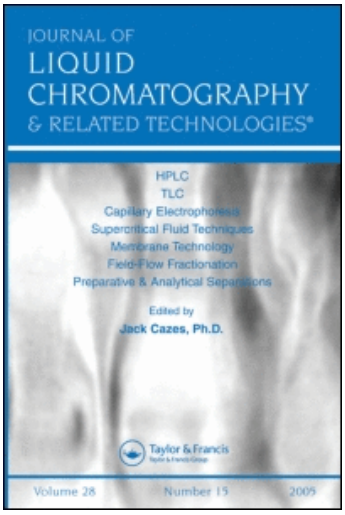

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Development and Validation of LC-MS/MS Method for the Simultaneous Determination of Quinine and Doxycycline in Pharmaceutical Formulations

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Abstract: A fast, sensitive, and specific liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed and validated for the simultaneous determination of quinine and doxycycline in pharmaceutical formulations. The LC-MS/MS method was carried out on a Sun Fire Waters C_{18} column (50 mm × 3.0 mm I.D.) and the mobile phase consisted of acetonitrile:0.1% formic acid (75:25, v/v), run at a flow rate of 0.45 mL/min (split 1:3). The injection volume was 10 μL for both standard and samples. The triple quadrupole mass spectrometer equipped with an electrospray source in positive mode (ES+) was set up in multiple reaction monitoring mode (MRM), monitoring the transitions of 325.0 > 307.0 and 445.0 > 428.1, for quinine and doxycycline, respectively. The total analysis time was 2 min and the method was linear in the concentration range of 10–1500 ng/mL for both compounds. Method validation investigated parameters such as the specificity, linearity, precision, accuracy, and robustness, giving results within the acceptable range. Moreover, the proposed method was successfully applied for determination of quinine and doxycycline in nanocapsule formulations to support the quality control.

Keywords: Doxycycline, LC-MS/MS, Quinine, Validation

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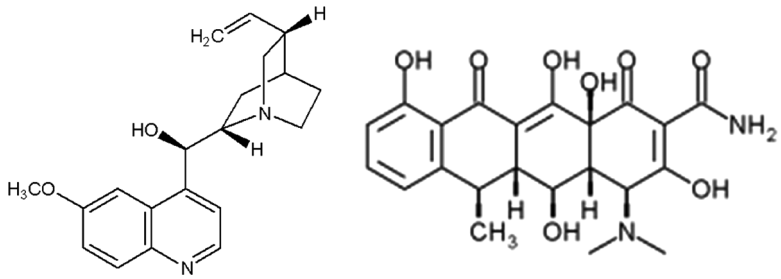
INTRODUCTION

Malaria is one of the most devastating tropical diseases caused by intracellular, protozoan parasites of the genus *Plasmodium* and more than 3 billion people live in malarial endemic regions. Five species of *Plasmodium* (*falciparum*, *vivax*, *ovale*, *malariae* and *knowlesi*) cause disease in humans and infection with *P. falciparum*, the most deadly of these parasites, results in more than 1 million deaths annually.[1,2] The World Health Organization (WHO) has declared malaria control a global development priority but this is not an easy task for many reasons. The antimalarial gap required to bring new and affordable drugs is large.[3]

P. falciparum, the most virulent of the plasmodial species, is increasingly difficult to treat because of the spread of parasite strains resistant to the former first line of antimalarials, chloroquine and sulfadoxine-pyrimethamine. Reduced susceptibility to quinine has also been observed, notably in Southeast Asia and South America, as well as the Pacific region and sub Saharan Africa. In areas where reduced susceptibility occurs, standard treatment regimens may no longer be adequate, and high concentrations of quinine, often combined with tetracycline or doxycycline, are implemented to achieve a clinical cure.[4]

Several HPLC methods are available for determination of quinine and doxycycline (Figure 1) separately in pharmaceutical formulations,[5–9] but there is no reported method for the simultaneous determination of both drugs.

It is essential to use well characterized and fully validated analytical methods to yield reliable results which can be interpreted satisfactorily. Analytical method validation includes all the experimental procedures and documentation, which demonstrates that a particular method used for quantitative measurement of analytes is reliable and suitable for the intended analytical applications. To prove this, a validation according to generally accepted guidelines from institutional bodies such as the



(a) (b)

Figure 1. Chemical structures of quinine (a) and doxycycline (b).

