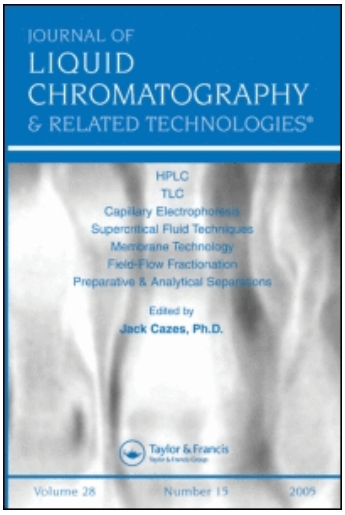

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Rapid Quantitation of Digoxin in Human Plasma and Urine Using Isotope Dilution Liquid Chromatography-Tandem Mass Spectrometry

Arnaud Salvador[a]; Cyriaque Sagan[b]; Jannick Denouel[c]
[a] Université Claude Bernard Lyon 1, Villeurbanne cedex, France [b] Novartis Pharma S.A.S, Rueil-Malmaison, France [c] CEPHAC Europe, Saint Benoît, France

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Rapid Quantitation of Digoxin in Human Plasma and Urine Using Isotope Dilution Liquid Chromatography-Tandem Mass Spectrometry

Arnaud Salvador
Université Claude Bernard Lyon 1, Villeurbanne cedex, France

Cyriaque Sagan
Novartis Pharma S.A.S, Rueil-Malmaison, France

Jannick Denouel
CEPHAC Europe, Saint Benoît, France

Abstract: A rapid LC/MS/MS assay to quantitate digoxin in human plasma and urine has been developed and validated using a Packard liquid handling systems-Multiprobe® II AMP8E1. Following automatic solid phase extraction on Waters Oasis HLB 30 mg 96 plates, digoxin and its internal standard (Digoxin D3) were analyzed by reverse phase chromatography (LUNA C_{18}, 5 μm, 150 × 2 mm), and introduced into the mass spectrometer (Sciex - API 3000) via the turboIonspray ion source operating in positive mode. The assay was validated for human plasma and human urine over a concentration range of 0.2–20 ng/mL and 1–100 ng/mL, respectively, using 0.5 mL of sample. The between day and within day coefficients of variation for all matrices were $<17\%$ at the concentrations of lower limit of quantification (LLOQ), and $<13\%$ at other quality control concentrations. The average recovery was 80.3% from plasma and 74.3% from urine. No matrix effect was observed. Freeze-thaw stability, stability of digoxin in matrix, and stability of extracted samples were also evaluated. The mean concentration after three freeze-thaw cycles was within $\pm 3.2\%$ of the nominal value in plasma and

Address correspondence to Arnaud Salvador, Université Claude Bernard Lyon 1, UMR 5180, 43 Boulevard du 11 novembre 1918, 69622, Villeurbanne cedex, France. E-mail: arnaud.salvador@univ-lyon1.fr

within ± 11.4% of the nominal value in urine. After storage for 4 hours at room temperature, greater than 97.2% of digoxin remained in plasma and 96% remained in urine. Digoxin in extract was stable in the autosampler at +5°C for 77 hours in plasma and 80 hours in urine.

Keywords: Digoxin, Mass spectrometry, Human plasma, Human urine, High performance liquid chromatography

INTRODUCTION

Digitalis glycosides are among the most useful groups of drugs in therapeutics. Cardiotonic glycosides have been used for more than 200 years for the treatment of congestive heart failure, with digoxin being one of the most frequently prescribed. Digoxin (see Figure 1) is obtained from the leaves of Digitalis lanata. It has positive inotropic and negative dromotropic actions. In cardiac failure, the positive inotropic effect results in increased cardiac output, decreased end systolic volume, decreased heart size, and

