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Development and validation of an LC–MS method with electrospray ionization for quantitation of digoxin in human plasma and urine: Application to a pharmacokinetic study

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ABSTRACT

A highly sensitive and specific LC–MS method was developed and validated for the quantification of digoxin in human plasma and urine using β-dihydrodigoxin as internal standard (IS). The assay procedure involved extraction of digoxin and IS from human plasma with chloroform–isopropanol (95:5, v/v). Chromatogrphic separation was achieved on a Spherisorb ODS2 column using a gradient mobile phase with 5 mmol/L ammonium acetate in water with 1% acetic acid and acetonitrile. The mass spectrometer was operated in the selected ion monitoring mode using the respective $[M+K]^+$ ions, m/z 819.4 for digoxin and 826.4 for IS. The method was proved to be accurate and precise at linearity ranges of 0.12–19.60 ng/mL in plasma with a correlation coefficient (r^2) of ≥0.9968 and 1.2 ng/mL in urine. The limit of quantification achieved with this method was 0.12 ng/mL in plasma and 1.2 ng/mL in urine. The intra- and inter-assay precision and accuracy values were found to be within the assay variability limits as per the FDA guidelines. The developed assay method was successfully applied to a pharmacokinetic study in human volunteers following intravenous administration of digoxin.

1. Introduction

Cardiac glycosides (digoxin and digitoxin) have been used for more than 200 years for their positive inotropic effects and for treatment of heart failure, atrial fibrillation, atrial flutter, and paroxysmal atrial tachycardia [1]. Digoxin is by far the most prescribed cardiac glycoside in the world, however, it has narrow therapeutic margin and there are many factors that may increase the sensitivity of a patient to toxic side effects such as age, hypokalaemia, hypomagnesaemia, renal or liver failure and the dosing of other drugs [2,3]. Therefore, therapeutic drug monitoring for digoxin is essential in clinical practice for efficacy as well as avoiding digoxin toxicity.

Immunoassays are commonly used in clinical laboratories for determination of serum or plasma digoxin concentrations. Unfortunately, digoxin immunoassays are affected by both endogenous and exogenous compounds. Endogenous compounds are termed digoxin-like immunoreactive substances (DLIS), which are found in elevated concentrations in volume-expanded patients [4,5]. In addition, biological activities of some metabolites of digoxin (e.g., digoxigenin) are low relative to the parent compound; however, their immunoreactivities in some digoxin immunoassays may be greater than that of digoxin [6,7]. Thus, the immunoassays could result in false positives [8]. Because of the high sensitivity, specificity and without interfering form DLIS, LC–MS technique is developed for the determination of digoxin [9–12]. However, the previously published methods require either large plasma volumes or a tandem mass spectrometer. Some methods utilizing solid-phase column extraction were expensive and time-consuming [13–15]. In addition, only one recent LC–MS method to determine digoxin in human urine has been published [16].

In order to fill this methodological gap, we developed a sensitive and selective LC–MS method for the determination of digoxin in human plasma and urine. The method was applied to a pharmacokinetic study in healthy subjects following single intravenous administration of 0.5 mg digoxin.

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2. Experimental

2.1. Chemicals

Solvents used were of HPLC quality and chemicals were of analytical grade. Methanol and acetonitrile were purchased from Baker (Gross-Gerau, Germany), ammonium acetate, acetic acid, isopropanol, chloroform and ammonium chloride from Merck (Darmstadt, Germany). Digoxin was obtained from Sigma. The internal standard (IS) *d*5-dihydrodigoxin (Fig. 1) was synthesized and provided by Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology (Stuttgart, Germany).

2.2. Standard solutions

The stock standard solutions of digoxin and IS *d*5-dihydrodigoxin (1 mg/mL) were prepared in methanol. Calibration standards were prepared by dilution of the stock solutions with methanol.

