduct ion on sensitivity

Quantifying digoxin at blood concentrations of 0.5 ng/mL or lower proved difficult with the conditions (biological sample and injection volumes among other parameters) and methods previously reported (Table 1) that are based on detection of the $[M+NH_4]^+$ ion for digoxin quantification. One previous report [2] inspired us to investigate ion full scan mass spectra (Q1) of digoxin (500 ng/mL dissolved in 60:40 (v/v) acetonitrile:water) in negative ion mode. Interestingly, in this experiment, the intensity of the $[M+HCOO]^-$ ion stood out from the other fragments including

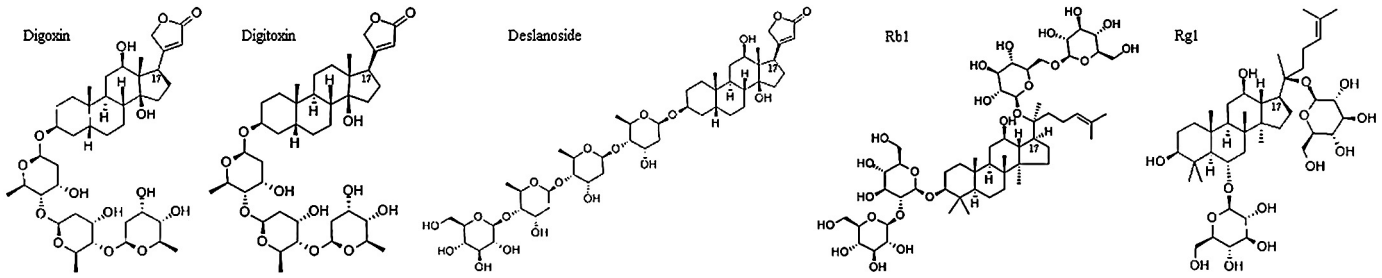

Fig. 1. Structures of the five compounds tested.

$[M-H]^-$ that was reported to be responsible for the high sensitivity [2].

As alternative internal standards, four additional steroid saponins were tested, namely digitoxin, deslanoside, and ginsenosides Rg1 and Rb1. For the digitaloid ones, intensities of formate-adduct ions were found stronger than deprotonated ions, while for ginsenosides, the intensities of formate-adducts and deprotonated ions were comparable and much lower than that of digitaloid ones. The structures of these four compounds and digoxin are all based on a steroid ring, and the major differences are the group attached to C17 and number and location of the attached glycosides. The digitaloid compounds have an electron-rich conjugated lactonic ring, while the ginsenosides have other substituents instead of the conjugated lactone ring (Fig. 1) that is more sterically hindered and less electron-rich. A possible explanation for the stronger adduct intensities observed with the digitaloid drugs is an electron transfer reaction between the conjugated lactone and formate.

A comparison with earlier work that used $[M+NH_4]^+$ for digoxin quantification (Fig. 2) highlighted the sensitivity of the reported methods, which is ordered $[M+HCOO]^-$ (Fig. 2d) > $[M-H]^-$ (Fig. 2c) > $[M+NH_4]^+$ (Fig. 2b). Under the optimized conditions developed in this study, the intensity of the $[M+HCOO]^-$ ion was approximately 16–18 times higher than the $[M-H]^-$ ion (Fig. 2), so we recommend that consider using adduct ions when other fragment ion is not sensitive enough for quantification.

3.1.2. The effect of mobile phase formic acid concentration

It has been reported that formic acid significantly increases either the negative or the positive ion ESI responses of many structurally different analytes [14]. To our knowledge, the most commonly used formic acid concentration used for digoxin determination is 0.1% (v/v) which is likely to be at the lower end on the concentration range [2,5–13]. After the $[M+HCOO]^-$ adduct was chosen for subsequent investigation, a mobile phase consisting of 0.1% acetonitrile:formic acid (60:40, v/v) resulted in a relatively low ion intensity that was comparable to the $[M+NH_4]^+$ ion. Furthermore, with these conditions, there was carryover from previous chromatographic separations at the retention time of digoxin in subsequent injections. Higher concentrations of formic acid did not increase the adduct intensity or eliminate the carryover. The commonly used mobile phase additive ammonium formate was then investigated at 0.2, 1, 2, 5 and 10 mM, but this also failed to increase the intensity of formate-adducts.