International Conference on Harmonization (ICH) or the Federal Drug Administration (FDA) is carried out. Fundamental parameters that require determination are specificity, linearity, accuracy, and precision.[10,11]

Liquid chromatography with UV detection (LC-UV) has been used for quality control of most of the pharmaceuticals due to its simplicity, high resolution, and satisfactory precision and accuracy. Otherwise, LC coupled with mass spectrometry (LC-MS) is a well established analytical method for the rapid identification and characterization of components in sample mixtures and has been widely used in clinical studies. Nowadays, this method has been increasingly applied for the analysis of pharmaceuticals, as it provides better efficiency of drug quantitation with a high degree of specificity and sensitivity. Moreover, as a consequence of the mass selectivity, it was expected that the time for the method development and sample turnover could be significantly reduced.[12–16]

This paper reports the development and validation of an LC-MS/MS method for the quantitation of quinine and doxycycline by specificity, linearity, accuracy, precision, limit of detection, limit of quantitation, and robustness. Moreover, it shows the applicability of the proposed method for the potency evaluation of both drugs in nanocapsule formulations.

EXPERIMENTAL

Chemicals and Reagents

Quinine and doxycycline reference substances were purchased from Sigma (Sigma, St Louis, USA) and Zhejiang Chem-tech (Zhejiang, China), respectively. All of the excipients from the nanocapsules formulations were obtained from different distributors: Epikuron 170 (Lucas Meyer, Hambourg, Alemanha), Miglyol 810 (Brasquim, Brazil), Poli (ε-caprolactona) (PCL) Mw = 65000 (Aldrich, Strasburg, França), and Polissorbato 80 (Delaware, Brazil). HPLC grade acetonitrile, acetone, methanol, formic, phosphoric, and acetic acid were purchased from Tedia (Fairfield, USA). All chemicals used were special analytical grade. For all the analyses, ultrapure water (Millipore, Bedford, MA, USA) filtered through a 0.22 μm membrane was used.

Apparatus and Analytical Conditions

LC-MS/MS

The LC-MS/MS method was performed on a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) equipped with a SCL-10A$_{VP}$ system controller,

LC-10 AD_{VP} pump, DGU-14A degasser, CTO-10 AD_{VP} column oven. The peak areas were integrated automatically by computer using a Masslynx (v. 3.6) software program. The experiments were carried out on a reversed phase sun fire (Waters, Milford, USA) C_{18} column (50 mm × 4.6 mm ID, with a particle size of 4 μm and pore size of 100 Å). A security guard holder (4.0 mm × 3.0 mm ID) was used to protect the analytical column. The LC system was operated isocratically at controlled temperature (35°C) using a mobile phase of acetonitrile/formic acid 0.1% (75:25, v/v). This was filtered through a 0.45 μm membrane (Millipore, Bedford, USA) and run at a flow rate of 0.45 mL/min (split 1:3). The injection volume was 10 μL for both standard and samples. The triple quadrupole mass spectrometer (Micromass, Manchester, UK), model Quattro LC, equipped with an ESI source using a crossflow counter electrode run in positive mode (ESI+), was set up in multiple reaction monitoring (MRM) mode, monitoring the transitions of 325.0 > 307.0 (quinine) and 445.0 > 428.1 (doxycycline).

For the optimization of mass spectrometer conditions, a mixed standard solution (1000 ng/mL) containing quinine and doxycycline was directly introduced and the following parameters were selected: cone gas and desolvation gas set at 40 and 400 L/h, respectively. Capillary voltage, extractor voltage, RF lens voltage, source temperature, and desolvation temperature were 3.00 kV, 5 V, 0.2 V, 120°C, and 400°C, respectively. The dwell time was set at 0.5 seconds; the collision gas pressure (argon) was 2.3×10^{-3} mbar. The cone voltage was 40 V (quinine) and 35 V (doxycycline) and the collision energy was 45 (quinine) and 20 V (doxycycline). Data acquisition and analysis were performed using the software Masslynx (v. 3.6) running under Windows XP on a workstation IBM PC.

Procedure

Preparation of Stock Solutions

The stock solutions of quinine and doxycycline were prepared by weighing 10 mg of the reference standards and the equivalent amount of the pharmaceutical sample, transferring each one to individual 10 mL volumetric flasks and diluting to volume with acetonitrile: water (50:50, v/v), obtaining a concentration of 1 mg/mL. The prepared stock solutions were stored at 2–8°C protected from light. Working standard solutions and samples of pharmaceutical formulations of quinine and doxycycline were prepared daily by diluting the stock solution to an appropriate concentration in acetonitrile:water (50:50, v/v).

