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A rapid and sensitive LC/MS/MS assay for quantitative determination of digoxin in rat plasma

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Abstract

Digoxin is a cardiac glycoside that is widely used for the treatment of congestive heart failure. To evaluate pharmacokinetics of digoxin in rats, a sensitive LC/MS/MS assay was developed and validated for the determination of digoxin concentration in rat plasma. For detection, a Sciex API3000 LC/MS/MS with atmospheric pressure ionization (API) mass spectrometry turbo ion spray inlet in the positive ion-multiple reaction monitoring mode was used to monitor precursor → product ions of *m/z* 798.6 → 651.6 for digoxin and *m/z* 577.6 → 433.3 for oleandrin, the internal standard (IS). The standard curve was linear ($r^2 \geq 0.999$) over the digoxin concentration range of 0.1–100 ng/ml in plasma for digoxin. The mean predicted concentrations of the quality control samples deviated by $< 5.8\%$ from the corresponding nominal values; the intra-assay and inter-assay precision of the assay were within 8.6% relative standard deviation. At the lower limit of quantitation (LLQ) of 0.1 ng/ml, the mean deviation of predicted concentrations from the nominal value was within 3.7%. The extraction recoveries of digoxin and internal standard were 82.7 ± 3.9 and $105.9 \pm 2.3\%$, respectively. The present method was successfully applied to characterization of pharmacokinetic profiles of digoxin in rats after oral administration.

Keywords: Pharmacokinetic; Digoxin; Rats

1. Introduction

Digoxin (Fig. 1) is a cardiac glycoside that is widely used for the treatment of congestive heart failure. Digoxin has a very narrow therapeutic index and thus, digoxin therapy requires strict monitoring of blood levels to minimize toxicity [1,2]. Digoxin has been shown to be a substrate of *P*-glycoprotein (P-gp) both in vitro [3] and in vivo [4]. Drug–drug interactions of digoxin with P-gp modulators have been well documented. For example, P-gp inhibitors, such as verapamil [5] and quinidine [6], have been shown to increase blood concentrations of digoxin. On the other hand, rifampin, a P-gp inducer, increased the

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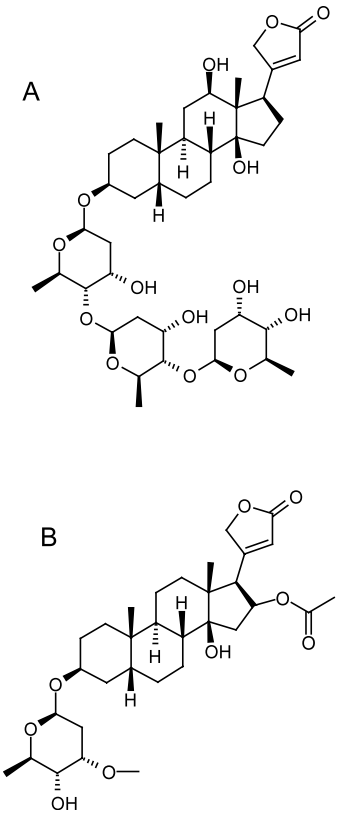

Fig. 1. Chemical structures of digoxin (A) and oleandrin (B) (internal standard).

intestinal P-gp expression and led to a significant decrease in oral exposure of digoxin [7]. Therefore, identification of drug candidates that are neither substrates nor inhibitors of P-gp would reduce the likelihood of a clinical drug–drug interaction. Implementation of small animal models (e.g. rats) to study digoxin interactions has been difficult mainly because of lack of a sensitive and easy to use bioanalytical methodology for digoxin quantitation.

There are numerous methods reported for digoxin quantitation, including radioimmunoassay (RIA), high performance liquid chromatography (HPLC) assay with RIA or fluorescence detection and LC/MS or LC/MS/MS assay. RIA is currently the most commonly used method for digoxin quantitation in biological matrices [8,9]. While this method is sensitive and used frequently in clinical and non-clinical studies, it has been reported that RIA is not specific and cross-reacts with digoxin metabolites and endogenous digoxin-like substances [10–12]. HPLC methods are capable of avoiding interference, but are generally not sensitive enough to quantitate digoxin at lower levels (e.g. <1 ng/ml). For digoxin interaction studies, [^{3}H]digoxin is often preferred even though significant effort has to be devoted to digoxin peak resolution and radioactive safety precautions. There are several reports describing LC/MS or LC/MS/MS methods for the identification and quantitation of digoxin, digoxin metabolites and digoxin-like substances in biological matrices [12,13]. For digoxin quantitation, these methods are generally sensitive but require large sample volume (0.5–4 ml plasma), which is not feasible in small animal models (e.g. rats).

