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Simple liquid chromatography–tandem mass spectrometry method for determination of novel anti-methicillin-resistant *Staphylococcus aureus* fluoroquinolone WCK 771 in human serum

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Abstract

A simple, rapid, specific, precise, accurate and sensitive method for determination of WCK 771 in human serum has been developed. The method uses high performance liquid chromatography with tandem mass spectrometric detection. Sample preparation involves protein precipitation method by addition of acetonitrile. Gatifloxacin was used as internal standard. The response was found to be linear from 0.312 to 40 μg/ml of serum with correlation coefficient greater than 0.99. Limit of detection and lower limit of quantification for WCK 771 was found to be 0.078 μg/ml and 0.312 μg/ml, respectively. The intra-day precision and accuracy from analysis of quality control (QC) samples at four concentrations was in the range of 2.36–2.58% and from 96.71 to 103.2%, respectively. The inter-day precision and accuracy from analysis of quality control samples at four concentrations was in the range of 3.14–6.82% and from 96.84 to 105.76%, respectively. WCK 771 was found to be stable for 24 h at auto-injector environment. WCK 771 was also found to be stable for 2 h in serum at 25 ± 3 °C and for 3 months at −20 °C. Mean absolute recovery at four different concentrations was 86.92% with standard deviation of 1.79. Throughput of the method is approximately one sample every 4 min. The method was also reproduced with monkey serum. The method was employed for estimation of drug serum levels during pre-clinical and clinical trials.

Keywords: WCK 771; Liquid chromatography–tandem mass spectrometry; Human serum

1. Introduction

Chemically, WCK 771 is a *S*-(−)-9-fluoro-6,7-dihydro-8-(4-hydroxypiperidin-1-yl)-5-methyl-1-oxo-1*H*,5*H*-benzo[*i*,*j*]quinolizine-2-carboxylic acid L-arginine salt tetrahydrate [1–3]. WCK 771 is a novel anti-methicillin-resistant *Staphylococcus aureus* (MRSA)/anti-vancomycin-resistant *S. aureus* (VRSA) agent [4–6]. WCK 771 possesses potent MRSA-fluoroquinolone-*R* activity [7]. WCK 771 possesses superior anti-anaerobic activity as compared to other fluoroquinolones [8]. WCK 771 is also found to be potent both *in vitro* and *in vivo* against *Staphylococcus pneumoniae* [9]. Due to such wide spectrum of activities WCK 771 is being developed for several clinical indications. It was important to have an assay method for estimation of WCK 771 in serum of different species for pharmacokinetic and toxicological studies. The aim of this study was to develop and validate such an assay method. Several methods have been reported for estimation of fluoroquinolones in biological matrices; most of them used high performance liquid chromatographic methods using ultraviolet or fluorimetric detectors [10–15]. The reason might be that most of the successful fluoroquinolones like norfloxacin, ciprofloxacin, ofloxacin, enrofloxacin, pefloxacin, sparfloxacin were developed in 1980s, when the use of liquid chromatography–tandem mass spectrometry was limited. Currently used atmospheric pressure ionization technique for hyphening liquid chromatography with mass spectrometer became commercially available in late 1980s and became part of modern bioanalytical laboratories in 1990s [16]. Applications of liquid chromatography–tandem mass spectrometry have

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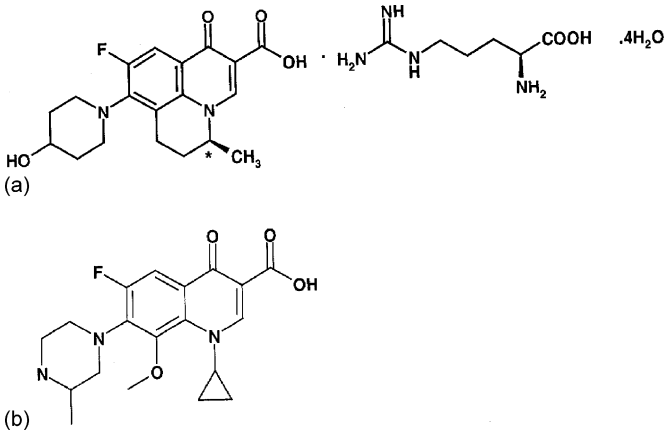