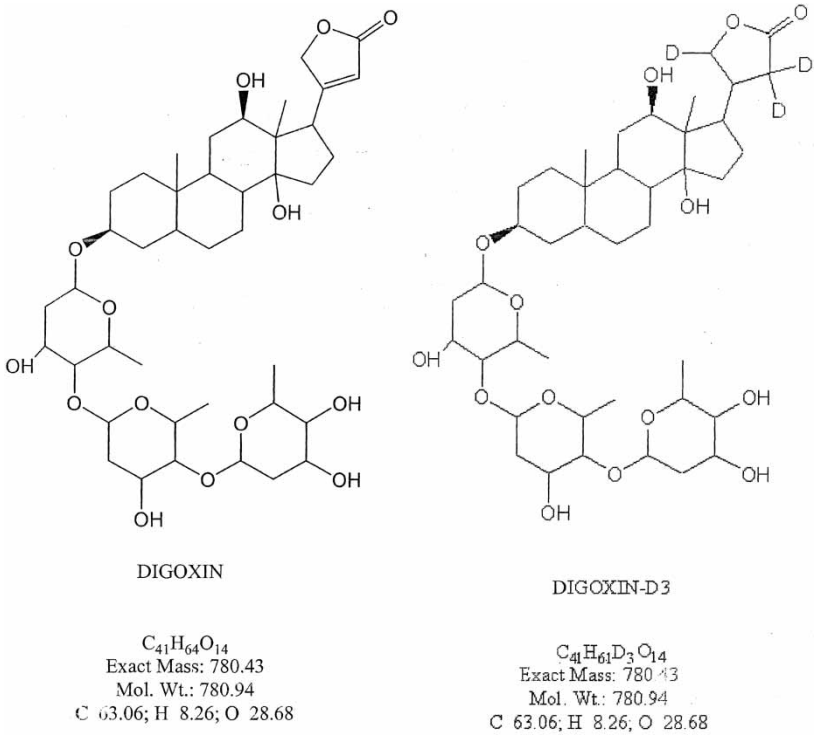

Figure 1. Structure of Digoxin and Digoxin-D3 (Internal standard).

decreased end diastolic pressure and volume. Digoxin is also given to slow the ventricular rate in the management of atrial fibrillation. Digoxin commonly produces side effects because the margin between therapeutic and toxic doses is narrow.[1,2] In general, the adverse reactions of digoxin are dose dependent and occur at doses higher than those needed to achieve a therapeutic effect. Due to the low therapeutic index of digoxin, dosing is important, therefore, accurate measurement of digoxin concentrations is required.

Determination of digoxin in plasma or urine is commonly performed by immunoassay due to its sensitivity, speed, and low costs; however, immunoassays have been reported to be subject to cross reactivity with the active and inactive metabolites of digoxin.[3–6] The combination of immunoassay with a separation technique, such as chromatography, can result in enhanced selectivity and sensitivity.[6–11] Quantification of digoxin can also be performed by liquid chromatography with ultraviolet (UV) or fluorescence detection.[12] However, due to a low extinction coefficient, UV is not sensitive enough, and, thus, requires a fluorescence derivatization of the samples.[11,13]Thanks to its high sensitivity and specificity, liquid chromatography coupled to mass spectrometry (LC-MS/MS) is a good alternative for the detection of digoxin or cardiac glycosides in biological samples.[14–17]The currently published LC-MS-MS methods use manual extraction and involve a relatively long analysis time. For the measurement of digoxin levels in clinical studies, a rapid method is essential, in order to perform the analysis of hundreds of samples per day. The aim of the present study was to develop a rapid LC/MS/MS assay with a labeled internal standard to quantitate digoxin in human plasma and urine.

EXPERIMENTAL

Chemicals

Digoxin was provided by Sigma (St. Louis, Mo, USA) (purity 98.2%); internal standard (Digoxin-D3) was provided by ArtMolecule (Poitiers, France) (purity 99%). All reagents were of analytical grade. Acetonitrile and ammonium acetate were obtained from J.T. Baker Co (Phillipsburg, NJ, USA). Methanol was obtained from Carlo Erba (Val de Reuil, France). Buffer solution pH 6 was purchased from SDS (Valdonne, France). Distilled water was purified using a Millipore system Milli Q (Molsheim, France).

Chromatography

The HPLC system consisted of a Agilent (Massy, France) 1100 series pump and a HTS Pal autosampler from CTC analytics AG (Zwingen, Switzerland),

with the sample cooler set at 10°C. Chromatographic separations were performed on a 5 μm Luna C_{18} column, 150 mm × 2 mm i.d. (Phenomenex, Macclesfield Cheshire, England), operated at 30°C, with a flow rate of 250 μL/min. An isocratic elution method was used with ammonium acetate solution 5 mM/acetonitrile (60:40, v/v).

Mass Spectrometry

A Sciex API 3000 triple quadrupole mass spectrometer (MDS Sciex, Toronto, Canada) was coupled to the HPLC system through a Sciex TurboIonSpray source (TIS) operated in positive mode. Instrument control, data acquisition, and processing were performed using the associated Analyst 1.1 sofware. The mass spectrometer was initially calibrated using polypropylene glycol as standard (Applied Biosystems, Foster City, CA, USA), setting the resolution as peak width at half height, in the range 0.7 ± 0.1 amu.

The nebulizer and the curtain gas flows (nitrogen) were set at 5 and 8 arbitrary units (AU), respectively. The TurboIonSpray source was operated at 400°C with the auxiliary gas flow (nitrogen) set at 8 L/min. The TurboIonSpray voltage was set at 4500 V and the orifice voltage and ring voltages at 16 and 340 V. Multiple reaction monitoring (MRM) experiments in the positive ionization mode were performed to detect ion transitions at m/z: 798.5/651.4 $[M + NH_4]^+$ (Digoxin) and 801.6/654.5 $[M + NH_4]^+$ for internal standard (Digoxin-D3), with a dwell time of 400 ms per transition. Product ions used for monitoring were selected based on their significance in the MS/MS spectra. The collision energy (19eV) was optimized, using the autotune feature of the software.