Sample Preparation

The nanocapsule formulations were prepared by nanoprecipitation of preformed polymer.[17] Briefly, an acetone solution containing triglycerides, quinine, doxycycline, and poly (ε-caprolactone) was added into an aqueous solution containing polysorbate 80. Acetone was removed and the suspension concentrated by evaporation (bath at 40°C) under reduced pressure (4 bar). The final formulation was adjusted to obtain a drug suspension of 2 mg/mL.

Validation of the Method

The method was validated in samples of pharmaceutical formulations with the label claim of 2 mg/mL by the determination of the following parameters: specificity, linearity, precision, accuracy, limit of detection (LOD), limit of quantitation (LOQ), and robustness, following ICH guidelines.[18]

Specificity

The evaluation of specificity was performed by analyzing solutions of a placebo containing the same excipients of the nanocapsules formulation. The samples were chromatographed to determine the extent to which mobile phase components and excipients could contribute to the interference with the analytes. The results were compared with LOQ (10 ng/mL).

Linearity and Range

Linearity was determined by constructing three calibration curves. For the construction of each calibration curve, seven standard concentrations of quinine and doxycycline in the range of 10–1500 ng/mL (10, 100, 250, 500, 750, 1000, and 1500 ng/mL) were prepared in acetonitrile:water (50:50, v/v). Before injection of the solutions, the column was equilibrated for at least 20 min with the mobile phase flowing through the system. The peak area ratio of the drug against the respective standard concentrations was used for plotting the graph and the linearity evaluated by a weighted (1/x) least squares regression analysis.

Precision

The precision of the method was determined by repeatability and intermediate precision. Repeatability was examined by six evaluations of the same concentration sample of quinine and doxycycline (1000 ng/mL), on

the same day, under the same experimental conditions. The intermediate precision of the method was assessed by carrying out the analysis on two different days (inter-days) and also by another analyst performing the analysis in the same laboratory (between-analysts).

Accuracy

The accuracy was evaluated applying the proposed method to the analysis of the in-house mixture of the formulation excipients with known amounts of the quinine and doxycycline, corresponding to the concentrations of 80, 100, and 120%. The accuracy was calculated as the percentage of the drug recovered from the formulation matrix.

Limit of Quantitation and Limit of Detection

The limit of quantitation (LOQ) was taken as the lowest concentration of analyte in a sample that could be determined with acceptable precision and accuracy, and the limit of detection (LOD), was taken as the lowest absolute concentration of analyte in a sample that could be detected but not necessarily quantified.

Robustness

The robustness of an analytical procedure refers to its ability to remain unaffected by small and deliberate variations in method parameters and provides an indication of its reliability for the routine analysis. The robustness was determined by analyzing the same samples (1000 ng/mL) under a variety of conditions of the method parameters, such as flow rate and mobile phase composition, column temperature, and injection volume.

Analysis of Pharmaceutical Formulation

Samples of pharmaceutical formulations of quinine and doxycycline were prepared daily by diluting the stock solution to an appropriate concentration (1000 ng/mL) in acetonitrile:water (1:1, v/v). An aliquot of 10 μL was injected for the analysis and the amount of quinine and doxycycline per formulation calculated against the reference standard.

RESULTS AND DISCUSSION

To obtain the best chromatographic conditions, different columns and mobile phases consisting of acetonitrile-water or methanol-water were

tested to provide sufficient selectivity and sensitivity in a short separation time. Modifiers such as ammonium acetate, formic, and acetic acid were tested. Formic acid was selected because it was easily miscible with organic solvent and led to improved peak symmetry and ionization efficiency of quinine and doxycycline. The best signal was achieved using acetonitrile:formic acid (75:25, v/v) with a flow rate of 0.45 mL/min (split 1:3).

In the present study, electrospray (ESI) was used as the LC-MS/MS interface because the efficiency of ionization of quinine and doxycycline were higher than atmospheric pressure chemical ionization (APCI). The mass spectrometric response of the analytes were measured by using selected reaction monitoring in which the mass spectrometer is tuned to several sets of ions (multiple reaction monitoring, MRM). In this method, a set of precursor ion/product pairs was monitored. The protonated molecular ions $[M + H]^+$ of quinine and doxycycline on the full scan mass spectra were m/z 325.0 and 445.0, respectively. Moreover, the collision energy in Q2 produced significant fragments for quinine (183.7, 160.0, 252.7, and 307.0) and doxycycline (154.0, 338.7, 410.1, and 428.1). The MS/MS transition $325.0 > 307.0$ and $445.0 > 428.1$ were selected since the ion scan product with m/z 307.0 and 428.1 presented a higher abundance and stability for the quinine and doxycycline, respectively.