Fig. 1. Chemical structures of WCK 771 (a) and gatifloxacin (b).

been reported for determination of fluoroquinolones in pig kidney tissue, fish tissue and sea foods [17,18]. These methods use either solid phase extraction or liquid-liquid extraction procedures to obtain required sensitivity. Due to extraction procedures involved these methods are tedius, cumbersome, time consuming and needs skilled manpower. Also due to longer chromatographic run times throughput of these methods is lower. This article describes very simple and high throughput method for estimation of WCK 771 in human serum.

2. Experimental

2.1. Chemicals

WCK 771 and gatifloxacin were prepared and provided by Chemistry group, Drug Discovery, Wockhardt Research Center. Both the compounds were characterized for their identity and purity. Chemical structures of WCK 771 and gatifloxacin (Internal standard, I.S.) are shown in Fig. 1. HPLC grade acetonitrile (Ranbaxy Fine Chemicals Limited, India), trifluoroacetic acid (Merck-Schuchardt, Germany) and double distilled water passed through Purelab classic (US Filters, USA) were used during studies.

2.2. Equipment

A HP 1100 HPLC (Hewlett Packard GmbH, Germany) consisting of vacuum degasser, quaternary pump, auto-injector and thermostatted column compartment was used. The chromatographic system consists of a C18 column (YMC-ODS-AM, 150 mm × 2.1 mm ID, particle size 5 μm) (YMC Co. Limited, Japan) and mobile phase (0.05% trifluoroacetic acid: acetonitrile; 40:60, pH of the mobile phase 2.2) delivered at 0.25 ml/min. The thermostatted column compartment was maintained at 35 °C. The auto-injector was set-up to make 5 μl injection every 3.5 min with needle-wash after each injection. The outlet of column was directly connected to electrospray probe. Mass spectrometric determination was performed on Quattro-II (Micromass UK Limited, UK), a triple quadrupole mass spectrometer operating in positive ion electrospray mode. The source block temperature and desolvation temperature were 100 °C and 300 °C, respectively. Nitrogen was used as drying gas and electrospray nebulising gas at the flow of 300 lit/h and 15 lit/h. The ESI capillary potential was set at 3.6 kV and cone voltage was 24 V. Collision cell was maintained at 2.7×10^{-3} mbar and collision energy used was 15 V and 18 V for WCK 771 and gatifloxacin, respectively. Argon was used as collision gas. Detector was photo multiplier tube maintained at 650 V. Quantitation was performed using selected reaction monitoring (SRM) of the transitions 361 → 343 for WCK 771 and 376 → 332 for gatifloxacin (I.S.) with dwell time 0.5 s. Mass calibration, data acquisition and quantitative analysis were performed using Windows NT-based Masslynx 3.3 software.

2.3. Sample preparation

An aliquot (50 μl) of serum was diluted with equal quantity of blank serum. To it 500 μl of acetonitrile containing 5 μg/ml gatifloxacin (I.S.) was added. The mixture was vortexed for 30 s and centrifuged at 10000 rpm at 4 °C for 10 min. Supernatant (5 μl) was injected for analysis.

2.4. Selectivity

To determine any interference of endogenous matrix components, serum obtained from six different sources was processed without addition of I.S. and analyzed. Also a major metabolite observed in serum was spiked in the lower limit of quantification (LLOQ) calibration standard to determine any effect of metabolite on analysis.

2.5. System suitability

Performance of the method was determined by injecting spiked (20 μg/ml) serum sample in triplicate. Relative standard deviation of ratio of analyte peak area to I.S. peak area was calculated. The system was considered suitable for further analysis if the relative standard deviation was less than 5%.