To evaluate digoxin pharmacokinetics in rats, a sensitive and robust LC/MS/MS method was developed and fully validated. The method was successfully applied to digoxin pharmacokinetic interaction studies in rats and can be easily extended to other animal species. The current method offers a number of advantages over existing methods, such as shorter analysis time, smaller sample volume (200 μl blood), amenable to serial sampling studies and devoid of extensive sample cleanup.

2. Experimental

2.1. Materials and reagents

Digoxin (purity, 96.4%) and internal standard, oleandrin (purity, 99%), were obtained from Sigma Chemical (St. Louis, MO). Methanol (Omnisolve, HPLC grade) was purchased from EM Science (Gibbstown, NJ). Rat plasma was supplied by Bioreclamation Inc. (Hicksville, NY). HPLC grade acetonitrile was obtained from Burdick and Jackson (Muskeson, MI). Water was purified by a Mill-Q-System from Millipore Corp. (Milford, MA). Ammonium formate (Avocado Research Chemicals, Ltd., Wordhill, MA) and formic acid (J.T. Baker, Phillipsburg, NJ) were of analytical-grade. Ammonium chloride was obtained from EM Science.

2.2. Instrumentation

The HPLC system consisted of a Perkin-Elmer (Norwalk, CT) LC200 HPLC pump and autosampler. The mass spectrometer was a Applied Biosystems MDS Sciex (Toronto, Canada) API-3000 Triple Quadrapole mass spectrometer (MS/MS) with a heated nebulizer interface (500 °C). Data were collected and processed using Sciex Analyst 1.1 data collection and integration software on an IBM compatible computer.

2.3. Chromatographic conditions

The HPLC column (50 × 2.0 mm) was a 3-micron YMC ODS AQ analytical column from Waters Corp. (Milford, MA). Column temperature was held at room temperature. The mobile phase consisted of acetonitrile and 5 mM ammonium formate (pH 3.4) (50:50, v/v) and was filtered through a 0.2-micron nylon filter before use. The chromatography was performed isocratically at a flow rate of 0.2 ml/min at room temperature. Total run time was 4 min for each injection.

2.4. MS/MS detection

Precursor ions for analytes and internal standards were determined from mass spectra obtained during infusion of neat solutions into the API-3000 mass spectrometer. Using API turbo ionspray (TISP) source, the mass spectrometer was operated in the positive ionization mode with the collision gas off. Under these conditions, the analytes yielded predominantly ammonium adduct ion at m/z 798.6 for digoxin and protonated molecular ion at m/z 577.6 for internal standard. Each of the precursor ions were subjected to collision-induced dissociation to determine the resulting product ions. The product ion spectra for digoxin and oleandrin are shown in Figs. 2 and 3, respectively. The primary fragments for digoxin and internal standard were attributed to the loss of one sugar group. Product ions resulting from this fragmentation pattern were chosen for MS/MS detection of each of the analytes at m/z 651.6 (digoxin) and 433.3 (internal standard). Interface independent instrument parameters were optimized during the infusion of a solution of digoxin through the TISP interface with HPLC mobile phase. These were (arbitrary units) CAD: 4, DP: 46, FP: 230, EP: −10, CE: 19, CXP, 32 and DF: −400. The electron multiplier was set at 2200 V. Settings were adjusted to maximize the response for the digoxin precursor/product ion combination of m/z 798.6 → 651.6.

Heated nebulizer parameters were optimized based on the MS/MS responses obtained during repetitive injections of digoxin (10-μl injections of a 100 ng/ml solution) in the presence of LC mobile phase. The LC/MS/MS system was operated in a flow injection analysis configuration (i.e. the HPLC columns were removed from the system and the analyte was injected directly in mobile phase flow then into the MS system) during the optimization experiments. After optimization, ionization of analytes was carried out using the following settings: source temperature, 500 °C; ion source voltage, 5000 V; nebulizer and curtain gases (nitrogen) 11 and 11, respectively; the flow rate of heated gas (gas 2) was operated at 5 l/min. Multiple reaction monitoring (MRM) mode was used in MS acquisition method for quantitation of digoxin. The dwell time was 150 ms.