2.3. Sample preparation

Samples (2 mL of plasma or 0.2 mL of urine) were spiked with 15 μL of IS (0.4 ng/μL) and 500 μL of ammonium chloride solution and extracted for 10 min with 7 mL of chloroform–isopropanol (95:5, v/v) at pH 9.5. After centrifugation 4000 × *g* for 10 min, the upper phase was discarded and the organic phase transferred to another vial and evaporated to dryness in a stream of nitrogen. The residue was dissolved in 50 μL of methanol–5 mmol/L ammonium acetate–acetic acid (79:20:1, v/v/v). An aliquot of 40 μL was used for LC–MS analysis.

2.4. LC–MS analysis

A HP Series 1100 LC–mass-selective detection (MSD) system (Agilent, Waldbronn, Germany) with binary pump, degasser, autosampler and mass selective detector equipped with an electrospray ion source was used. Chromatographic separation was achieved on a Spherisorb ODS2 column (5 μm × 150 mm i.d.), particle size 5 μm, Waters, Milford, MA, USA) maintained at 40 °C in a column oven. The mobile phases for HPLC were: (A) 5 mmol/L ammonium acetate in water with 1% acetic acid and (B) acetonitrile. The digoxin and IS were separated with a gradient mobile phase at a flow rate of 1 mL/min with a run time of 15 min. The following gradient was used: 0–3.0 min, 28–60% B; 3.0–7.5 min, 60% B; 7.5–8.0 min, 60–80% B; 8.0–10.4 min, 80% B (0.7 mL/min); 10.4–10.5 min, 80–28% B, followed by a 4.5-min post-run interval at 28% B until the next sample was injected.

The mass spectrometer was tuned with the autotune procedure provided by the ChemStation software (Rev. A.06.01). Electrospray parameters were as follows: capillary voltage 5000 V, drying gas flow 11 L/min nitrogen, drying gas temperature 350 °C, nebulizer pressure 60 pounds per square in. gauge (psig) and fragmentor 160 V. Peak width was set at 0.25 min. The mass spectrometer was operated in the selected ion monitoring mode (SIM resolution high) using the respective $[M+K]^+$ ions, mass-to-charge (m/z) 819.4 for digoxin and m/z 826.4 for *d*5-dihydrodigoxin.

2.5. Standardisation and validation

Drug-free plasma and urine were obtained from healthy volunteers who had not taken any medication at least 3 days before blood and urine collection. Standard curves were prepared by adding 10 μL of standard solutions with increasing amounts of digoxin to 2 mL of drug-free plasma or 0.2 mL of urine and extracting the samples as described above. The final concentrations obtained were 0.12, 0.20, 0.78, 1.96, 7.80 and 19.60 ng/mL in plasma for digoxin and 1.2, 2.0, 7.8, 19.6, 78.0 and 196.0 ng/mL in urine. Standard curves were evaluated by weighted ($1/x$) linear regression based on internal standard calibration and were obtained by plotting peak–area ratios against the concentration of digoxin. The concentration of digoxin in unknown samples was obtained from the regression line.

After the successful validation of the assay for plasma was developed and validated, an abbreviated validation strategy for urine would be used in the study.

The reproducibility and accuracy of the method was established by analysing quality control samples prepared by adding known amounts of digoxin to 20 mL of drug-free plasma or 2 mL of urine which were divided into aliquots and stored at −20 °C. The final concentrations were 0.12, 0.35, 1.96 and 7.80 ng/mL in plasma for digoxin and 3.5, 19.6 and 78 ng/mL in urine. Quality control samples were always extracted and analyzed together with the samples.

The intra-assay precision and accuracy was assessed by measuring the concentration of digoxin in six aliquots of the different quality control samples extracted and analyzed on a single day. Inter-assay precision and accuracy were determined from the results of the different quality control samples which were extracted and analyzed sixfold on 3 different days. The limit of quantification (LOQ) was determined as the lowest concentration with a coefficient of variation (CV) and a bias of <20% ($n = 6$).

Extraction recoveries were determined by comparing the peak areas from extracted standards in human plasma to the peak areas of unextracted standards at three different quality control samples (0.35, 1.96 and 7.80 ng/mL).