2.6. Linearity

Linearity of WCK 771 was determined in the range of 0.312–40 μg/ml. Calibration standards were prepared and run on three different days. Calibration graphs were constructed using weighted linear regression (1/concentration^2) of the analyte/I.S. peak area ratios versus nominal analyte concentrations.

2.7. Limit of detection (LOD) and lower limit of quantification

LOD of the method was determined by analyzing five preparations of spiked serum (0.078 μg/ml). Mean and relative standard deviation of estimated concentration was determined. Similarly, five preparations of spiked serum (0.312 μg/ml) were analyzed for determination of LLOQ.

2.8. Intra-day precision and accuracy

Intra-day precision and accuracy of the method was determined by analyzing serum quality control (QC) samples five times at four different concentrations (0.625, 2.5, 10 and 20 μg/ml). Quality control values were calculated from standard calibration curve containing eight different concentrations spanning the concentration range (0.312–40 μg/ml).

2.9. Inter-day precision and accuracy

Inter-day reproducibility of the method was determined by analyzing serum quality control samples at four different concentrations (0.625, 2.5, 10 and 20 μg/ml) on four different days against the standard calibration curve.

2.10. Post-preparative stability

Serum quality control samples at three concentrations were assayed at initial time and after 2, 4 and 24 h of storage in the auto-injector at 25 °C.

2.11. Freeze–thaw stability

Serum was spiked with analyte at four different concentrations (0.625, 2.5, 10 and 20 μg/ml). Aliquots were withdrawn immediately after spiking and after 1, 2 and 3 freeze–thaw cycles. These aliquots were processed and assayed against calibration standards. The freeze–thaw cycle consisted of freezing at −20 °C and thawing to room temperature.

