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A fast reverse-phase high performance liquid chromatographic tandem mass spectrometry assay for the quantification of clindamycin in plasma and saliva using a rapid resolution package

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ABSTRACT

A new method for the quantitative analysis of clindamycin in human plasma and saliva by liquid chromatography/electrospray ionisation tandem mass spectrometry (LC/ESI-MS/MS) has been developed using a rapid resolution C18 column (2.1 mm × 30 mm × 3.5 μm). A simple deproteinization procedure was applied to the samples before analysis. Multiple reaction monitoring (MRM) mode of precursor–product ion transitions for clindamycin (425.1/126.1) and the internal standard, lincomycin (407.2/126.0) was used. Chromatographic separation was achieved at 0.6 ml/min in less than 1.5 min, with improved peak resolution and sensitivity between drug and internal standard.

The assay exhibited a linear dynamic range between 0.05 and 15.0 μg/ml and gave a determination coefficient of 0.991 or better. The limit of quantification of the method was 10 ng/ml in both biological samples. Intra-day and inter-day precision ranged from 7.5% to 11.5%. Good accuracy was observed for both the intra-day and inter-day assays (R.S.D. below ±4%). The suitability of the developed method for the analysis of clindamycin in plasma and saliva samples was demonstrated by the measure of clindamycin in samples taken up to 6 h after oral and intravenous administration of this drug in infectious patients.

1. Introduction

Clindamycin (methyl 7-chloro-6,7,8-trideoxy-6-[(2S,4R)-1-methyl-4-propylpyrrolidine-2-carboxamido]-1-thio-1-threo-D-galacto-octopyranoside monohydrochloride) (Fig. 1) is an antibiotic of the "lincosamide" class. Despite over 25 years of widespread clinical use, clindamycin retains potent activity against many aerobic and anaerobic gram-positive and gram-negative pathogens. Moreover, clindamycin remains the drug of choice for pulmonary infections caused by anaerobic pathogens [1]. Since its introduction, the optimal dosing regimen for clindamycin has been a subject of considerable debate, because the pharmacodynamic characteristics of this agent have been poorly defined. Intravenous clindamycin is typically administered at a dose of 600 mg every 6–8 h, although regimens employed in clinical trials have ranged from 300 mg every 8 h to 1200 mg every 12 h for both intra-abdominal and pulmonary infections [2]. In humans, absorption of clindamycin is rapid and virtually complete (90%) following oral administration [3,4]. Concentrations of clindamycin in the serum increase linearly with increased dose, and levels exceed the minimum inhibitory concentration for most indicated organisms for at least 6 h following administration of the recommended dose. Clindamycin is widely distributed throughout the body and has an average biological half-life of 2.4 h. The major bioactive metabolites excreted in urine and feces are clindamycin sulfoxide and N-desmethylclindamycin [5].

A variety of studies done during the past 10–15 years have demonstrated that the success of a specific dose of drug is dependent on both a measure of drug exposure, such as the serum peak level, the area under the serum concentration versus time curve (AUC), and the duration of time serum levels exceed certain concentrations, and a measure of the potency of the drug against the infecting organisms (e.g., MIC or minimum bactericidal concentration). These so-called pharmacokinetic/pharmacodynamic (PK/PD) parameters can be major determinants of the in vivo efficacy of antimicrobial agents. The specific parameters most commonly correlated with outcome include the ratio of peak to minimum inhibitory concentration (peak/MIC ratio), the ratio of the 24-h area under the curve to MIC (24 h AUC/MIC ratio), and the duration of time serum levels exceed the MIC expressed as the percentage of the dosing interval [6]. Studies in humans are more limited by the availability of pharmacological tools that allow the measure of PK/PD parameters, such as optimal sampling, valid analytical procedures, and the development of adequate population pharmacokinetic modelling.