A previously reported sensitive method investigated the rapid pharmacokinetics of four unspecified generic compounds using

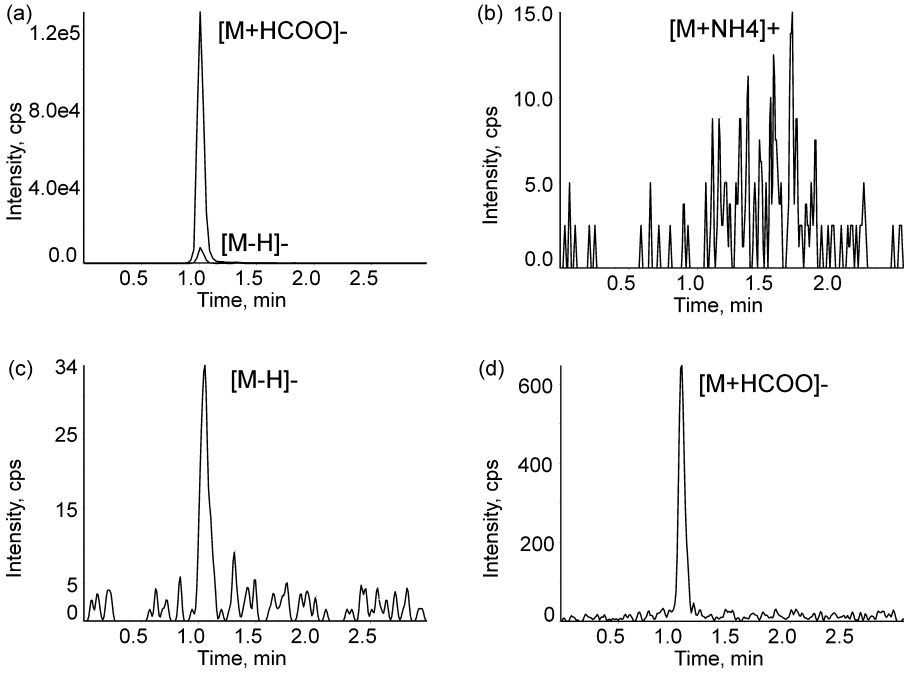

Fig. 2. Comparison of the $[M+NH_4]^+$, $[M+HCOO]^-$ and $[M-H]^-$ ion intensities. (a) Digoxin = 0.5 μg/mL, mobile phase = acetonitrile/0.002% formic acid, 60/40 (v/v). (b) Digoxin = 0.5 ng/mL, mobile phase = acetonitrile/2 mM ammonium formate, 60/40 (v/v). (c) Digoxin = 0.5 ng/mL, mobile phase = acetonitrile/0.002% formic acid, 60/40 (v/v). (d) Digoxin = 0.5 ng/mL, mobile phase = acetonitrile/0.002% formic acid, 60/40(v/v). This figure shows that the intensity of $[M+HCOO]^-$ is 16–18 times stronger than $[M-H]^-$, and the intensity of $[M+NH_4]^+$ is weaker still.