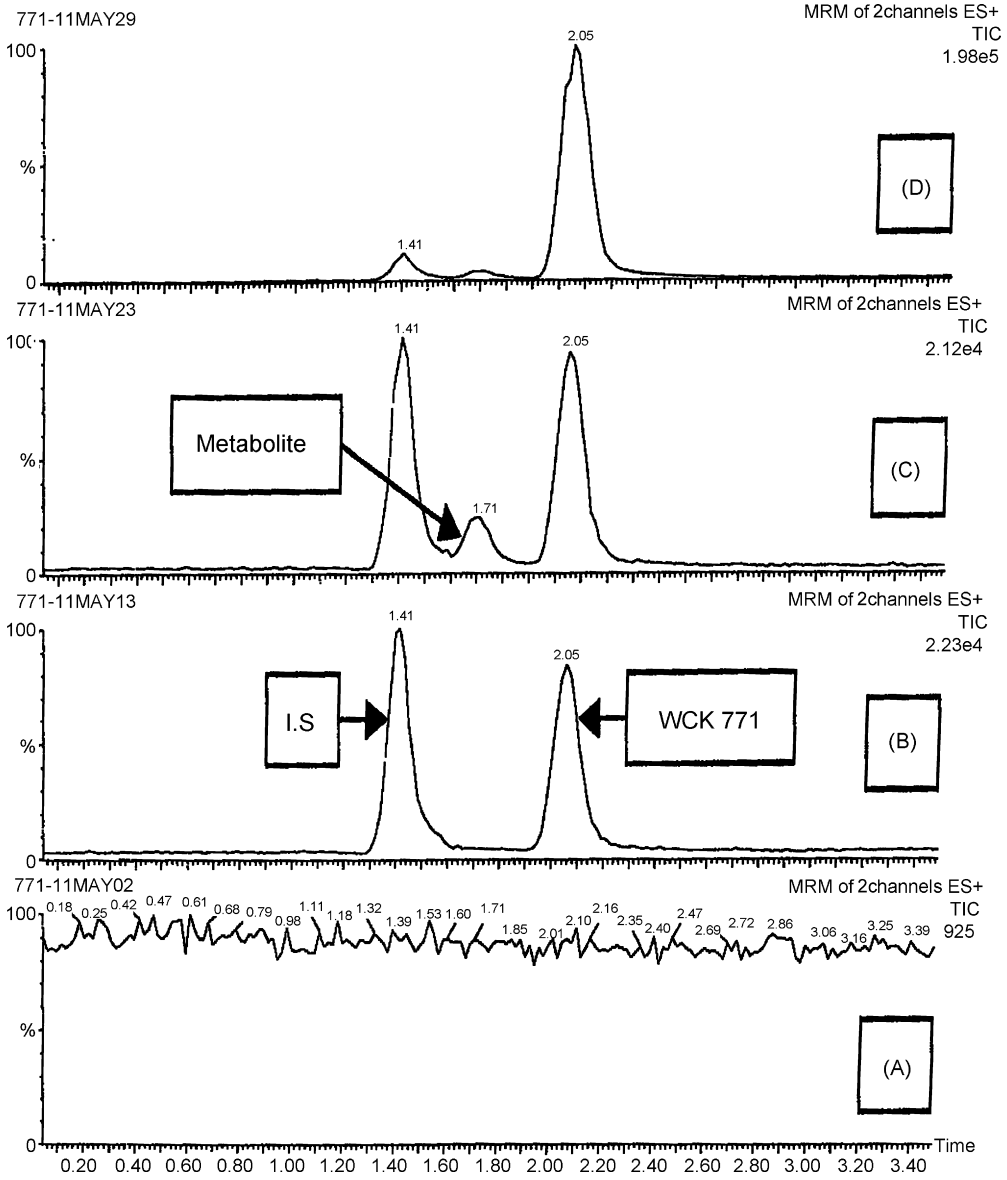

Fig. 2. Overlaid total ion chromatograms of: blank serum (A); serum spiked with WCK 771 (1.25 μg/ml) and I.S. (B); serum spiked with WCK 771 (1.25 μg/ml), metabolite and I.S. (C); and study sample (D).

Table 1
Intra-day precision and accuracy

Nominal concentration (μg/ml)	Found concentration mean ($n=5$) (μg/ml)	RSD (%)	Accuracy (%)
0.625	0.64	2.39	103.2
2.5	2.55	2.55	101.9
10	10.03	2.36	100.3
20	19.34	2.58	96.7

2.12. Short-term (bench top) stability

Short-term stability was carried out at room temperature. Serum was spiked with analyte at four different concentrations (0.625, 2.5, 10 and 20 μg/ml). Aliquots were withdrawn immediately after spiking and after 2 h. The withdrawn samples were processed immediately and assayed.

2.13. Long-term stability

Long-term stability was carried out at −20 °C and −70 °C. Serum was spiked with analyte at four different concentrations (0.625, 2.5, 10 and 20 μg/ml). Aliquots were withdrawn immediately after spiking and after 1, 2 and 3 months. The withdrawn samples were immediately processed and assayed.

2.14. Absolute recovery

Absolute recovery was determined at four different concentrations (0.625, 2.5, 10 and 20 μg/ml) using water as matrix. Aqueous samples were processed and analyzed in same fashion as that of serum samples. Peak areas obtained with aqueous samples were compared with peak areas obtained with serum samples.