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Different analytical approaches were employed in the determination of clindamycin in patient samples. In general, for therapeutic drug monitoring (TDM) in patients it would be desirable to have a sensitive and specific analytical method that involve an automated and fast sample preparation step and has a high sample turnover. Currently, several commercially available immunoassays such as fluorescence polarization immunoassays, microparticle enzyme immunoassay (MEIA) and enzyme immunoassay technique and immunophilling-binding assays (RRA) fulfil these requirements and are employed for TDM of some drugs. However, no such method is available for TDM of clindamycin. Several methods have been described in the literature with this aim, including microbiological assays, radioimmunoassays [7] and gas chromatographic assays [8]. However, these methods are ether non-specific or time-consuming. Different chromatographic methods for the quantitation of clindamycin in biological samples have been already available. Some methods require pre-treatment of clindamycin followed by chromatographic separation and detection by ultraviolet (UV) detection [9–13]. Although these methods meet many or all generally accepted criteria for bioanalytical methods validation, they are often cumbersome, require tedious pre-treatment procedures; with liquid–liquid pre-treatment and washing steps or a combination of liquid–liquid pre-treatment and solid-phase pretreatment, and require very long chromatographic time to resolve clindamycin from several endogenous peaks.

Other methods using HPLC coupled to mass spectrometers. Disadvantages of these methods are their inadequate internal standardization [14,15], the use of long-time gradient elution procedure [16], and the need of a liquid–liquid pre-treatment procedure before HPLC analysis [17].

The aim of the present study was to develop a simple HPLC assay for clindamycin quantitation en biological samples (plasma and saliva) that was precise and accurate at lower concentrations and was capable of a high throughput.

2. Experimental

2.1. Chemicals, reagents and solutions

Clindamycin (purity >99.0%) was a gift of Pfizer (New York, US). Lincomycin (I.S.) (purity >99.0%) (Fig. 1B) were provided by Fluka (Hannover, Deutschland). Acetonitrile, methanol (HPLC grade), were obtained from Merck (Darmstadt, Germany). Finally, Fluka (Hannover, Deutschland) provided trifluoroacetic acid. Ultra-pure water was obtained from a Wasserlab (Pamplona, Spain) system.

2.2. Standard solutions and samples

Stocks solutions of clindamycin and lincomycin with a concentration of 1 mg/ml were prepared separately by dissolving 10 mg of each analyte in methanol. Intermediate stock standards of 1, 10 and 100 μg/ml were prepared using methanol. Finally, eight standard solutions of clindamycin (1, 2, 10, 20, 50, 100, 200, and 300 μg/ml) were made by further dilution of the intermediate stock solution with appropriate volumes of methanol. The standard solution of lincomycin (2.5 μg/ml) was similarly prepared. Standard and stock solutions of clindamycin and lincomycin were stored at −80 °C.

Calibration pools of 0.05, 0.1, 0.5, 1, 2.5, 5, 10, and 15 μg/ml were prepared by diluting 0.5 ml of each spiking solution to 10 ml with potassium EDTA control human plasma in 10 ml volumetric flasks. Plasma quality control pools of 0.15, 0.75 and 7.5 μg/ml were prepared by diluting 0.5 ml of 3, 15 and 150 μg/ml spiking solution to 10 ml with potassium EDTA control human plasma in 10 ml volumetric flasks. Calibrator and quality control samples are placed into 1.2 ml propylene tubes and frozen at −80 °C until use.