2.6. Stability

The stability of digoxin in plasma was evaluated with four studies: a short-term stability study, a long-term stability study, a freeze–thaw study and stability in processed sample. Plasma blank samples were spiked with digoxin at concentration of 0.35, 1.96 and 7.80 ng/mL and each concentration was measured five times. Plasma samples were extracted and subsequent HPLC analysis was carried out as described previously. Short-term stability test was performed at room temperature. Plasma samples spiked with digoxin were kept at room temperature for 12 h, extracted and then analyzed. The long-term stability study was carried out with plasma blank samples spiked with digoxin, which were stored at −80 °C for 1 month. For freeze–thaw stability spiked samples were analyzed immediately after preparation and on a daily basis after repeated freeze–thaw cycles at −80 °C on 3 consecutive days. Finally, the processed quality control samples ready for injection were kept at ambient temperature for 8 h before analysis.

2.7. Matrix effects

The matrix effects were investigated using six independent sources of plasma. The peak areas of extracted blank plasma samples which were spiked with standard solution at three final concentrations (0.35, 1.96 and 7.80 ng/mL) and IS (15 μL of a 0.4 ng/μL) were compared with peak areas of the same concentration of standard solution diluted in methanol. In addition, the matrix effects were investigated using six independent sources of urine. The three final digoxin concentrations using extracted blank urine were 3.5, 19.6 and 78.0 ng/mL and IS (15 μL of a 0.4 ng/μL). The matrix effects were evaluated according to the ratios of peak areas of digoxin or IS in spiked plasma or urine post-extraction to those in methanol.

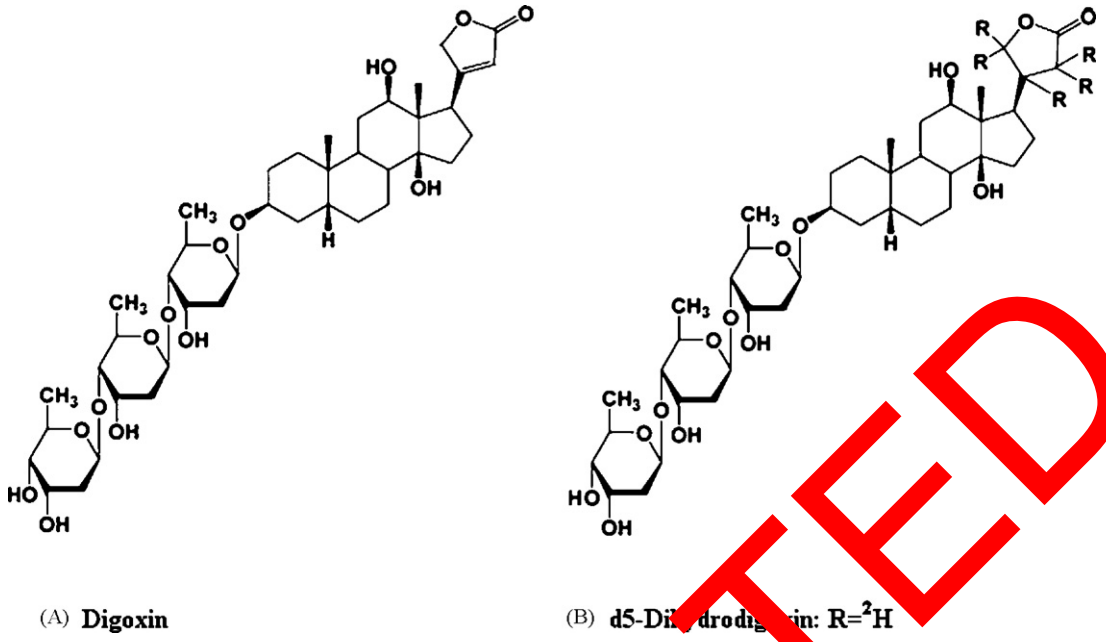

Fig. 1. Chemical structures for (A) digoxin an dihydrodigoxi).