3. Results and discussions

Due to the high specificity of tandem mass spectrometer (selected reaction monitoring) as detector, no interfering peaks were observed during analysis of blank serum samples from six different sources. Though the metabolite (sulphate conjugate) produces identical SRM transition (after cleavage of conjugate group) as of analyte, it did not interfered with the analyte peak (RT 2.05 min) due to different chromatographic retention (1.71 min). Similarly, I.S. elutes at different retention time (1.41 min) and does not compete for ionization of analyte. A representative total ion chromatogram of blank serum, serum spiked with WCK 771 (1.25 μg/ml) and I.S., serum spiked with WCK 771 (1.25 μg/ml), metabolite and I.S. and study sample is shown in Fig. 2. Average peak area ($n=5$) of WCK 771 at LLOQ did not deviate significantly (less than 5%) in presence of metabolite. System suitability sample was run everyday in beginning of validation sequence. Relative standard deviation of ratio of analyte peak area to I.S. peak area was always found to be less than 5%, which ensured adequate system performance. A linear response was observed in the concentration range of 0.312–40 μg/ml with correlation coefficient for three consecutive calibration standards analysis 0.9997, 0.9985 and 0.9993. A regression equation obtained was $y = 1.95487x + 18.6507$. During analysis of three calibration curves on three different days none of the level including LLOQ deviated more than 15% of the nominal concentration. LOD of the method was found to be 0.078 μg/ml, where the peak response was found to be greater than three times of baseline noise. LLOQ of the method was the lowest concentration (0.312 μg/ml) of calibration standards, which can be determined with acceptable precision (RSD < 20%) and accuracy (accuracy ± 20%). Intra-day precision and accuracy data is shown in Table 1. The method was found to be precise (RSD well below acceptable limit of 15%) and accurate (nominal concentration ± 15%). Inter-day precision and accuracy data is shown in Table 2. The method was found to be reproducible on 3 days with precision and accuracy. Post-preparative stability was inferred from serum quality control samples at three concentrations, which were prepared and assayed at initial time

Table 2
Inter-day precision and accuracy

Day	Nominal concentration (μg/ml)			
	0.625	2.5	10	20
	Found concentration (μg/ml)			
1	0.64	2.58	10.25	19.82
2	0.63	2.58	10.01	18.53
3	0.72	2.22	10.84	19.31
4	0.65	2.50	10.17	19.82
Mean	0.66	2.47	10.32	19.37
RSD (%)	5.74	6.82	3.51	3.14
Accuracy (%)	105.8	98.8	103.2	96.8

Table 3
Post-preparative stability data

Nominal concentration (μg/ml)	Found concentration (μg/ml)				Mean	Accuracy (%)
	0 h	2 h	4 h	24 h		
0.625	0.63	0.73	0.61	0.64	0.65	104
2.5	2.45	2.71	2.45	2.68	2.57	102.8
10	10.02	9.75	9.51	10.75	10.01	100.1

Table 4
Freeze–thaw stability data

Nominal concentration (μg/ml)	Found concentration (μg/ml)				Mean	Accuracy (%)
	Initial	Cycle-1	Cycle-2	Cycle-3		
0.625	0.64	0.65	0.69	0.70	0.68	108.8
2.5	2.58	2.70	2.63	2.73	2.69	107.6
10	10.25	10.12	10.59	10.64	10.45	104.5
20	19.82	17.86	20.96	19.66	19.49	97.45

Table 5
Short-term stability data

Nominal concentration (μg/ml)	Found concentration (μg/ml)	
	Initial	After 2 h
0.625	0.65	0.64
2.5	2.22	2.37
10	10.84	11.16
20	19.31	20.37

Table 7
Pharmacokinetic parameters in healthy human volunteers (600 mg, intravenous infusion, $n = 6$)

C_{max} (μg/ml)	23.21 ± 3.75
AUC (0–24 h) (μg h/ml)	144.12 ± 32.23
AUC (0–∞) (μg h/ml)	154.51 ± 35.55
T1/2 (h)	6.06 ± 1.48
Cl (L/h/kg)	4.05 ± 0.9
Vd (L/kg)	34.86 ± 8.96