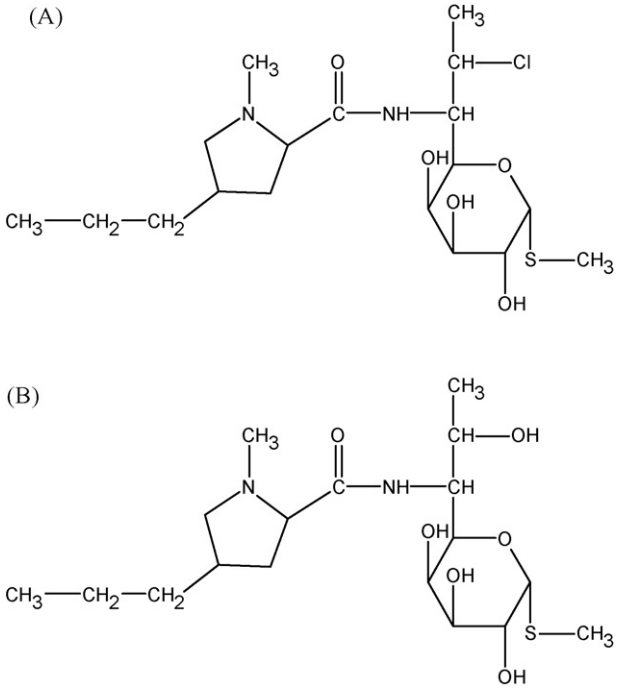

Fig. 1. Chemical structures of clindamicyn (A) and lincomycin (B).

2.3. Sample preparation

Plasma samples (0.5 ml) were transferred to eppendorf tube and spiked with the internal standard (25 μl of 2.5 μg/ml of lincomycin). Then, 1 ml methanol was added to the tubes. After vortex-mixed well for 2 min, the tubes were centrifuged at 10,000 × *g* for 10 min. The supernatants were transferred to limited volume autosampler vials, capped and placed on the HPLC autosampler. A 5-μl aliquot of the supernatant was injected onto HPLC column.

2.4. Apparatus and chromatographic conditions

The apparatus used for the HPLC analysis was a Model 1200 series Rapid Resolution liquid chromatographic system coupled with an triple quadrupole mass spectrometer (Agilent, Waldbronn, Germany). Data acquisition and analysis were performed with a Hewlett-Packard computer using the Masshunter program (version B.01.03). Separation was carried out at 50 °C on a rapid resolution, 30 mm × 2.1 mm column packed with C_{18}, 3.5 μm silica reversed-phase particles (Zorbax SB-C_{18} column®) obtained from Agilent (Agilent, Waldbronn, Germany). A security guard cartridge with the same package preceded this column. Mobile phase was a mixture of methanol–trifluoroacetic acid 0.01% (40:60, v/v). Separation was achieved by isocratic solvent elution at a flow-rate of 0.6 ml/min. Quantitation was done using multiple reaction monitoring (MRM) mode to monitor precursor → product ion transition of m/z 425.1 → 126.1 for clindamycin, and 407.2 → 126.0 for lincomycin.

2.5. Quantitation

Each calibration curve consisted of eight calibration points (0.05, 0.1, 0.5, 1, 2.5, 5, 10 and 15 μg/ml). Calibration curves were determined by least square linear regression analysis (weighting $1/X^2$) of peak area ratio of clindamycin and lincomycin versus the corresponding clindamycin concentration.

2.6. Validation

A thorough and complete method validation of clindamycin in human plasma and saliva was done following the US FDA guideline [18]. The method was validated for selectivity, sensitivity, interference check, linearity, precision and accuracy, recovery, matrix effect, cross-specificity, stability and dilution integrity.

The method was validated by analysis of human plasma and saliva quality control samples prepared at three concentrations spanning the calibration range (0.15, 7.5 and 7.5 μg/ml) and calibrator samples prepared as is above indicated. Three samples of each quality control pool, and calibration samples were analyzed on six different analytical batches. On batch 1 the number of samples each quality control pool was five. The linearity of the method was assessed by along six different analytical batches. To be acceptable the calibration curve had to have a correlation coefficient (r) of 0.995 (determination coefficient of 0.990) or better and the back-calculated concentration of the calibrator samples were ±15% deviation from the nominal value except at limit of quantitation, which was set at ±20%. Precision of a method was expressed as the percentage of the coefficient of variation of replicate measurements. Accuracy was measured according to the following equation:

$$\text{Percentage difference from theoretical value} = \left[\frac{X - C_T}{C_T}\right] \times 100$$

where X is the determined concentration of a quality control and C_T is the theoretical concentration. To be acceptable, the measures should be lower than 15% at all concentrations.

The overall recovery for clindamycin and lincomycin was calculated by comparing the peak-area ratios of spiked samples before and after the extraction in different lots of plasma at three concentration levels.