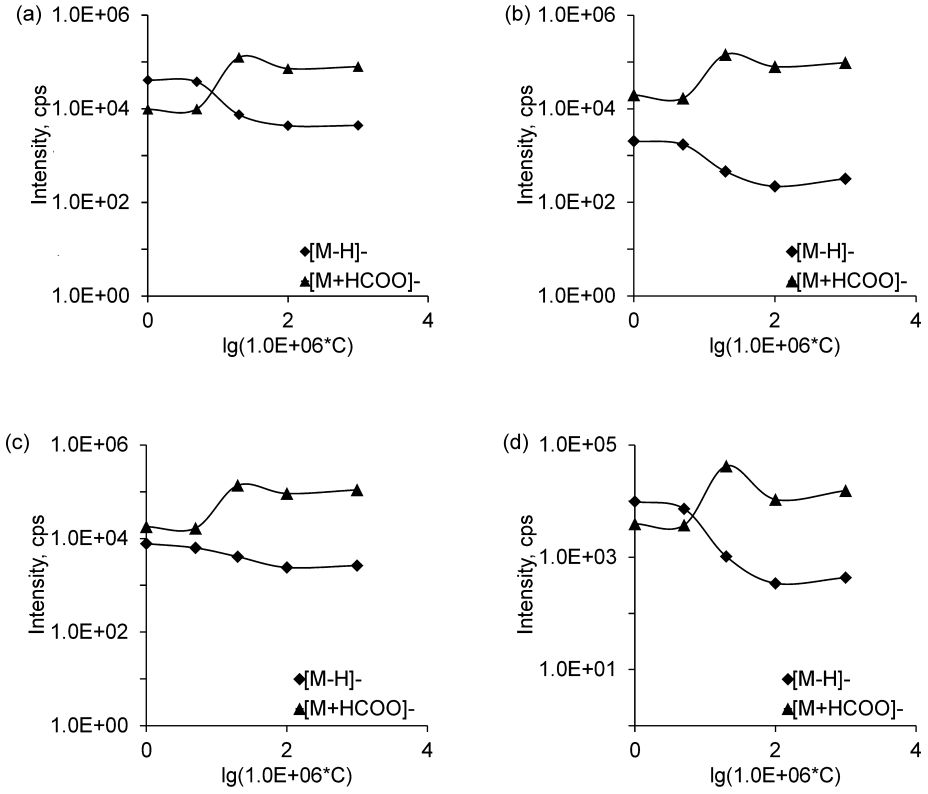

Fig. 3. Optimization of formic acid concentration in the mobile phase for LC/MS/MS detection of digoxin and three other steroid saponins. Ionization was inhibited when formic acid exceeded 0.002%, with $[M-H]^-$ especially affected.

a formic acid concentration of 0.0015%, therefore we decided to lower the concentration in our method to 0, 0.0005, 0.002, 0.01 and 0.1% (Fig. 3a). Interestingly, the trend in $[M+HCOO]^-$ ions was very different to that of $[M-H]^-$, with a peak intensity obtained with 0.002% formic acid, while the $[M-H]^-$ ion intensity was much lower at this formic acid concentration. So it suggested that formic acid concentration over 0.002% might inhibit the ionization [15,16]. The peak $[M+HCOO]^-$ ion intensity could be due to both an enhanced ionization at 0.002% formic acid, and an increased supply of $HCOO^-$. To evaluate the impact of formic acid concentration on adduct formation and mass ionization, three other steroid saponins were investigated. The results obtained were similar to those obtained with digoxin (Fig. 3b–d), which further indicates that ionization is inhibited when mobile phase formic acid concentrations exceeded 0.002%. In addition to enhancing formate ion intensity, a formic acid concentration of 0.002% also eliminated the carryover and residual intensity from previous injections. In conclusion, a concentration of 0.002%, and certainly less than the commonly used 0.1%, should be used to make the most of the highly sensitive LC/MS method.

3.1.3. Optimization of extraction

The application of Oasis HLB μElution plate usually involves five steps: (i) conditioning with organic solvent, (ii) equilibrating with aqueous phase, (iii) loading sample, (iv) washing with buffer solution and (v) finally eluting with some selective solution. During the optimization of extraction, we found that loading and eluting were the main influencers of digoxin recovery. Separating the loading and elution into two equal portions achieved an 80% recovery compared a recovery of only 35% with a single portion for each step.

Table 3
Calibration curve.

Nominal concentration (ng/mL)	Observed concentration (ng/mL)	RE (%)
0.2	0.199	−0.5
0.5	0.507	1.4
1	1	0
2	2	0
5	5.07	1.4
10	10.2	2
20	19.9	−0.5
50	48.1	−3.8

3.2. Method validation

3.2.1. Specificity

Under the optimized method conditions, digoxin and IS were fully separated with retention times of 1.0 and 1.4 min, respectively (Fig. 4). No interference from endogenous metabolites was observed.

3.2.2. Calibration curve, linearity and LOQ

All calibration curves showed good linearity within the assay range of 0.2–50 ng/mL and gave a correlation coefficient of not less than 0.9998 by weighted ($1/x^2$) linear regression. The smallest concentration that gave a signal-to-noise value exceeding 10 (0.2 ng/mL) was defined as the LOQ. The RE of each concentration in the calibration curve was −3.8% to 1.4%. Detailed data are shown in Table 3.

3.2.3. Precision and accuracy

Intra- and inter-day precision and accuracy data demonstrated that the developed method was highly accurate, with RSD of 1.5% to 6.7% and RE of −5.64% to 3.67% (Table 4).