2.8. Pharmacokinetic study

The LC–MS assay developed was used to investigate the pharmacokinetic parameters in 8 healthy volunteers afte sin-gle intravenous administration of 0.5 mg digoxin over a per d

min. Blood s mples were drawn before drug intake and at dif-rent time poi s (10, 20, 30, 40, 45 min and 1, 1.25, 1.5, 2, 3, 4, 5 9, 12 h) af dosing for digoxin. Urine was collected before dru dmini tion and at 0–72 h after administration. The study had bee approved by the local ethics committee (Tongji Medical

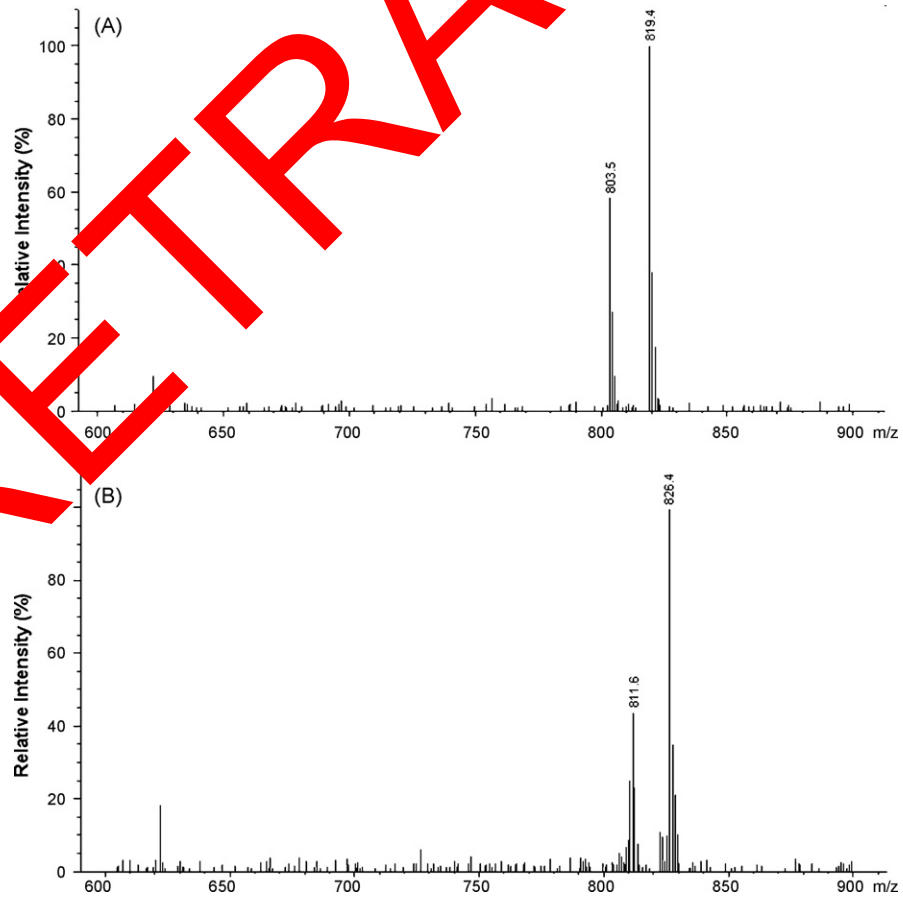

Fig. 2. Production mass-spectra of $[M+K]^+$ of (A) digoxin and (B) *d*5-dihydrodigoxin (IS).

College, Huazhong University of Science and Technology, Wuhan, China). All volunteers gave their written informed consent prior to participation in the study.