The matrix effect over clindamycin and lincomycin was determined by comparing the responses of the post-extracted plasma standard QC samples ($n = 3$) with the response of analytes from neat standard samples at equivalent concentrations.

The selectivity of the assay was determined by the individual analysis of blank samples. The retention times of endogenous compounds in the matrix were compared with those of clindamycin and lincomycin.

LOD was defined as the sample concentration resulting in a peak area of three times the noise level. LOQ was defined as the lowest drug concentration, which can be determined with an accuracy and precision <20%. In this work LOD of the assay method was determined by analysis of the peak baseline noise in 10 blank samples.

The stability of clindamycin in both frozen plasma and saliva samples (−80 °C) over 3 months, and in processed samples left at room temperature (20 ± 3 °C) over 24 h, was also studied.

2.7. Application of the method

To demonstrate the reliability of this method for the study of clindamycin pharmacokinetics, this assay was applied to the TDM of clindamicyn in plasma and saliva samples obtained from a cohort of 68 infectious patients treated with a different clindamycin regimens. Venous blood samples were withdrawn in EDTA tubes immediately prior to dosing and at 0.5, 1, 2, 3, 4 and 6 h postadministration. They were stored frozen (−20 °C) until analysis. Additionally, in all subjects, saliva (1 ml) was collected after stimulation of saliva flow with citric acid (10 mg on the tongue) at each of the following nominal times after oral drug intake: 0.5, 1, 2, 3, 4 and 6 h. Saliva was collected by asking the patient to spit into a cup or was aspirated with a syringe from the oral cavity, and store frozen until analysis.

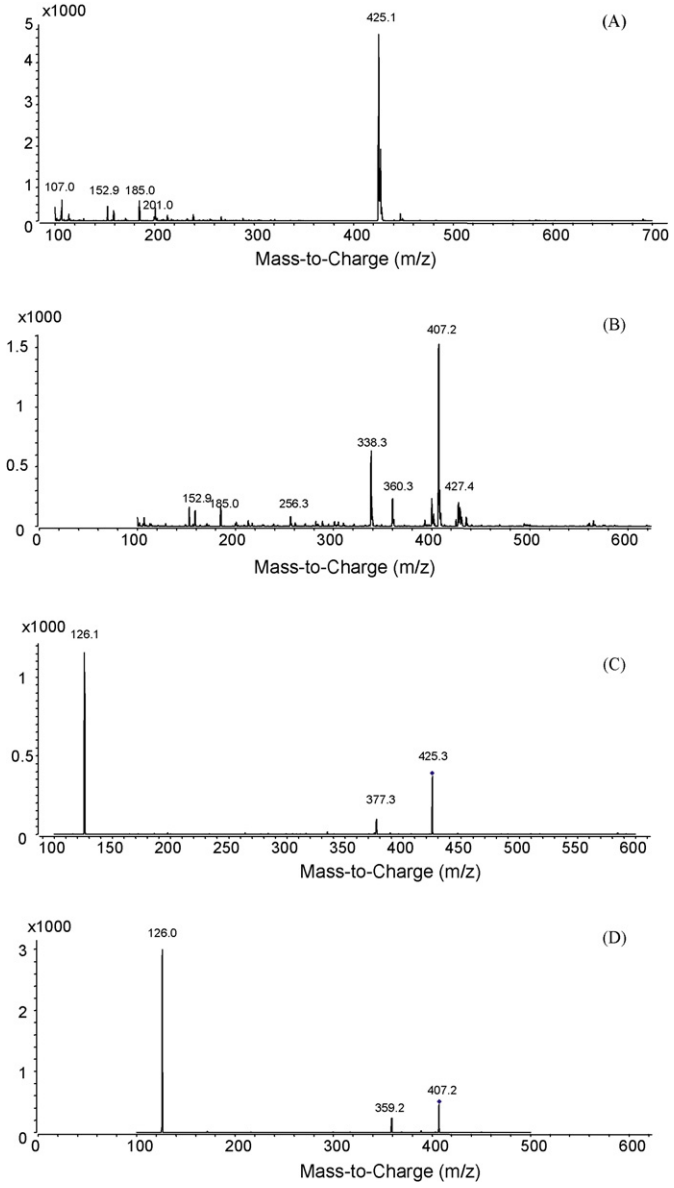

Fig. 2. ESI-MS and ESI-MS/MS spectra of clindamycin (A and C) and lincomycin (B and D), obtained after direct infusion of standard solutions of 10 μg/ml of clindamycin and lincomycin.