and after 2, 4 and 24 h of storage in the auto-injector at 25 °C. No significant degradation could be detected in the samples left on the auto-injector for 24 h. Post-preparative stability data is shown in Table 3. The analyte was found to be stable for three freeze–thaw cycles as found concentration remained in the range of ±15% of nominal concentration. Freeze–thaw stability data is shown in Table 4. The analyte was found to be stable in serum for 2 h at room temperature (25 ± 2 °C). Found concentrations after 2 h did not deviate significantly from the nominal concentrations (±15%). Short-term (bench top) stability data is shown in Table 5. The analyte was found to be stable in serum for 3 months when stored at −20 °C. After 3 months, found concentration remained in the range of 15% of nominal concentration. Long-term stability data is shown in Table 6. Absolute recovery of analyte at 0.625, 2.5, 10 and 20 μg/ml was found to be 88.52 ± 9.38, 86.11 ± 1.71, 84.88 ± 0.74 and 88.89 ± 0.98%, respectively. This indicates insignificant matrix effect during the analysis.

The described and validated method was employed in estimation of WCK 771 in human serum during phase-I clinical trials to evaluate safety and pharmacokinetics of single and BID i.v. doses of WCK 771 in normal, healthy, adult, Indian male subjects. WCK 771 was administered as an i.v. infusion over 1 h. At each dose level, two subjects received placebo and six subjects received WCK 771. The PK parameters were assessed using non-compartmental model using WinNonlin 2.1 software, the results are shown in Table 7. A pharmacokinetic profile of single i.v. dose (600 mg) is shown in Fig. 3. Performance of the validated method was monitored by injecting six QC samples (two samples of three different concentrations, 0.625, 2.5 and 20 μg/ml) intermittently and at the end of run during clinical trial sample analysis. Analysis was considered correct if at least found concentrations of four QC samples were within ±15% of nominal concentration and failing two QC samples were not of the same concentration. During production, 114 samples including calibration standards and QC samples were assayed in one run fulfilling above criteria, this envisages ruggedness of the method. The method was reproduced on another recently procured tandem mass spectrometer (API 3000, Applied Biosystems, USA). Linear response was observed with correlation coefficient of 0.9995. Found concentrations on two different

Table 6
Long-term stability data

Nominal concentration (μg/ml)	Found concentration (μg/ml)	
	Initial	After 3 months
0.625	0.60	0.59
2.5	2.32	2.46
10	9.29	11.46
20	17.94	22.44

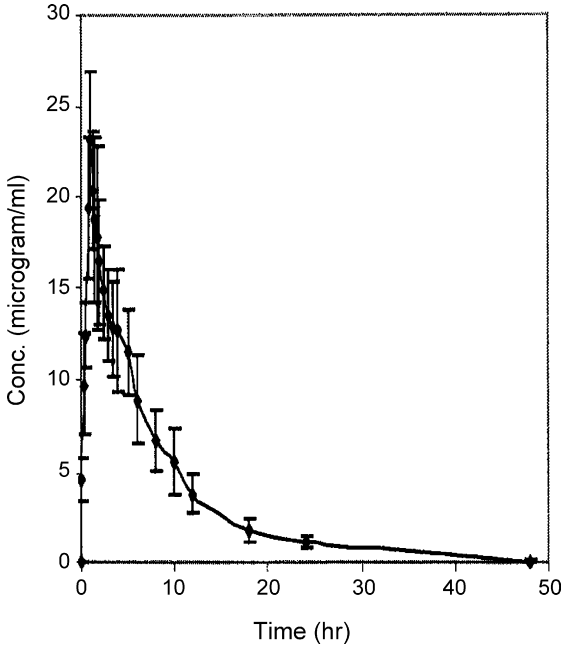

Fig. 3. Human PK profile of single i.v. dose (600 mg) ($n = 6$).