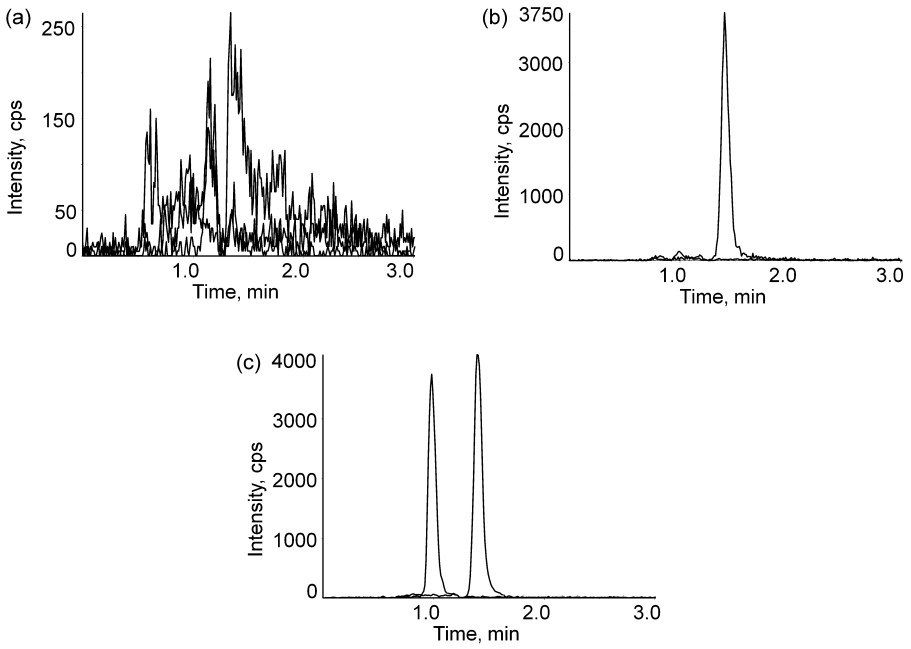

Fig. 4. Typical chromatograms showing specificity.

Table 4
Results of intra- and inter-day precision and accuracy assay.

Normal concentration (ng/mL)	First day			Second day			Third day		
	Observed concentration (ng/mL)	RSD (%)	RE (%)	Observed concentration (ng/mL)	RSD (%)	RE (%)	Observed concentration (ng/mL)	RSD (%)	RE (%)
0.5	0.497	6.7	−0.56	0.513	3.6	2.6	0.518	4.8	3.76
5	4.93	2.1	−1.4	4.98	3.7	−0.36	5.05	6.1	1.12
50	47.2	3.0	−5.64	46.9	3.0	−6.12	47.6	1.5	−4.96

Table 5
Matrix effect and recovery of digoxin assay in rat plasma.

QC samples (ng/mL)	Matrix effect (%)	Recovery (%)
0.5	97.80	87.81
5	100.75	88.01
50	100.75	80.22

3.2.4. *Effects of matrix and recovery*

The effects of the matrix and recovery were evaluated using blank rat plasma matrix from different individuals, which gave a digoxin recovery of 80.22–88.01%. The matrix effect of QC samples ranged from 97.80% to 100.75%, indicating no apparent ionization enhancement or suppression. Detailed data are shown in Table 5.

3.2.5. *Stability*

No significant differences between normal and observed concentrations were apparent, indicating that digoxin was stable in biological matrix (Table 6).

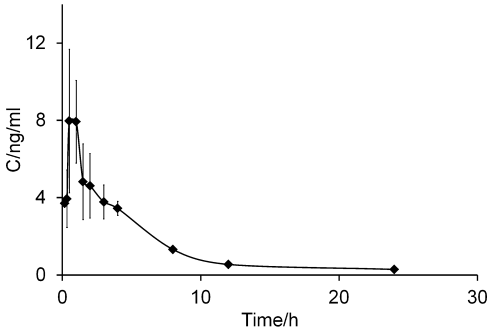

Fig. 5. Pharmacokinetics in rat following a single oral administration of digoxin. C_{max} and C_{min} of digoxin are 7.97 ng/mL (at 0.5 h) and 0.29 ng/mL (at 24 h), respectively.

3.3. *Application (digoxin quantitation)*

The developed digoxin assay was successfully applied to determine profiles of digoxin plasma concentration versus time in three rats following an oral administration of a single dose of 0.05 mg/kg.

Table 6
Stability of digoxin assay in rat plasma.

Stability	*n*	1 ng/mL			40 ng/mL		
		Observed concentration (ng/mL)	RSD (%)	RE (%)	Observed concentration (ng/mL)	RSD (%)	RE (%)
Short-term	3	1.01	6.82	0.63	40.30	1.31	0.75
Long-term	3	1.06	3.92	6.33	40.40	2.11	1.00
Three freeze–thaw cycles	3	1.06	3.56	6.33	40.13	3.63	0.33

The mean ($n = 3$) profile (Fig. 5) gave a digoxin C_{max} and C_{min} of 7.97 ng/mL (at 0.5 h) and 0.29 ng/mL (at 24 h), respectively.

3.4. Conclusion

These results demonstrated the usefulness of the $[M+HCOO]^-$ adduct and the benefit of extremely low formic acid concentrations for digoxin determination, and a sensitive method was developed and validated. Compared with several previously reported sensitive methods, this novel approach is more convenient due to requiring a smaller biological sample and injection volume, shorter analysis time, and more rapid and simpler sample extraction procedure courtesy of the use of Oasis HLB μElution plates for sample preparation. The optimized method was successfully applied to study rat pharmacokinetics following a single oral administration.

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