The coupling of LC with MS/MS detection in the MRM mode showed high specificity because only the ions derived from the analytes of interest were monitored. Therefore, the comparison of the chromatograms of the blank and LOQ (10 ng/mL) indicated that no interferences were detected from mobile phase and excipients of the formulation.

A typical chromatogram obtained by the proposed LC-MS/MS method, with the resolution of the symmetrical peak corresponding to quinine and doxycycline are shown in Figure 2. The low retention times of 2.0 minutes allows a rapid determination of the drugs, which is an important advantage for the routine analysis.

The linearity was determined by three determinations of the concentrations in the range of 10–1500 ng/mL. The values of the determination coefficient for quinine ($r^2 = 0.9957$) and doxycycline ($r^2 = 0.9989$) indicate significant linearity of the calibration curves for the method.

The precision evaluated as the repeatability of the method was studied by calculating the relative standard deviation (RSD) for six determinations of the concentration of 1000 ng/mL performed on the same day and under the same experimental conditions (intra-day, Table 1). The RSD values obtained were 1.23% and 1.05% for quinine and doxycycline, respectively.

The intermediate precision was assessed by analyzing two samples of the pharmaceutical formulation on three different days (inter-day,

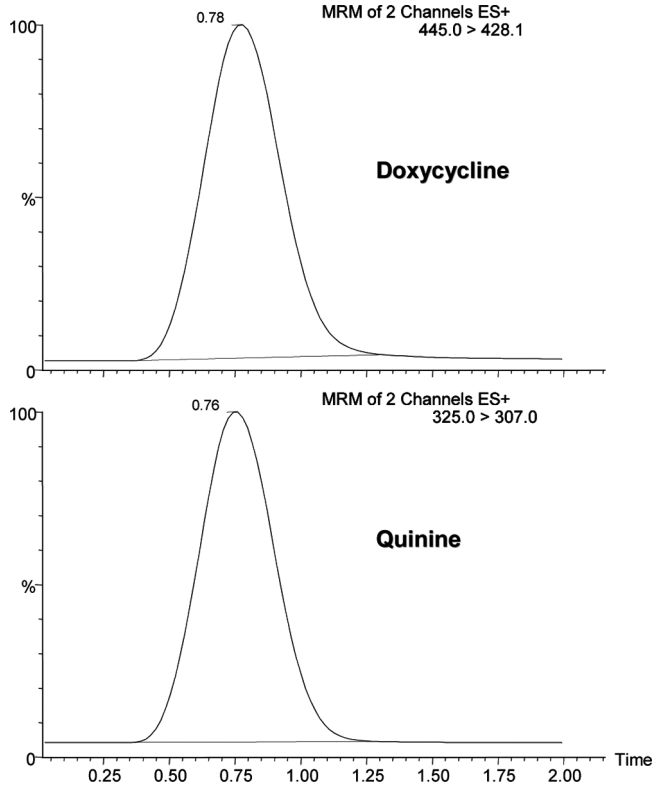


Figure 2. Representative LC-MS/MS chromatogram of quinine (10 ng/mL) and doxycycline (10 ng/mL).

Table 1. Intra-day precision for the determination of quinine and doxycycline in samples of pharmaceutical formulations

	Quinine			Doxycycline		
Sample	Amount (ng/mL)	Found (ng/mL)	(%)	Amount (ng/mL)	Found (ng/mL)	(%)
1	1000	1014.6	101.46	1000	1022.7	102.27
2	1000	994.1	99.41	1000	1015.5	101.55
3	1000	998.8	99.88	1000	996.1	99.61
4	1000	1025.7	102.57	1000	1001.4	100.14
5	1000	1011.3	101.13	1000	998.9	99.89
6	1000	1021.4	102.14	1000	1001.4	100.14
Mean	–	1010.98	101.10	–	1006.0	100.60
RSD (%)	–	1.23	1.23	–	1.05	1.05

Table 2. Between-day precision for the determination of quinine and doxycycline in samples of pharmaceutical formulation

		Quinine				Doxycycline			
Sample	Day	Found (ng/mL)[a]	(%)	Mean (%)	RSD (%)	Found (ng/mL)[a]	(%)	Mean (%)	RSD (%)
A	1	1003.4	100.34	99.54	1.92	1009.0	100.90	100.59	0.29
	2	1009.3	100.93			1003.1	100.31		
	3	973.6	97.36			1005.5	100.55		
B	1	997.7	99.77	99.36	1.78	1004.4	100.44	99.41	1.37
	2	1008.9	100.89			999.2	99.92		
	3	974.3	97.43			978.7	97.87		

[a]Mean of three replicates.