Pharmacokinetic parameters were calculated from plasma concentration–time data by non-compartmental methods. The maximum plasma concentrations (C_{max}) were obtained from observed data. The area under the plasma concentration versus time curve (AUC) from 0 to the last measurable concentration (C_t), AUC_{0-t}, was calculated with the linear trapezoidal rule. The area under the plasma concentration versus time curve from 0 to infinity ($AUC_{0-\infty}$) was calculated as $AUC_{0-t} + C_t/\lambda_z$, where λ_z is the slope of the log-linear regression of the terminal concentration data points. The terminal elimination half-life ($T_{1/2}$) was calculated as $(\ln 2)/\lambda_z$. Total clearance (CL_{total}) was calculated as dose/$AUC_{0-\infty}$. Renal clearance (CL_r) was calculated as Ae_{0-12h}/AUC_{0-t}, where Ae_{0-12h} is the cumulative amount of drug excreted in the urine in the 12 h following intravenous administration of digoxin. Nonrenal clearance (CL_{nr}) was calculated as $CL_{total} - CL_r$. The Drug and Statistics Software (DAS, Version 2.0) (Mathematical Pharmacology Professional Committee of China, China) was used.

3. Results and discussion

3.1. Sample preparation

Liquid–liquid extraction [9–11] or solid-phase extraction (SPE) [13–15] has been described for the determination of digoxin. We have used liquid–liquid extraction with chloroform–isopropanol (95:5, v/v), the mean extraction efficacies from plasma for digoxin (0.35, 1.96 and 7.80 ng/mL) and IS were found to be (81.7 ± 3.5)%, (85.6 ± 6.9)%, (82 ± 3.8)% and (87.2 ± 4)%, respectively.