Standards and Quality Control Samples

Two stock solutions of digoxin were prepared independently by dissolving accurately weighed standard compounds in methanol to yield a concentration of 200 μg/mL. One solution was used to spike plasma and urine calibration samples, and the other was used to prepare the quality control (QC) samples. Both stock solutions were diluted further with methanol/water (50:50, v/v).

For plasma, the preparation of calibration standards, quality control samples, validation, and tests samples were performed using human plasma with lithium heparin as anticoagulant. A 25 μL volume of digoxin spiking solution was added to 500 μL of plasma and urine. In plasma, the calibration standard concentrations (at seven levels) of digoxin ranged from 0.200 to 20.0 ng/mL, and quality control (QC) samples were prepared at 0.200, 0.500, 10.0, and 16.0 ng/mL. In urine, the calibration standard concentrations

(at seven levels) of digoxin ranged from 1.00 to 100 ng/mL, and quality control (QC) samples were prepared at 1.00, 3.00, 50.0, and 80.0 ng/mL.

Extraction

Extraction was performed using a Packard liquid handling system Multiprobe® II AMP8E1 (Perkin Elmer, Torrance, CA, USA). For plasma and urine, the samples were thawed at room temperature and vortexed, then centrifuged for 5 minutes at 3500 rpm at approximately +4°C. To 500 μL of the sample, 50 μL of internal standard solution (100 ng/mL for plasma and 500 ng/mL for urine) was added, followed by 450 μL of buffer solution pH 6. The sample was then loaded (900 μL) onto an Oasis HLB 30 mg 96 well plate (Waters, Milford, MA, USA), preconditioned with 1 mL of methanol and 1 mL of water. Following rinsing with 1 mL of methanol/water (40:60, v/v), analytes were eluted with 1 mL of methanol in a 2 mL 96 deep well plate. The eluate was then evaporated to dryness under a gentle stream of nitrogen at approximately +40°C. The residue was dissolved in 100 μL and 500 μL of mobile phase for plasma and urine, respectively. An aliquot of 20 μL was injected into the LC/MS/MS system.

Validation Procedures

The validation experiments were designed with the reference to "Guidance for Industry-Bioanalytical Method Validation" recommended by the Food and Drug Administration (FDA) of the United States.[18] To evaluate the precision and accuracy of the method, four runs were performed on four separate days. Each run consisted of two sets of calibration standards, six replicates of each QC concentration for the intra-batch or two replicates of QC concentration for inter-batches, evaluation samples, blanks, and a zero (blank + internal standard). The stability of the samples under various conditions was evaluated as a part of the validation, using QC samples ($n = 6$) prepared at the low and the high concentrations of 0.500 and 16.0 ng/mL in plasma and 3.00 and 80.0 ng/mL in human. The parameters assessed during method validation are discussed in the following sections.

RESULTS AND DISCUSSION

Sample Preparation Method

Dogoxin is relatively hydrophobic, which allows for good extraction using either liquid-liquid or solid phase extraction (SPE) techniques. A previously described[12,14] single step, liquid-liquid extraction method using a mixture

of chloroform:2-propanol according was not retained, as it is more difficult to automate than SPE. Thus, HLB30 mg 96 plates were used for sample extraction. In order to clean the samples, the proportion of methanol used for the washing step was optimized. It was shown that digoxin remained adsorbed on the SPE support until washed with a methanol:water mixture (v/v) containing at least 60% of methanol, and it is completely eluted using pure methanol. It was, therefore, decided to wash the extraction plate with a methanol:water (40:60, v/v) mixture, and elute the compounds with pure methanol.

Liquid Chromatography and Mass Spectrometry

The use of a labelled compound instead of structural homologue such as oleandrin[14] or methyldigoxin[17] allowed a fast isocratic separation without a long equilibration time between injections. A C_{18} Luna column was chosen based on the resulting good chromatographic peak shape and short retention time. A typical high throughput method has an analysis time of under 2 minutes, with peaks eluting to the void volume of the column. For this case, in order to avoid experiencing an unfavourable matrix effect, it was preferable to have the compound elute after 2.5 minutes, with a total run time of 5 minutes. There conditions allow for the analysis of approximately 288 samples per day (~1500 samples per week), which corresponds to three 96 well plates per day.

The electrospray ionisation (ESI) mass spectra obtained by infusion of digoxin in methanol/water (50:50, v/v) via a tee junction with mobile phase, with and without ammonium acetate, are shown in Figure 2. Without ammonium acetate, the ammonium, sodium, and potassium adducts of the molecular ion were observed for digoxin (m/z 798, m/z 803, and m/z 819, respectively). Protonation, which generally represents the main ionization process in ESI, was not observed, even with addition of acetic acid or formic acid in the mobile phase. Increasing or decreasing the declustering potential also failed to generate the proton adduct. Fragmentation of ammonium and sodium adducts are shown in Figures 2c and 2d. No dominating daughter ion for the sodium adduct was observed even at high collision energy.