3. Results and discussion

Generally, TDM of drugs in humans involves analysis of a large number of samples and therefore requires simple, rapid and reliable analytical methods. Although there have been several reports documenting HPLC assays for the determination of the plasma concentration of clindamycin, these methods have some limitations. Longer sample pre-treatment duration, complex column switching, poor peak resolution, and inadequate internal standardization are the main drawbacks of the previous published methods [9–17]. As result these methods are time-consuming when large number of samples are assayed.

In this work, HPLC–MS/MS operation parameters were carefully optimized for the monitorization of clindamycin in plasma and saliva samples form infectious disease patients. ESI-MS of clindamycin (Fig. 2A), shows a characteristic protonated isotope pattern at m/z 425.1. After scanning the fragment at m/z 425.1 with collision-induced dissociation (CID), the product ion spectrum (Fig. 2C) showed the prominent fragment ions at m/z 377.3, and

126.1. The fragment ion at m/z 126.1 corresponds to the 3-propyl-N-methylpyrrolidine ion after loss of the rest of the molecule, while the ion at m/z 377.0 corresponds to the loss of $HSCH_3$ from the precursor. The ESI-MS of the chemical compound selected as internal standard, lincomycin is showed in Fig. 2B. ESI-MS shows the characteristic protonated ion $[M+H]^+$ at m/z 407.2 corresponding to their molecular formula. As can bee seen in Fig. 2D, the MS/MS spectra of lincomycin have very similar characteristic ions to observed in clindamycin mass spectra such as the loss of $HSCH_3$ and 3-propyl-N-methylpyrrolidine. Therefore, the transition to m/z 126 could be used for the MS detection of both clindamycin and lincomycin.

The procedure employed in this work offers a rapid way to isolate clindamycin from the plasma matrix. Although matrix ionization suppression is considered to be likely a problem when using the protein precipitation method for sample preparation, this pre-treatment method has been chosen as the sample preparation procedure due to its simplicity. However, it has to remember that the mass spectrometry system was easy contaminated after analysis of a lot of biological samples when organic precipitation is employed as drug sample pre-treatment procedure. In order to avoid mass spectrometry contamination with earlier eluted endogenous components of the sample matrix, a switch technique was developed. The first 0.1 min of eluate was switched away form the mass spectrometry detector and the eluate from 0.1 to 1.5 min were allowed to enter into mass spectrometric system.

Several packages were assayed to obtain an adequate chromatographic performance for clindamycin quantitation in human plasma. Finally, a rapid resolution column packed by 3.5 μm ultrapure silica particles performed with sterical protection the siloxane bond, that improved package stability, was employed. With the use of this column liquid chromatographic performance showed a remarkably improved since excellent peak shape is obtained for clindamycin and lincomycin without apply a gradient elution to separate the drug, internal standard and other minor elution peaks. Moreover, the retention time was decreased (clindamycin peak appeared at 1 min), and the peak was sharper, symmetric and displayed excellent baseline resolution from other peaks. Fig. 3 displays the chromatograms of real plasma and saliva samples. Blank samples did not show peaks corresponding to clindamycin and lincomycin retention times (panels A and C). The retention time of clindamycin and lincomycin was 1.22 ± 0.09 and 0.27 ± 0.02 min in plasma (panel B) and 1.1 ± 0.05 and 0.28 ± 0.03 min in saliva (panel D). As can be seen, in the chromatographic conditions described above clindamycin and lincomycin eluted with R_S value of 2.3. This R_S value reflected the high degree of selectivity that showed the chromatographic developed method. The symmetry factor of all peaks ranged from 0.9 to 1.1, values near to 1. The high selectivity degree of the method described here also allows us to simplify the extensive sample-preparation protocol that must be applied prior chromatographic analysis. It has to remember that previously developed techniques involved clean-up techniques with a combination of deproteinization and solid-phase or liquid–liquid pre-treatment procedures.