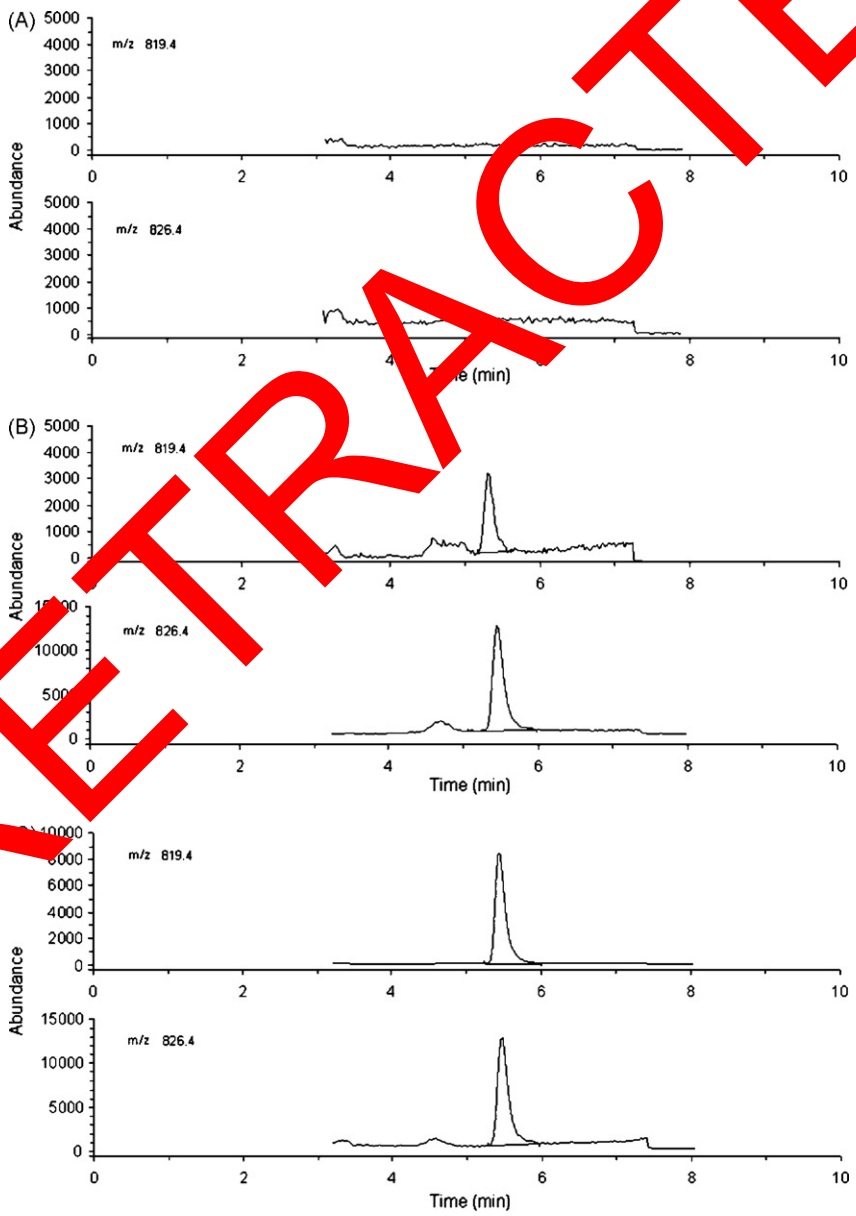

Fig. 3. Typical chromatograms obtained from human plasma samples: (A) blank plasma; (B) blank plasma spiked with standard solution (LOQ); (C) plasma sample from a subject 0.75 h after intravenous infusion of 0.5 mg digoxin with concentration of 7.51 ng/mL.

Table 1
Intra- and inter-assay precision and accuracy for the determination of digoxin in human plasma.

Concentration added (ng/mL)	Intra-assay ($n = 6$)			Inter-assay (each of 3 days, $n = 6$)		
	Concentration found (ng/mL) (mean ± SD)	CV (%)	Bias (%)	Concentration found (ng/mL) (mean ± SD)	CV (%)	Bias (%)
0.12	0.12 ± 0.01	11.1	−3.0	0.11 ± 0.01	12.5	−5.7
0.35	0.34 ± 0.03	8.6	−0.7	0.35 ± 0.03	7.8	−1.6
1.96	2.02 ± 0.06	3.2	3.7	2.01 ± 0.10	5.1	2.8
7.80	8.11 ± 0.26	3.2	3.9	8.07 ± 0.29	3.6	3.5

3.2. LC–MS analysis

The positive ion electrospray mass spectra of digoxin and IS show the protonated molecular ion $[M+K]^+$ as the base peak (Fig. 2), m/z 819.4 for digoxin and m/z 826.4 for IS.

HPLC separation was achieved in a total runtime of 15 min on a Spherisorb ODS2 column with ammonium acetate (5 mmol/L) in water with 1% acetic acid–acetonitrile gradient (Fig. 3C). Because of the highly selective detection method, there were no interfering peaks present in more than 6 blank samples investigated from healthy volunteers. An example of a blank plasma sample is shown in Fig. 3A, the lowest quality control sample (0.12 ng/mL) in Fig. 3B.

3.3. Assay validation

The linearity of the standard curves showed to be good over the entire concentration range measured: 0.12–19.60 ng/mL for digoxin in plasma and 1.2–196.0 ng/mL in urine. The correlation coefficients (r^2) ranged between 0.9968 and 0.9999 for digoxin in plasma and 0.9989–0.9994 in urine.

Accuracy and precision of the method were determined with quality control samples as described above. The relative standard deviation (RSD) and mean values of the deviation from the amount added (% bias) were calculated. The results for plasma are given in Table 1, for urine in Table 2. The data showed good precision of the method with an intra- and inter-assay RSD below 15% even at the LOQ (0.12 ng/mL). The intra- and inter-assay accuracy as expressed by the bias ranged between −5.7 and 3.9% for digoxin. Intra-assay precision and accuracy of the method were within 7.4% in urine.

The stability of digoxin in plasma was determined under various conditions according to the procedure described above. Short-term stability test performed at room temperature showed that three quality control samples were stable for 12 h (mean recoveries were 96.0, 102.5 and 98.0%, respectively). The long-term stability results indicated that digoxin samples were stable during 1 month, with an average recovery of 97.6, 101.5 and 102.6%, respectively. No significant change of digoxin concentration in plasma was detected after exposing samples to three freeze–thaw cycles and mean recovery was found to be 97.8%. Finally, the stability in the processed sample ready for injection was also determined. Result showed that three quality control samples were stable at least for 8 h with CVs below 9.1%.