By contrast, the product ion mass spectra of the digoxin ammonium adduct showed a fragment ion at m/z 651 as a base peak ion, corresponding to a sequential loss of one digitoxose and a protonated ion at m/z 781. Subsequent collision of the base peak led to sequential losses of the glycoside moieties by cleavage of the ether bonds between the glycosidic oxygen and the anomeric carbon, to give a series of ions at m/z 521, m/z 391. The ammonium adducts ions were selected as the precursor ions, and the most intense ion at m/z 651 was selected as the product ion to be monitored. In order to assure robustness and reproducibility of the method, ammonium acetate was added to the mobile phase. Under these conditions, only the ammonium adduct is produced.

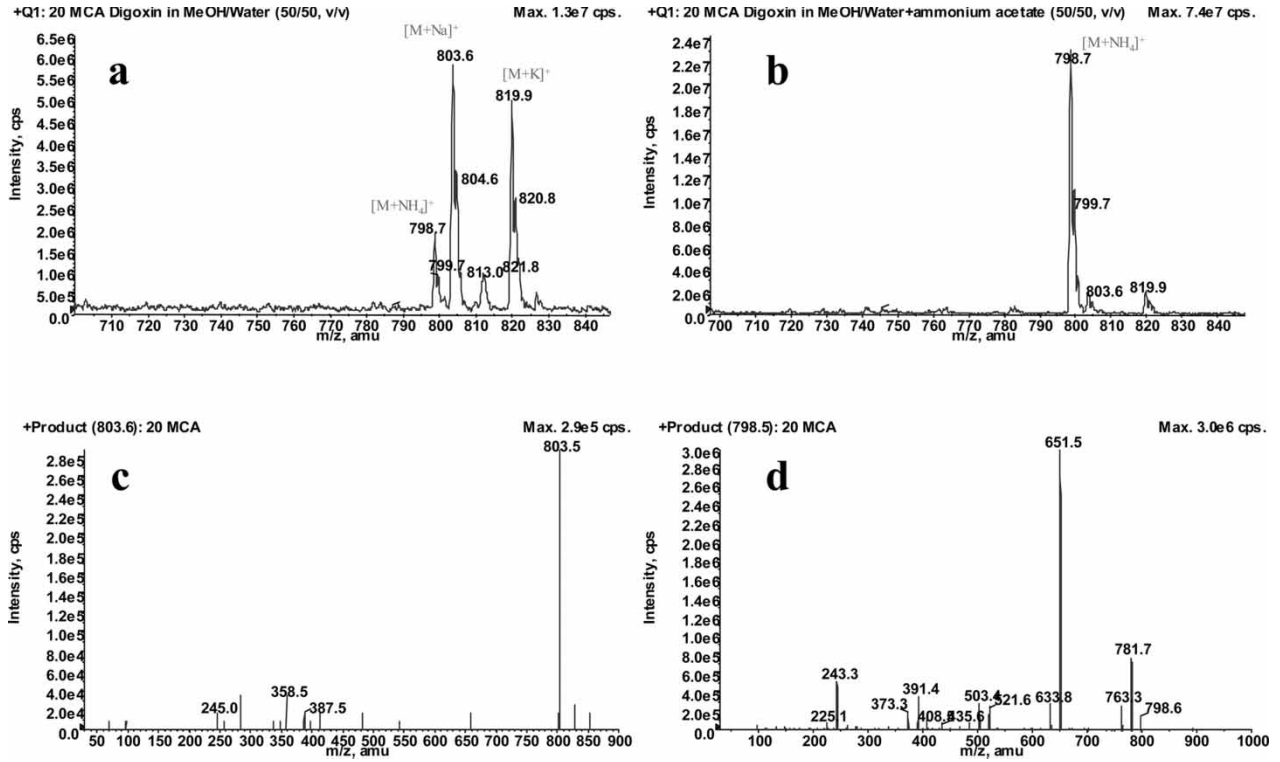

Figure 2. Mass spectra of digoxin: (a) Positive Q1 full scan in MeOH/water (50/50, v/v), (b) Positive Q1 Full scan in MeOH/Water + Ammonium acetate (50/50, v/v), (c) product ions scan of [Digoxin + $Na]^+$ adduct, (d) product ions scan of [Digoxin + $NH_4]^+$ adduct.

Linearity

A weighted $(1/x^2)$ least-squares linear regression of response vs. concentration was used for the calibration. A high degree of correlation in plasma was demonstrated in the range of 0.200 to 20.0 ng/mL, with typical correlation greater than 0.99, an average slope of 0.11684 ± 0.0082146 (mean $\pm$ SD, $n = 5$), and a precision of 7.0%. In urine, a high degree of correlation was also demonstrated in the range 1.00 to 100 ng/mL, with typical correlations greater than 0.99, an average slope of 0.021658 ± 0.00071639 (mean $\pm$ SD, $n = 5$), and a precision of 3.3%.

Precision and Accuracy

Six replicates of the QC samples at each concentration level were used to evaluate the intra-day precision and accuracy. Two replicates of the QC samples at each concentration level from three separate batches were used to evaluate the inter-day precision and accuracy. The intra-day mean accuracy in plasma and in urine was between −0.20% and 9.00% and between −0.33% and 8.60%, respectively (Table 1). The inter-day precision was between 1.78% and 9.58% (16.79% at the LLOQ level) and between 1.95%and 3.63%, for plasma and urine, respectively.