2.5. Preparation of standards and quality control (QC) samples

Digoxin stock solution was prepared in methanol at a concentration of 100 μg/ml. Primary digoxin solution was diluted with the mobile phase, 50:50 (v/v%) acetonitrile and 5 mM ammonium formate (pH 3.4) to 4 μg/ml. Secondary plasma standard solution was prepared by diluting the primary solution with rat plasma to give a concentration of 200 ng/ml. The working standard plasma solutions were serially diluted to provide concentrations of 0.1, 0.5, 2, 5, 25, 50 and 100 ng/ml of digoxin. Internal standard was prepared in acetonitrile at a concentration of 500 ng/ml. Digoxin stock solution for QC was prepared separately and QC plasma samples were prepared at 0.3, 5, 20 and 80 ng/ml in the same manner as plasma standard.

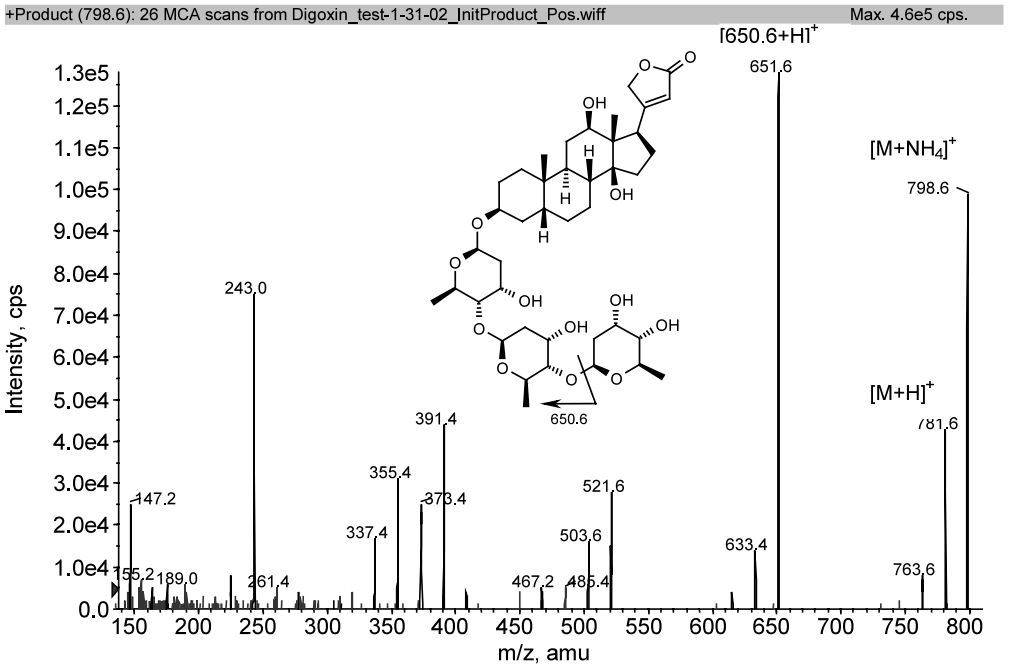

Fig. 2. Product ion mass spectrum of ammonium cluster $[M+NH_4]^+$ of digoxin at *m/z* 798.6.