Table 8
Intra-day precision and accuracy (monkey serum)

Nominal concentration (μg/ml)	Found concentration mean ($n = 5$) (μg/ml)	RSD (%)	Accuracy (%)
0.625	0.58	2.89	92.7
5.0	4.77	1.96	95.34
40.0	38.66	2.22	96.64

Table 9
Inter-day precision and accuracy (monkey serum)

Day	Nominal concentration (μg/ml)		
	0.625	5.0	40.0
	Found concentration (μg/ml)		
1	0.58	4.77	38.66
2	0.64	5.01	41.11
3	0.59	4.90	40.22
Mean	0.60	4.89	39.99
RSD (%)	5.41	2.45	3.10
Accuracy (%)	96.11	97.83	99.98

instruments were within acceptable limit (±15% of the average value), this shows that the method is rugged.

The method was reproduced with monkey serum and was validated for precision, accuracy, linearity range and stability (short-term, long-term, freeze–thaw and pre-preparative). Since the expected serum drug levels were high in toxicokinetics study, linearity was carried out in the concentration range 0.312–80 μg/ml. Correlation coefficients for three consecutive calibration standards analysis was 0.9999, 0.9995 and 0.9986. A regression equation obtained was $y = 0.648366x + 30.9493$. Method was found to be precise and accurate. Intra-day precision (% RSD) was found to be 2.89, 1.96 and 2.22 for concentrations 0.625, 5 and 40 μg/ml, respectively. Intra-day accuracy (%) was found to be 92.70, 95.34 and 96.64 for concentrations 0.625, 5 and 40 μg/ml, respectively. Intra-day precision and accuracy data is shown in Table 8. Inter-day ($n = 3$) precision (% RSD) was found to be in the range of 2.45–5.41 for concentrations 0.625, 5 and 40 μg/ml. Inter-day ($n = 3$) accuracy (%) was found to be in the range of 96.11–99.98 for concentrations 0.625, 5 and 40 μg/ml. Inter-day precision and accuracy data is shown in Table 9. Analyte was found to be stable in autoinjector environment for 24 h. Post-preparative stability data is shown in Table 10. Freeze–thaw stability, short-term (bench top) and long-term stability was carried out at concentration 1.25 μg/ml and 20 μg/ml. The analyte was found to be stable for three freeze–thaw cycles, 4 h at room temperature and for 3 months at −20 °C. Freeze–thaw stability, short-term and long-term stability data is shown in Tables 11, 12 and 13, respectively.

Table 12
Short-term stability data (monkey serum)

Nominal concentration (μg/ml)	Found concentration (μg/ml)	
	Initial	After 4 h
1.25	1.17	1.28
20	19.69	20.95

Table 13
Long-term stability data (monkey serum)

Nominal concentration (μg/ml)	Found concentration (μg/ml)	
	Initial	After 3 months
1.25	1.30	1.17
20	20.54	18.44

Table 10
Post-preparative stability data (monkey serum)

Nominal concentration (μg/ml)	Found concentration (μg/ml)			Mean	Accuracy (%)
	0 h	3 h	24 h		
1.25	1.18	1.32	1.15	1.22	97.33
20	20.63	21.53	19.69	20.62	103.08

Table 11
Freeze–thaw stability data (monkey serum)

Nominal concentration (μg/ml)	Found concentration (μg/ml)				Mean	Accuracy (%)
	Initial	Cycle-1	Cycle-2	Cycle-3		
1.25	1.22	1.23	1.29	1.18	1.23	98.40
20	21.4	20.53	20.72	20.09	20.69	103.43

4. Conclusion

A simple, rapid, selective, precise, accurate and rugged method for determination of WCK 771 in human serum was developed and validated as per guidelines. Serum concentrations of WCK 771 could be quantified in the range of 0.312–40 μg/ml., making it possible to use in pharmacokinetic studies after single and BID intravenous dosing during phase-I clinical trials in healthy human volunteers. Same method was easily transferred for estimation of WCK 771 in serum of other species like monkey. The method is comparatively simple because sample preparation does not involve any liquid-liquid or solid phase extraction procedure. Shorter time of analysis allows high-throughput quantitative analysis, making the method more productive and more cost effective.

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