Recovery

The extraction recovery of digoxin from the two different matrices was determined at three QC levels, by comparing the peak area ratios of digoxin to internal standard in samples that had been spiked with analyte prior to extraction with samples to which the analyte was added post-extraction. The internal standard was added to both sets of samples post-extraction. The extraction recoveries of digoxin from human plasma and urine were greater than 77% and 73%, respectively (Table 1). The dependence on concentration was negligible.

Matrix Effect and Selectivity

Due to the nature of electrospray ionization, matrix components eluting from the HPLC column into the mass spectrometer at the same time as the analyte and/or internal standard may affect the ionization of the compound of interest. This effect may be seen as either suppression or an enhancement of analyte and/or internal standard response, even if the matrix component is not present in the MRM channel monitored for the analytes or internal standard. If a matrix effect is observed, low concentrations will be more affected than higher concentrations. The matrix effect was evaluated by spiking blank

Table 1. Intra- and inter-day precision and accuracy in plasma and urine

	Intra-day variation (n = 6)			Inter-day variation (n = 6)			
Nominal concentration (ng/mL)	Determined concentration (Mean ± S.D.)	Precision (%)[a]	Accuracy (%)[b]	Determined concentration (Mean ± S.D.)	Precision (%)[a]	Accuracy (%)[b]	Recovery (n = 6) (%)[c]
Plasma							
0.200	0.218 ± 0.0151	6.93	9.00	0.224 ± 0.0376	16.79	12.00	
0.500	0.520 ± 0.0231	4.44	4.00	0.505 ± 0.0484	9.58	1.00	77.83
10.0	9.98 ± 0.164	1.64	−0.20	9.45 ± 0.405	4.29	−5.50	81.39
16.0	16.3 ± 0.242	1.48	1.88	15.7 ± 0.280	1.78	−1.88	81.57
Urine							
1.00	1.06 ± 0.0256	2.42	6.00	0.850 ± 0.0243	2.86	−15.00	
3.00	2.99 ± 0.0565	1.89	−0.33	2.75 ± 0.0729	2.65	−8.33	74.97
50.0	54.3 ± 1.21	2.23	8.60	43.5 ± 1.58	3.63	−13.00	74.28
80.0	80.7 ± 2.04	2.53	0.88	73.3 ± 1.43	1.95	−8.38	73.61

[a]Expressed as R.S.D.: (S.D./mean) × 100.

[b]Expressed as % difference: [(concentration found−concentration added)/concentration added] × 100.

[c]Expressed as mean peak area ratio of extracted samples/mean peak area ratio of the unextracted samples.

human matrix from 6 different individuals before extraction at the lower limit of quantification (LLOQ), and at the concentration used for the internal standard (n = 6). No matrix effect was observed in plasma or urine, as the accuracies are within ± 12.5% of the nominal concentration for each matrix (Table 2). Assay selectivity was assessed by analysing drug free pooled plasma and urine from six individual humans, and examining for peaks that interfered with digoxin and the internal standard. There were no chromatographically interfering peaks observed at the retention times of either digoxin or the internal standard in the samples, as shown by the double blank chromatograms in Figures 3 and 4.

Stability

The stability of digoxin in plasma and urine under various conditions was evaluated by comparing the concentrations of low and high stability QC samples to their respective nominal concentrations. The stability experiments were aimed at testing all possible conditions that the samples might

Table 2. Matrix effect of digoxin in plasma and urine

	Matrix effect (n = 6)		
Nominal concentration (ng/mL)	Determined concentration (Mean ± S.D.)	Precision (%)[a]	Accuracy (%)[b]
Plasma			
Matrix 1	0.191 ± 0.00462	2.42	−4.50
Matrix 2	0.202 ± 0.0194	9.60	1.00
Matrix 3	0.208 ± 0.0298	14.33	4.00
Matrix 4	0.225 ± 0.0515	22.89	12.50
Matrix 5	0.183 ± 0.00582	3.18	−8.50
Matrix 6	0.197 ± 0.0151	7.66	−1.50
Mean of matrix	0.201 ± 0.0146	7.26	0.50
Urine			
Matrix 1	0.998 ± 0.0366	3.70	−1.20
Matrix 2	0.984 ± 0.0434	4.41	−1.60
Matrix 3	0.976 ± 0.0261	2.67	−2.40
Matrix 4	0.974 ± 0.0357	3.67	−2.60
Matrix 5	0.984 ± 0.0667	6.78	−1.60
Matrix 6	0.958 ± 0.0327	3.41	−4.20
Mean of matrix	0.979 ± 0.0133	1.36	−2.1

[a]Expressed as R.S.D.: (S.D./mean) × 100.

[b]Expressed as % difference:[(concentration found−concentration added)/concentration added] × 100.