Assay performance of the present method was assessed by all the following criteria: linearity, accuracy, precision, LOD, LOQ, stability, and applicability pharmacokinetic studies and in therapeutic drug monitoring of clindamycin in infectious patients. The assays exhibited linearity between the response (y) and the corresponding concentration of clindamycin (x), over the 0.05–15 μg/ml range in the plasma and saliva samples. Results are presented in Table 1. For each point of calibration standards, the concentrations were back-calculated from the equation of the regression curves, and R.S.Ds. were computed. The obtained values are also reflected in Table 1. The calibration standard curve had a reliable reproducibility over the standard concentrations across the calibration range over six different batch analysis. The average regression ($n = 6$) was found to

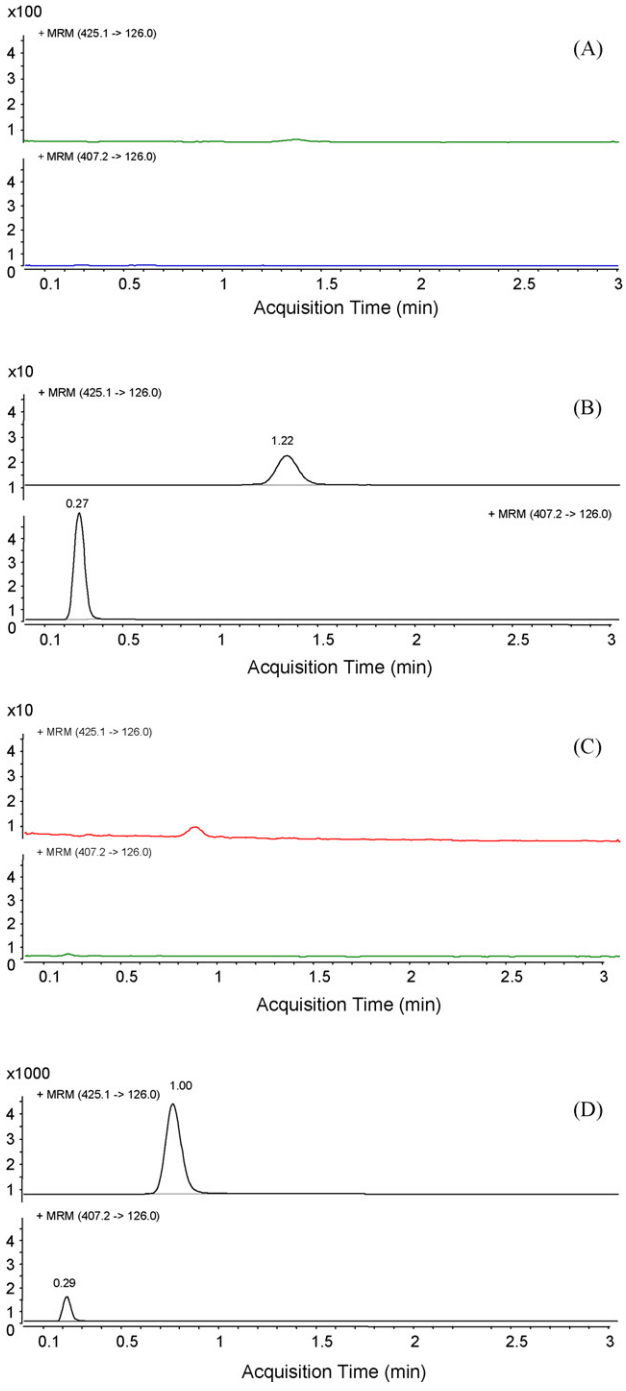

Fig. 3. Chromatograms of clindamycin (top) and lincomycin (bottom) from blank plasma sample (A), patient plasma sample extracted 4 h after clindamycin administration (clindamycin concentration: 0.17 μg/ml) (B), blank saliva sample (C), and saliva sample extracted from a infectious patient 4 h after clindamycin administration (clindamycin concentration: 3.15 μg/ml) (D).