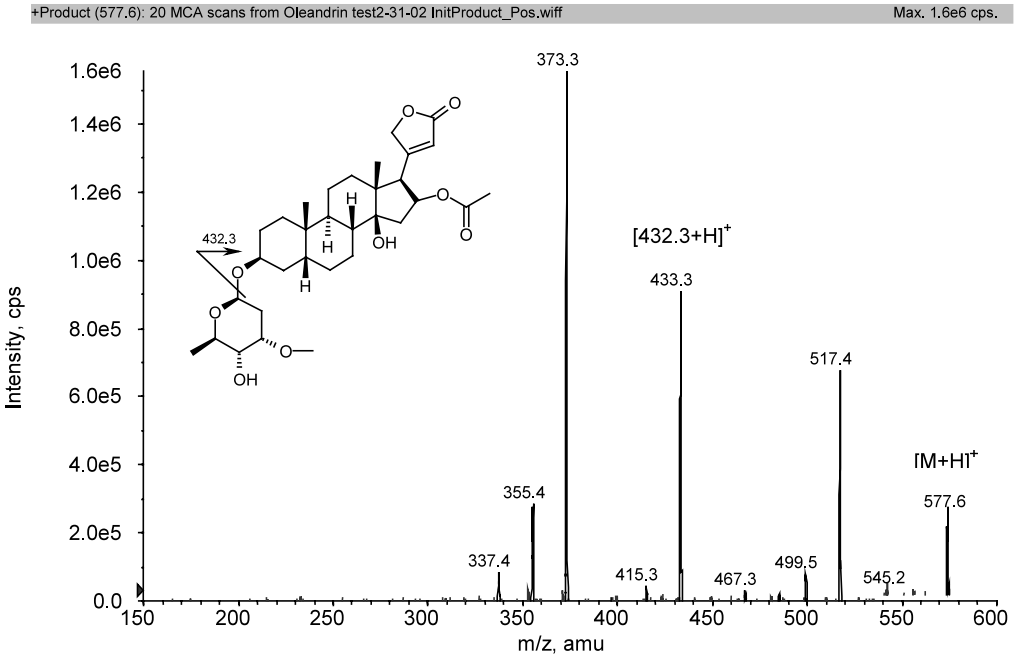

Fig. 3. Product ion mass spectrum of protonated molecule $[M+H]^+$ of oleandrin at *m/z* 577.6.

2.6. Sample preparation

A 0.1 ml aliquot of plasma sample was transferred to a clean screw capped tube. Ammonium chloride buffer (50 μl, pH 8.8) was added and followed by 300 μl of acetonitrile containing 500 ng/ml of IS. The tube was then vortexed. Methylene chloride (150 μl) was added and the sample was vortexed again for 2 min. After centrifugation at $20{,}200 \times g$ for 4 min, the upper organic layer was transferred to a 4 ml glass tube and evaporated to dryness under a stream of nitrogen at 40 °C. The dried residue was reconstituted with 50 μl of the mobile phase. A 10 μl aliquot of the sample was injected onto the LC/MS/MS.

2.7. Validation procedure

2.7.1. Calibration curve and linearity

The seven-point calibration curve was constructed by plotting peak area ratio (y) of digoxin to the internal standard versus digoxin concentrations (x). The regression parameters of slope, intercept and correlation coefficient were calculated by weighted ($1/x$) linear regression in Analyst 1.1 software used in Sciex API3000. The concentrations of calibration standards, analyzed in duplicate, were then back calculated. Linearity was evaluated by comparing the correlation coefficient (r^2), residuals and errors between theoretical and back-calculated concentrations of calibration standard samples.

2.7.2. Lower limit of quantitation

The lower limit of quantitation (LLQ) was evaluated by spiking digoxin at a concentration of 0.1 ng/ml with six different lots of drug-free rat plasma and assaying them as unknown samples against the standard curve.

2.7.3. Intra-assay and inter-assay precision and accuracy

Precision and accuracy were evaluated by determining the digoxin concentration in five replicates of QC samples at five different concentrations and one level of dilution QC samples daily for 3 separate days. QC samples at twice the upper limit of the standard curve (dilution QC samples at 200 ng/ml) were assayed to ensure that dilution of study samples did not affect accuracy and precision. The dilution QC samples were diluted 4-fold with rat K_3EDTA plasma prior to analysis and processed as other QC samples. Each run consisted of calibration standards in duplicate, QC and dilution QC samples in five replicates and blank plasma samples with and without internal standard in duplicate, The analysis was run daily for 3 separate days to evaluate assay performance.

The accuracy of the assay was evaluated by percent deviation (DEV) from nominal concentration using the formula: $\%\text{DEV} = 100 \times$ (mean back calculated concentration − nominal concentration)/nominal concentration. Intra- and inter-assay precision were obtained by one-way analysis of variance (ANOVA) testing, reported as percent RSD for each QC. Acceptable accuracy and precision were $< 15\%$ DEV and $< 15\%$ RSD at every concentration studied except for the lower limit of quantitation (LLQ) where 20% DEV and 20% RSD were acceptable.