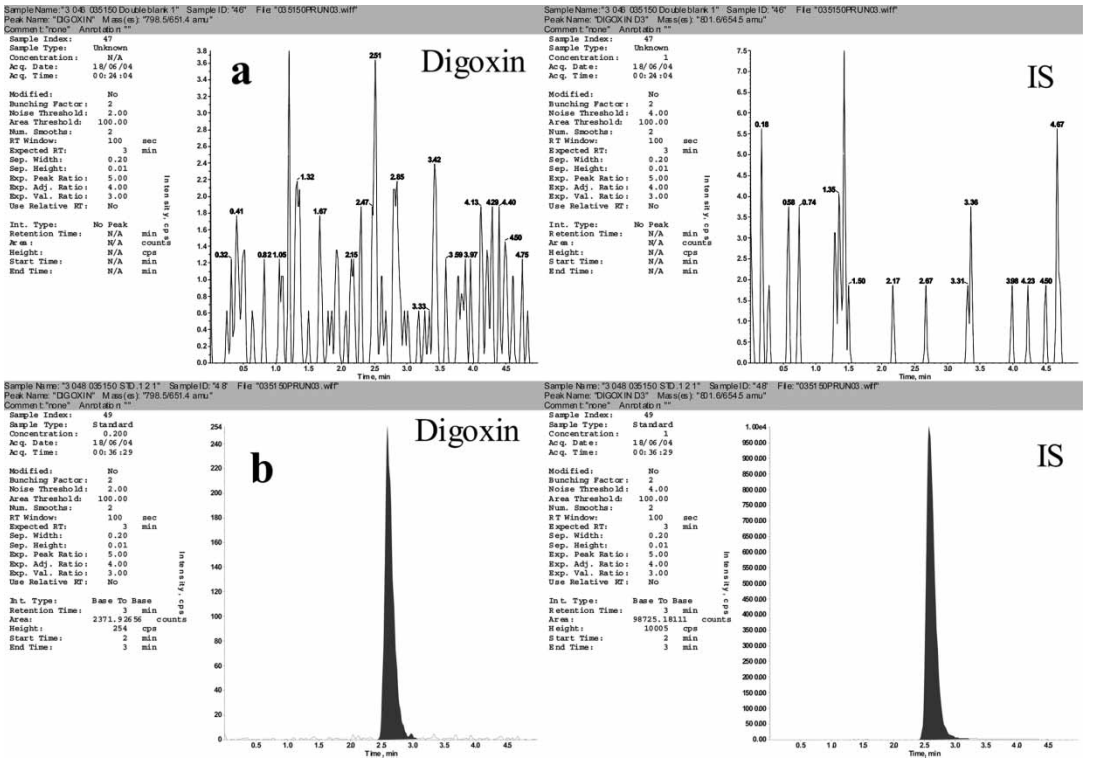

Figure 3. Representative chromatograms from the method validation in human plasma: (a) a double blank, (b) an LLOQ at 0.200 ng/mL. The following MRM transitions were monitored for digoxin and the internal standard (m/z 798.5 to m/z 651 and m/z 801.6 to m/z 654.5).