be ≥0.995. The % accuracy observed for the mean of back-calculated concentrations for three calibration curves was within −10.0–8.0%; while the % precision values ranged from 2.22 to 11.11. The LOD of clindamycin in plasma and saliva was 2.5 and 2.3 ng/ml ($S/N = 3$) and the estimated LOQs were calculated as low as 10.1 and 9.8 ng/ml ($S/N = 10$), which are better than the previously obtained values from plasma samples [16,17]. The obtained values were confirmed with a result of 10.2 and 9.9 ng/ml for plasma and saliva samples ($n = 12$), with R.S.D. ≤5%. Although the imprecision and inaccuracy are acceptable we set the lower concentration of the drug

Table 1
Standard calibration curves of clindamycin in plasma and saliva samples: calibration curves (mean slope, intercept, and correlation coefficient, $n = 6$) and precision and accuracy data of back-calculated concentrations of calibration samples.

	r	Slope	Intercept	Concentration (μg/ml)							
				0.05	0.1	0.5	1	2.5	5	10	15
Plasma											
n	6	6	6	6	6	6	6	6	6	6	6
Mean	0.9985	0.0917	−0.00187	0.045	0.11	0.49	1.08	2.45	5.12	10.02	15.93
S.D.	0.0014	0.0013	0.00012	0.001	0.01	0.05	0.08	0.22	0.32	1.01	1.33
CV (%)[a]	0.14	1.36	6.42	2.22	9.09	10.20	7.41	8.98	6.25	10.08	8.35
Accuracy (%)[b]				−10.00	10.00	−2.00	8.00	−2.00	2.40	0.20	6.20
Saliva											
n	6	6	6	6	6	6	6	6	6	6	6
Mean	0.9991	0.0925	−0.00162	0.052	0.09	0.51	1.03	2.53	4.95	10.12	14.98
S.D.	0.0124	0.0011	0.00015	0.002	0.01	0.04	0.05	0.25	0.36	1.05	1.02
CV (%)[a]	1.24	1.19	9.26	3.85	11.11	7.84	4.85	9.88	7.27	10.38	6.81
Accuracy (%)[b]				4.00	−10.00	2.00	3.00	1.20	−1.00	1.20	−0.13

r = correlation coefficient.
[a] Expressed as %R.S.D. (S.D./mean) × 100.
[b] Calculated as [(mean determined concentration − nominal concentration)/nominal concentration] × 100.

that could be assayed at 50 ng/ml, an adequate value to perform the evaluation of clindamycin pharmacokinetic profile in infectious patients.

The pre-treatment recoveries of clindamycin in plasma and saliva samples were 95.1 ± 2.28% and 89.2 ± 1.35%, respectively. Similar values were obtained for the internal standard (95.1 ± 2.51% and 92.1 ± 2.13% for plasma and saliva samples).

The evaluation of matrix effect from the influence of coeluting components on analyte ionization is needed from all LC–MS/MS method. The effect of the matrix on ionization efficiency, expressed as the ratio of the peak area of analytes spiked after pre-treatment to that of the neat standard solutions in the mobile phase, were negligible with less than 12.5% of loss in the recovery values of clindamycin and the internal standard.

Accuracy values were within acceptable limits (Table 2). The results for within-day and between-day precision for our sample are presented in Table 3 and the values ranged between 3.5% and 8.2%, 7.5% and 11.5%, respectively. The obtained values for the precision evaluation study in saliva were also acceptable.

Clindamycin was stable in plasma and saliva samples stored at −80 °C for at least 6 months. The stability of clindamycin in processed samples left at 4 ± 0.3 °C over 24 h was also studied from our laboratory quality control setup for the drug at concentrations of 0.15, 7.5 and 15 μg/ml. Clindamycin and the internal standard were also stable in these conditions.