Table 2). The RSD values obtained for each sample were 1.92 and 1.78% for quinine and 0.29 and 1.37% for doxycycline. Between analysts precision was determined by calculating the RSD for the analysis of two samples of the pharmaceutical formulation by two analysts; the values were found to be 0.75 and 1.62% for quinine and 1.12 and 1.07% for doxycycline (Table 3).

The accuracy was assessed from three replicate determinations of three different solutions containing 800, 1000, and 1200 ng/mL. The mean value of 101.40% and RSD of 1.50% for quinine and 98.77% and RSD of 2.86% for doxycycline were obtained (Table 4), showing that the method is accurate within the desired range.

The LLOQ evaluated in an experimental assay, with the precision of 2.64% and accuracy of 101.42% for quinine and precision of 2.93% and accuracy of 99.23% for doxycycline, was found to be 10 ng/mL and LOD was found to be 0.1 ng/mL for both products.

Table 3. Between-analysts precision for the determination of quinine and doxycycline in samples of pharmaceutical formulation

		Quinine				Doxycycline			
Sample	Analyst	Found (ng/mL)[a]	(%)	Mean (%)	RSD (%)	Found (ng/mL)[a]	(%)	Mean (%)	RSD (%)
A	1	1002.9	100.29	99.76	0.75	995.5	99.55	100.35	1.12
	2	992.3	99.23			1011.4	101.14		
B	1	1010.8	101.08	99.94	1.62	993.5	99.35	100.11	1.07
	2	987.9	98.79			1008.7	100.87		

[a]Mean of three replicates.

Table 4. Accuracy for the determination of quinine and doxycycline in samples of pharmaceutical formulations

Analyte	Theoretical amount (ng/mL)	Experimental amount (ng/mL)[a]	Accuracy (%)	RSD (%)
Quinine	800	800.66	101.40	1.50
	1000	1030.70		
	1200	1212.82		
Doxycycline	800	770.74	98.77	2.86
	1000	1018.75		
	1200	1177.10		

[a]Mean of three replicates.

In order to assess the robustness, different parameters were evaluated: flow rate, column temperature, injection volume, and changing the mobile phase composition. The results and the experimental range of the selected variables are given in Table 5, together with the optimized values.

Analysis of Nanocapsule Formulations

The LC-MS/MS method validated in this paper was also used for the potency evaluation of quinine and doxycycline in nanocapsule formulations as shown in Table 6. The results demonstrated that the proposed

Table 5. Chromatographic conditions and range investigated during robustness testing

Variable	Range investigated	Quinine (%)[a]	Doxycycline (%)[a]	Optimized value
Column temperature (°C)	30	97.19	97.71	35
	35	100.28	99.42	
	40	101.25	98.41	
Injection volume (μL)	5	104.14	105.22	10
	10	101.30	100.14	
	20	103.58	99.38	
Flow rate (mL/min)	0.40	103.07	104.36	0.45
	0.45	101.11	100.92	
	0.50	102.40	103.24	
Acetonitrile (%)	70	103.99	102.07	75
	75	100.73	99.78	
	80	98.18	101.65	

[a]Mean of three replicates.

Table 6. Determination of quinine and doxycycline in nanocapsules formulations by the LC-MS/MS method

Analyte	Sample	mg/mL	(%)[a]	RSD (%)
Quinine	1	2	100.51	0.88
	2	2	101.67	1.23
Doxycycline	1	2	102.70	1.77
	2	2	101.75	1.19

[a]Mean of three determinations.

LC-MS/MS method can be used for the determination of quinine and doxycycline without prior separation of the excipients of the formulation, with the advantage of a very short time of analysis (2 minutes), representing also an improvement for the quality control of pharmaceuticals, as the technique is highly selective and sensitive. The method could be used for routine and in-process quality control analysis according to the intended analytical application and laboratory structure.

CONCLUSION

The results of the validation studies show that the LC-MS/MS method are specific, accurate, and possesses significant linearity and precision characteristics without any interference from the excipients. LC-MS/MS has the advantages of a very short time of analysis and the relatively low flow rate, allowing the analysis of a large number of samples with less mobile phase. Moreover, the LC-MS/MS demonstrated high sensitivity and selectivity, representing an alternative for the simultaneous quality control analysis of quinine and doxycycline.

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