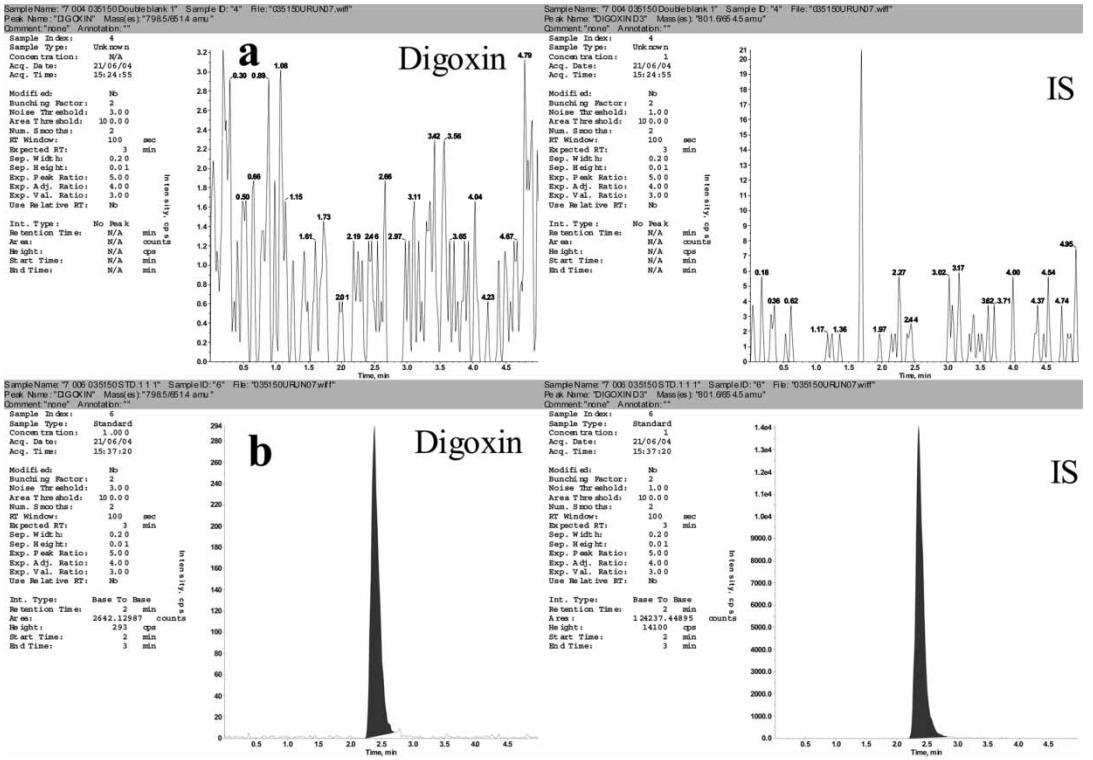

Figure 4. Representative chromatograms from the method validation in human urine: (a) a double blank, (b) an LLOQ at 0.200 ng/mL. The following MRM transitions were monitored for digoxin and the internal standard (m/z 798.5 to m/z 651 and m/z 801.6 to m/z 54.5).

experience between collection and to analysis. Stability results are summarized in Table 3. Three freeze thaw cycles and 4 h room temperature storage for low and high quality controls samples indicated that digoxin was stable in human plasma and urine under these conditions. QC stability samples were stable when stored frozen at −75°C for at least 41 days in plasma and 36 days in urine. Testing of autosampler stability, of quality control extract samples, indicated that digoxin was stable when kept in the autosampler for up to 77 hours for plasma and urine.

Table 3. Stability of digoxin in plasma and urine

Nominal concentration (ng/mL)	Determined concentration (Mean[a] ± S.D.)	Precision (%)[b]	Accuracy (%)[c]
Freeze-thaw stability			
Plasma			
0.500	0.484 ± 0.0112	2.31	−3.20
16.00	16.2 ± 0.163	1.01	1.25
Urine			
3.00	2.66 ± 0.0757	2.85	−11.33
80.0	76.3 ± 1.26	1.65	−4.63
Short-term stability (4 h at room temperature)			
Plasma			
0.500	0.486 ± 0.0106	2.18	−2.80
16.00	16.5 ± 0.458	2.78	3.13
Urine			
3.00	2.88 ± 0.114	3.96	−4.00
80.0	80.6 ± 1.82	2.26	0.75
Stability of extract			
Plasma (77 hours)			
0.500	0.510 ± 0.0292	5.73	2.00
16.00	16.9 ± 0.423	2.50	5.63
Urine (80 hours)			
3.00	3.04 ± 0.0601	1.98	1.33
80.0	80.4 ± 1.48	1.84	0.50
Long-term stability at −25°C			
Plasma (41 days)			
0.500	0.518 ± 0.0213	4.11	3.60
16.00	17.0 ± 0.245	1.44	6.25
Urine (36 days)			
3.00	2.87 ± 0.111	3.87	−4.33
80.0	77.8 ± 1.56	2.01	−2.75

[a] n = 6.

[b] Expressed as R.S.D.: (S.D./mean) × 100.

[c] Expressed as % difference:[(concentration found−concentration added)/concentration added] × 100.

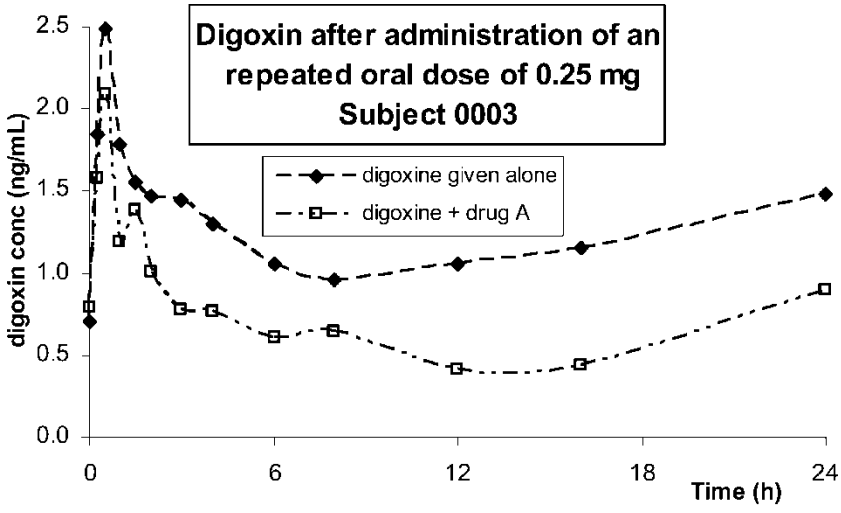

Figure 5. Mean plasma concentration profiles of digoxin after a single oral administration of 0.25 mg digoxin or single oral co-administration of 0.25 mg digoxin and 300 mg drug A.