The applicability of this method has been demonstrated in vivo by the determination of clindamycin in plasma and saliva samples from transplant subjects receiving oral daily doses (Fig. 4). The use of saliva in TDM has expanded rapidly in the past 30 years, especially for anticonvulsants. Saliva sampling is non-invasive and painless, and does not require specially trained personnel to obtain the samples. Clindamycin concentration–time profiles in saliva and plasma are shown in Fig. 4. Clindamycin concentration profiles for saliva paralleled the plasma concentration profiles with a fairly constant saliva/plasma concentration ratio throughout the 6-h sampling period. Therefore saliva may be used for the TDM of clindamycin in infectious patients.

Table 2
Accuracy of the method, expressed as relative error in %, for determining clindamycin concentrations.

Accuracy ($n = 5$)		
Concentration added (μg/ml)	Concentration found (Mean ± S.D.) (μg/ml)	Accuracy (%)
Plasma		
0.05	0.05 ± 0.004	2.00
0.15	0.15 ± 0.013	1.33
0.75	0.74 ± 0.052	−1.00
7.5	7.6 ± 0.542	1.12
Saliva		
0.05	0.05 ± 0.001	4.00
0.15	0.15 ± 0.025	0.60
0.75	0.74 ± 0.016	−1.00
7.5	7.51 ± 0.543	0.16

Table 3
Between and within-day variability of the HPLC method for determining clindamycin concentrations.

Concentration added (μg/ml)	Between-day variability ($n = 18$)		Within-day variability ($n = 5$)	
	Concentration found (mean ± S.D.) (μg/ml)	C.V. (%)	Concentration found (mean ± S.D.) (μg/ml)	C.V. (%)
Plasma				
0.05	0.05 ± 0.005	9.64	0.05 ± 0.004	7.45
0.15	0.15 ± 0.017	11.47	0.15 ± 0.013	8.55
0.75	0.73 ± 0.072	9.95	0.74 ± 0.052	7.00
7.5	7.90 ± 0.722	9.14	7.6 ± 0.542	7.14
Saliva				
0.05	0.06 ± 0.005	8.18	0.05 ± 0.001	2.30
0.15	0.15 ± 0.016	10.96	0.15 ± 0.025	1.66
0.75	0.75 ± 0.055	7.36	0.74 ± 0.016	2.13
7.5	7.55 ± 0.652	8.64	7.51 ± 0.543	7.22

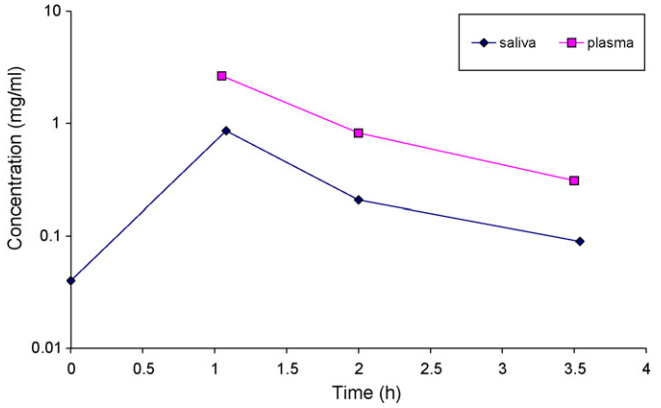

Fig. 4. Plasma and saliva concentrations–time profiles of clindamycin in one infectious patient after the intravenous administration of a dose of 600 mg clindamycin/6 h.

4. Conclusions

The analytical procedure described in this paper for the quantification of clindamycin in human plasma and saliva samples is simple, fast, selective and sensitive. The use of rapid resolution column and mass spectrometry detection allows the application of a minimal sample preparation procedure. Validation results prove that the developed method was accurate, reproducible and meets the recommendations of FDA guidelines. The method was successfully applied to analyze the clindamycin concentration–time profile in plasma and saliva samples in infectious patients.

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