Table 2
Intra-assay precision and accuracy for the determination of digoxin in human urine ($n = 6$).

Concentration added (ng/mL)	Concentration found (ng/mL) (mean ± SD)	CV (%)	Bias (%)
3.5	3.56 ± 0.11	3.0	1.8
19.6	20.94 ± 0.39	1.8	7.4
78.0	83.63 ± 2.29	2.7	7.2

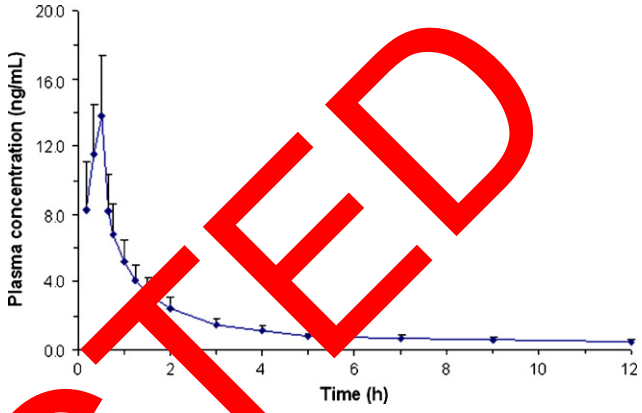

Fig. 4. Mean (±SD) plasma concentration-time profile of digoxin after intravenous infusion of 0.5 mg digoxin in 8 healthy volunteers.

3.4. Matrix effects

The mean matrix effects in plasma at three concentrations (0.35, 1.96 and 7.80 ng/mL) were 95.4, 96.7 and 97.8%, respectively. The mean matrix effects in urine at three concentrations were from 96.3 to 99.1%. The mean matrix effects for IS in plasma or urine were (96.1 ± 2.1)% and (97.2 ± 1.3)%, respectively. Matrix effects values less than 100% and those more than 100% express ionization suppression and ionization enhancement, respectively. The results showed that the analysis of digoxin and IS were not interfered with by endogenous substances in plasma or urine.

3.5. Assay application

The assay presented allowed for the determination of digoxin in human plasma and urine in a pharmacokinetic study. Mean plasma concentration–time curves of 8 volunteers following single intravenous administration of 0.5 mg digoxin are shown in Fig. 4. The corresponding pharmacokinetic parameters are summarized

Table 3
Pharmacokinetic parameters (mean ± SD) of digoxin after intravenous infusion of 0.5 mg digoxin in 8 healthy volunteers.

Parameter	Mean ± SD
AUC_{0-12} (ng h/mL)	20.39 ± 4.17
$AUC_{0-\infty}$ (ng h/mL)	25.74 ± 8.00
C_{max} (ng/mL)	13.81 ± 3.53
$T_{1/2}$ (h)	31.10 ± 12.75
Ae_{0-12h} (μg)	203.80 ± 24.77
Ae_{0-72h} (μg)	316.47 ± 36.11
CL_{total} (mL/min)	343.05 ± 73.37
CL_r (mL/min)	170.69 ± 32.69
CL_{nr} (mL/min)	172.35 ± 50.02

Parameters: AUC, area under the plasma concentration–time curve; C_{max}, maximum plasma concentration; $T_{1/2}$, terminal elimination half-life; Ae_{0-12h}, the cumulative amount of drug excreted in the urine (0–12 h); Ae_{0-72h}, the cumulative amount of drug excreted in the urine (0–72 h); CL_{total}, total clearance; CL_r, renal clearance; CL_{nr}, nonrenal clearance.

in Table 3. The total cumulative urinary excretion rate of digoxin up to 72 h was (63.3 ± 7.2)% of the administered dose, which indicated that CL_r was an important component of the total clearance of digoxin. In addition, digoxin had a relatively long half-life of over 30 h. The pharmacokinetic profile of digoxin in this study was similar to the previously published reports [17,18]. However, the C_{max} after intravenous administration with the same dosage was slightly higher (13.8 ng/mL) in this study than reported (9.1 ng/mL) in the literature [19], which could be explained by an infusion duration of 30 min instead of the 1 h used in this study.