Application

The method presented here was successfully used to quantify digoxin in plasma samples from a clinical interaction drug study. Figure 5 shows mean concentration plots for digoxin in plasma following 0.25 mg of digoxin taken orally once daily, or 0.25 mg of digoxin and 300 mg of drug A taken orally once daily. The validated LC-MS/MS methods was used to support clinical interactions drugs studies in human plasma and urine, with up to 250 samples analysed per day.

CONCLUSIONS

A sensitive LC-MS/MS method for the quantitative determination of digoxin using SPE 96 plates was validated in both human plasma and urine. The calibration curves showed goodness of fit over the concentration range of 0.200–20.0 ng/mL in plasma and 1.00 to 100 ng/mL in urine. Within-and between- run precision and accuracy for calibration standards and QCs met FDA acceptance criteria for bioanalytical method validations. Digoxin was shown to be stable in plasma and urine during typical sample storage conditions. The validated LC-MS/MS method was used to support clinical studies in human plasma and urine.

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REFERENCES

1. Scherrmann, J.M.; Venet, R.; Menin, Y.; Bourdon, R. Optimization of the dosage of digoxin: Proposition of a minimal exploratory protocol–optimisation de la posologie de la digoxine proposition d'un protocole exploratoire minimal. Therapie **1985**, *40* (2), 73–79.
2. Godfraind, T. In *Pharmacologie des Concepts Fondamentaux aux applications Thérapeutiques*; Schorderet, M., Ed.; Frisons-Roche/Slatkine: Paris, 1989; 167–175.
3. Stoll, R.G.; Christensen, M.S.; Sakmar, E.; Wagner, J.G. The specificity of the digoxin radioimmunoassay procedure. Commun. Chem. Pathal. Pharmacol. **1972**, *4*, 503–510.
4. Stone, J.A.; Soldin, S.J. An update on digoxin. Clin. Chem. **1989**, *35*, 1326–1331.
5. Baselt, R.C.; Cravey, R.H. *Disposition of toxic Drugs and chemicals in Man*, 4th Ed.; Chemical Toxicology Institute: Foster City, 1995; 802–810.
6. Embree, L.; McErlane, K.M. Comparison of digoxin analysis by high-performance liquid chromatography/post-column derivatization and fluorescence polarization immunoassay. Xenobiotica **1990**, *20* (6), 635–643.
7. Freytag, J.W.; Lau, H.P.; Wadsley, J.J. Affinity-column-mediated immunoenzymometric assays: Influence of affinity-column ligand and valency of antibody-enzyme conjugates. Clin. Chem. **1984**, *30* (9), 1494–1498.
8. Reh, E.J. Determination of digoxin in serum by on-line immunoadsorptive clean-up high-performance liquid chromatographic separation and fluorescence-reaction detection. Chromatogr. B **1988**, *43*, 119–130.
9. Stone, J.A.; Soldin, S.J. Improved liquid chromatographic/immunoassay of digoxin in serum. Clin. Chem. **1988**, *34* (12), 2547–2551.
10. Embree, L.; McErlane, K.M. Development of a high-performance liquid chromatographic-post-column fluorogenic assay for digoxin in serum. J. Chromatogr. B **1989**, *496* (2), 321–334.
11. Graefe, K.A.; Tang, Z.; Karnes, H.T. High-performance liquid chromatography with on-line post-column immunoreaction detection of digoxin and its metabolites based on fluorescence energy transfer in the far-red spectral region. J. Chromatogr. B **2000**, *745* (2), 305–314.
12. Kwong, E.; McErlane, K.M. Analysis of digoxin at therapeutic concentrations using high-performance liquid chromatography with post-column derivatization. J. Chromatogr. **1986**, *381* (2), 357–363.
13. Tzou, M.C.; Sams, R.A.; Reuning, R.H. Specific and sensitive determination of digoxin and metabolites in human serum by high performance liquid chromatography with cyclodextrin solid-phase extraction and precolumn fluorescence derivatization. j. Pharm. Biomed. Anal. **1995**, *13* (12), 1531–1540.
14. Tracqui, A.; Kintz, P.; Ludes, B.; Mangin, P. High-performance liquid chromatography-ion spray mass spectrometry for the specific determination of digoxin and some related cardiac glycosides in human plasma. J. Chromatogr. B **1997**, *692* (1), 101–109.

15. Higashi, T.; Nakayama, N.; Shimada, K.; Kasai, H.; Nakazawa, H. High-performance liquid chromatography-tandem mass spectrometry of cardiac steroids. J. Liq. Chromatogr. & Rel. Technol. **1999**, *22* (15), 2283–2296.
16. Arao, T.; Fuke, C.; Takaesu, H.; Nakamoto, M.; Morinaga, Y.; Miyazaki, T. Simultaneous determination of cardenolides by sonic spray ionization liquid chromatography-ion trap mass spectrometry—A fatal case of oleander poisoning. J. Anal. Toxicol. **2002**, *26* (4), 222–227.
17. 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.
18. Food and Drug Administration of the United States, Guidance for industry-Bioanalytical Method Validation, US Department of Health and Human Services, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medecine (CVM), May 2001.

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