2.7.4. Specificity

Six blank plasma samples from six lots of rat plasma were processed with and without the internal standard to evaluate presence of interfering peaks.

2.7.5. Recovery

For the recovery study, digoxin and the internal standard were spiked before and after extraction to account for potential matrix effects on ionization efficiency in electrospray. The recovery of digoxin from plasma was evaluated at three different concentrations in triplicate at 5, 25 and 50 ng/ml. The stock solution of digoxin was prepared in 40% acetonitrile in 5 mM of ammonium formate ($\text{pH} = 3.4$) and serially diluted to three different concentrations and used for spiking. The recovery of the internal standard was evaluated using a concentration of 500 ng/ml in acetonitrile, the same concentration as used in the assay.

2.7.6. Stability

Bench-top stability was assessed by leaving the QC samples at two different concentrations at room temperature for 4 h. Freeze/thaw stability was assessed over three cycles. QC samples at two different concentrations were thawed at room temperature and refrozen at −20 °C over three cycles and assayed. The stability of reconstituted samples in autosampler vials was assessed at ambient temperature for over 24 h. The freezer storage stability of digoxin in plasma at −20 °C was evaluated by assaying QC samples on day 0 and week 4. Freshly processed standard samples were used to quantitate all the QC samples. All stability QC samples were analyzed in triplicate.

2.7.7. Animal study

Male Sprague–Dawley rats (body weight 200–250 g) were obtained from Charles River Laboratory (Wilmington, MA). Rats were dosed at 0.2 mg/kg and the dosing vehicle consisted of 40% ethanol and 60% water. A minimum of 40% ethanol was needed to provide a clear dosing solution of digoxin. The final dosing volume was 5 ml/kg for oral containing 0.04 mg/ml digoxin (via oral gavage). The amount of ethanol given did not produce any abnormal behavior in these rats. The samples were collected for 10 h after the dose. EDTA was used as an anticoagulant and ≈0.2 ml aliquots of blood samples were collected at each time point. Plasma samples were obtained by centrifugation at 4 °C and 3000 rpm for 15 min and stored at −20 °C prior to analysis.

3. Results and discussion

3.1. Chromatography and specificity

Under optimized HPLC and MS conditions, digoxin and the internal standard were baseline separated with the retention times of 0.86 and 2.37 min, respectively (Fig. 4). Since no late-eluting peaks were observed, regeneration of the column using a gradient elution step was not necessary. The total run time was 4 min and much shorter than previously published methods. Blank rat plasma from six lots showed no significant interfering peaks at the retention times of digoxin and the internal standard (Fig. 4). Since plasma samples were precipitated with acetonitrile and simultaneously extracted with methylene chloride, the background was very low and reconstituted samples did not cause any type of tubing or needle sprayer clog due to non-precipitated proteins [14].

3.2. Calibration curve, linearity and LLQ

The calibration curve was linear over the concentration range of 0.1–100 ng/ml of digoxin in rat plasma with correlation coefficients $r^2 \geq 0.999$ and consistent slope values when evaluated by weighed $(1/x)$ linear regression. Residuals were randomly distributed when plotted against concentration. Table 1 shows the results of calibration accuracy in the 3 day validation study. The calibration curves were accurate with $<7.2\%$ deviation from the nominal values and precision was within 8.1%. At a digoxin plasma concentration of 0.1 ng/ml, the accuracy was within 3.7% and the precision was within 7.0%. Therefore, the LLQ of digoxin assay in rat plasma was established at 0.1 ng/ml. A typical chromatogram of an LLQ sample is shown in Fig. 4.

3.3. Accuracy and precision

Table 2 shows the within- and between-assay accuracy and precision data. The method was found to be highly accurate with $<5.8\%$ deviation from the nominal values and highly precise with between-run precision $<3.4\%$ and within-run precision $<8.6\%$ at each concentration of QC sample tested.

3.4. Recovery

To account for the effect of the plasma matrix on ionization efficiency in the ion spray, digoxin and the internal standard were spiked in blank rat plasma before and after sample extraction. The recoveries of digoxin and the internal standard were consistent at $82.7 \pm 3.9\%$ and $105.9 \pm 2.3\%$ across the tested range.