4. Conclusions

A method has been developed for determination of digoxin in human plasma and urine using liquid–liquid extraction and HPLC–mass spectrometry. Sensitivity, selectivity and reproducibility allow for the application in pharmacokinetic studies. In 8 volunteers the most relevant pharmacokinetic parameters of digoxin have been evaluated following intravenous dosing.

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References

[1] P. Manunta, M. Ferrandi, Hypertension 47 (2006) 343.
[2] M.M. Matzuk, M. Shlomchik, L.M. Shaw, Ther. Drug Monit. 13 (1991) 215.
[3] M. Barclay, E. Begg, N. Z. Med. J. 116 (2003) U704.
[4] A. Dasgupta, Am. J. Clin. Pathol. 118 (2002) 132.
[5] A. Dasgupta, Toxicol. Rev. 25 (2006) 273.
[6] J.J. Miller, R.W. Straub, R. Valdes Jr., Clin. Chem. 40 (1994) 1898.
[7] B. Bednarczyk, S.J. Soldin, I. Gasinka, M. D'Costa, L. Perrot, Clin. Chem. 34 (1988) 393.
[8] R. Valdes Jr., Clin. Chem. 31 (1985) 1526.
[9] A. Tracqui, P. Kintz, B. Ludes, P. Mangin, J. Chromatogr. B Biomed. Sci. Appl. 692 (1997) 101.
[10] M. Yao, H. Zhang, S. Chong, M. Zhu, R.A. Morrison, J. Pharm. Biomed. Anal. 32 (2003) 1189.
[11] J. Smalley, A.M. Marino, B. Xin, T. Olah, P.V. [illegible]mane, J. Ch[illegible]atogr. B Analyt. Technol. Biomed. Life Sci. 854 (2007) 260.
[12] J. Ni, H. Ouyang, M. Aiello, C. Seto, L. Borb[illegible]a, T. Sakuma, R. E[illegible] D. Welty, A. Acheampong, Pharm. Res. 25 (2008) 1572.
[13] K. Mitamura, A. Horikawa, Y. Yam[illegible], Y. Ikeda, [illegible]iii, K. Shim[illegible], Biol. Pharm. Bull. 30 (2007) 1653.
[14] L. Frommherz, H. Köhler, B. [illegible]kmann, M[illegible]ehr, J. Bo[illegible], J. Legal Med. 122 (2008) 109.
[15] Y. Hashimoto, K. Shibakawa, [illegible]akad[illegible], Miyata, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. [illegible]9 (200[illegible]
[16] B.J. Kirby, T. Kalho[illegible]M. Hebert, T. [illegible]terling, J.D[illegible]adkat, Biomed. Chromatogr. 22 (2008) 712.
[17] B.J. Gurley, A[illegible]val[illegible]W. Barone, D.K. [illegible]ms, P. Breen, C.R. Yates, L.B. Stuart, M.A. Hubb[illegible], Y. Tong, Cheboyina, Drug Metab. Dispos. 35 (2007) 240.
[18] M. Feuring, Y. Lee, L.H. [illegible]wski, N. Michiels, M. De Smet, A.K. Majumdar, K.J. Petty, M.P. Goldberg, M.G. [illegible]phy, K.M. Gottesdiener, M. Hesney, L.E. Brackett, M. [illegible]enning, J. Clin. Pharmacol. [illegible]3 (2003) 912.
[19] [illegible]Tsutsumi, T. Kotegawa, M. Kuranari, Y. Otani, T. Morimoto, S. Matsuki, S. Nakano, J. Clin. Pharmacol. 42 (2002) 1159.