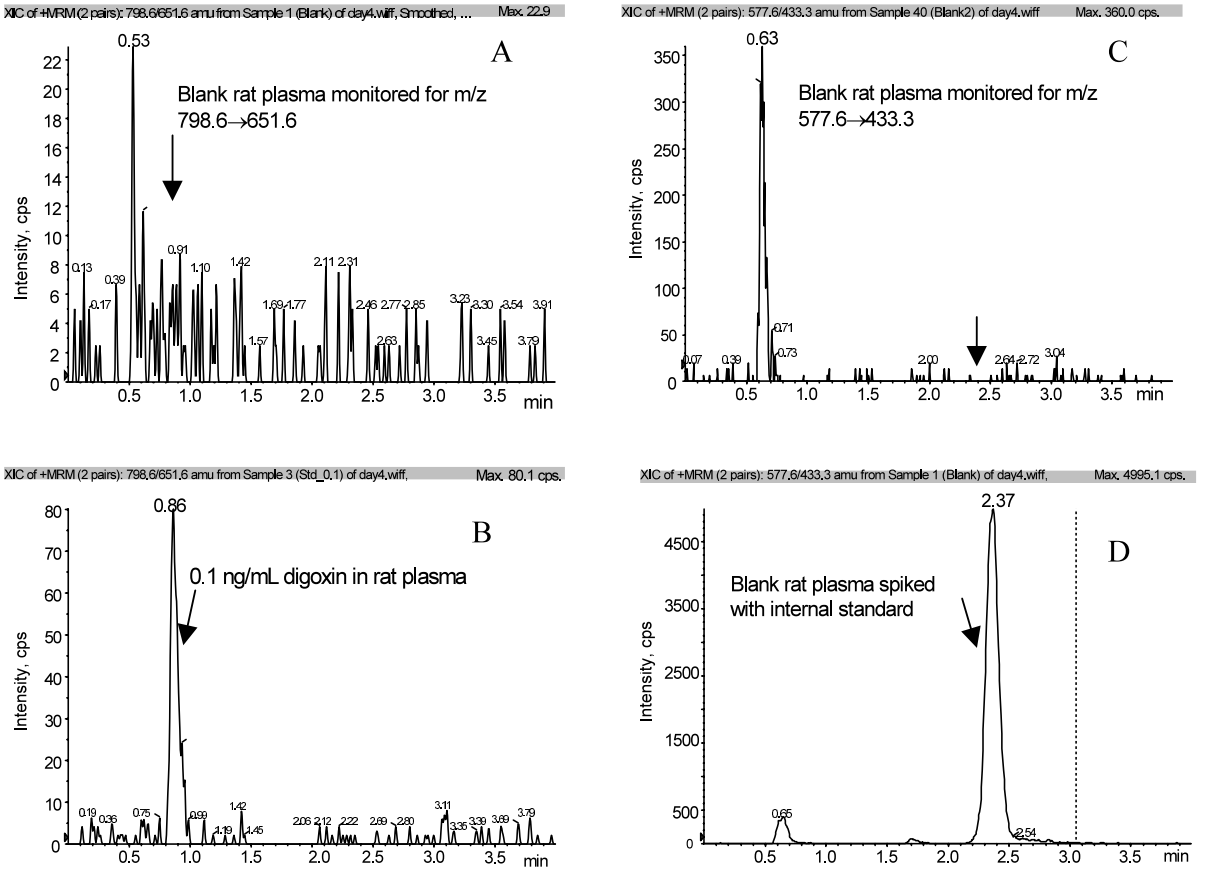

Fig. 4. Respective selective reaction monitoring chromatograms of rat plasma at *m/z* 798.6→651.6 or *m/z* 577.6→433.3 obtained from (A) blank rat K_3EDTA plasma containing 500 ng/ml IS; (B) rat K_3EDTA plasma containing digoxin at lower limit of quantitation of 0.1 ng/ml and IS at 500 ng/ml; (C) blank rat K_3EDTA plasma without IS; (D) blank rat K_3EDTA plasma containing IS at 500 ng/ml. (Arrow indicates the position of digoxin or IS peak.)

Table 1
Deviation from the nominal concentration (DEV) and relative standard deviation (RSD) of calibration standards of digoxin in rat plasma from three validation runs

Nominal concentration (ng/ml)	Mean observed concentration (ng/ml)	DEV (%)	RSD (%)
0.100	0.103	3.0	8.1
0.500	0.536	7.2	4.6
2.00	1.91	−4.8	6.1
5.00	4.85	−3.1	2.3
25.0	23.6	−5.5	4.3
50.0	51.0	1.9	7.2
100	100.7	0.7	3.4

Table 2
Accuracy and precision of digoxin assay in rat plasma

	Nominal concentration (ng/ml)				
	0.300	5.00	20.0	80.0	200.0*
Accuracy (n = 15)					
Mean observed concentration (ng/ml)	0.295	4.83	21.2	82.8	201.9
(DEV %)	−1.60	−3.49	5.77	3.53	0.97
Precision					
Inter-assay (RSD %, $n = 15$)	2.20	0.00	3.37	1.57	3.05
Intra-assay (RSD %, $n = 15$)	8.62	4.22	6.50	1.37	7.19

* Dilution QC samples with dilution factor of 4.

Table 3
Stability of digoxin in rat K_3EDTA plasma

Sample condition	Nominal concentration (ng/ml)			
	5.00		80.0	
	Observed	% DEV	Observed	% DEV
Freshly prepared	4.83	−3.49	82.8	3.5
4 h at room temperature	4.43	−11.3	70.6	−11.7
Autosampler 24 h stability	4.57	−8.7	83.3	4.2
4 weeks at −20 °C	4.70	−5.9	77.2	−3.6
Freeze/thaw cycle No. 1	4.68	−6.4	71.2	−11.0
Freeze/thaw cycle No. 2	4.72	−5.6	78.3	−2.2
Freeze/thaw cycle No. 3	4.57	−8.3	71.8	−10.3

All QC samples were analyzed in triplicate. Mean values are reported.

3.5. Stability

No significant loss of digoxin ($\leq 11.7\%$) was observed after storage of plasma at room temperature on the bench-top for at least 4 h (Table 3). Processed samples were stable up to 24 h in the autosampler tray (Table 3). Plasma samples were stable at −20 °C for at least 4 weeks with no significant loss ($\leq 5.9\%$, Table 3). Plasma samples were stable over at least three freeze/thaw cycles (Table 3).

3.6. Application

The method was successfully applied to the determination of digoxin plasma concentration levels in rats following oral administration of a single 0.2 mg/kg dose. From our MRM chromatograms of plasma obtained from pre and post dose rats, it is shown that no significant interfering peaks were detected at the retention times of peaks of interest, as well as in the ion channel, in the

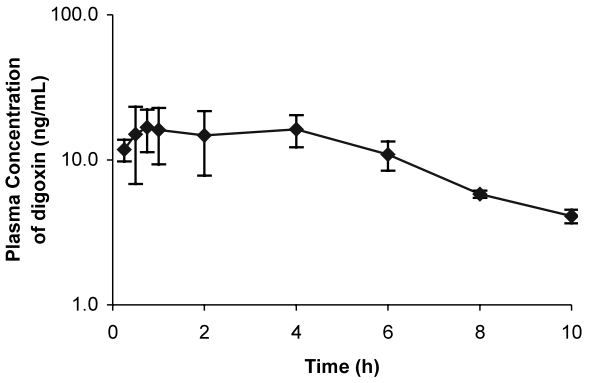

Fig. 5. Mean plasma concentrations versus time profile of digoxin in rats ($n = 3$) following a single oral dose of 0.2 mg/kg of digoxin.

predose samples. The mean (S.D.) ($n = 3$) plasma concentration versus time profile for digoxin is depicted in Fig. 5. C_{max}, AUC_{0-t}, T_{max} of digoxin was 21.7 ± 1.8 ng/ml, 120.1 ± 13.1 ng/ml*h and 2.17 h, respectively.

4. Conclusions

An LC-MS/MS assay for the quantitation of digoxin plasma concentration was developed and validated. The new assay was rapid, sensitive, specific, accurate and reproducible. In addition, the assay required very small volume of plasma, which allows serial sampling in small laboratory animals. The new method was successfully applied to the characterization of pharmacokinetics of digoxin in rats after oral administration.

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