

Development of the Liquid Chromatography Tandem Mass Spectrometry Method for Determination of Chloroquine and Desethylchloroquine in Human Plasma

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ABSTRACT

Objective: Several studies reported a relationship between adverse effect and elevated blood concentration of chloroquine (CQ) and its active metabolite, desethylchloroquine (DCQ). Therefore, therapeutic drug monitoring of these drugs might be useful. This study aims to develop and validate a liquid chromatography tandem mass spectrometry (LC-MS/MS) method for the determination of CQ and DCQ in human plasma.

Methods: Chromatographic separation was carried out on the Synergi® 2.5µm polar RP (150 x 4.6 mm.I.D.) and used 0.3% formic acid/acetonitrile at 70/30,v/v as a mobile phase. The mass spectrometry was operated with positive electro spray ionization and multiple reactions monitoring (MRM) mode. The mixture of methyl *t*-butyl ether and isooctane at 90/10,v/v with adjusted pH to 12 by ammonium hydroxide were used for extraction. The method was developed and fully validated according to USFDA guidelines.

Results: The ion transitions were 320.01 to 247.01 and 142.12 m/z for CQ and 292.0 to 179.01 and 114.10 m/z for DCQ. The lower limit of quantification (LLOQ) were 0.2 and 0.4 ng/mL for CQ and DCQ, respectively. This method had acceptable accuracy and precision with good linearity ($r^2 > 0.997$) and high recovery of extraction (89.34-108.42%). The ranges of quantification were 0.2-1,000 ng/mL for CQ and 0.4-1,000 ng/mL for DCQ. Neither anticoagulant nor matrix had any effect which interfered with this analysis.

Conclusion: A highly selective, sensitive and reproducible LC-MS/MS method to detect CQ and DCQ level in human plasma was developed and validated. This method was successfully applied to determine the steady state concentration of CQ and DCQ in plasma of rheumatoid arthritis patients.

Keywords: LC-MS/MS, chloroquine, desethylchloroquine

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INTRODUCTION

Chloroquine (CQ) was originally used for malarial prevention and treatment. Later on, its anti-inflammatory property was disclosed, thus its use was expanded to treat various inflammatory diseases such as rheumatoid arthritis (RA) and lupus erythematosus.¹

Although CQ has long been used, its mechanism of action is not well understood. CQ is well absorbed when

administered orally. The bioavailability is nearly 80%. Its volume of distribution is high and its protein binding capacity is approximately 55%. CQ is metabolized by the cytochrome P450 (CYP) system in which CYP2C8, CYP3A4 and CYP2D6 are the major enzymes. CQ is de-ethylated to desethylchloroquine (DCQ), its major active metabolite.² The elimination half-life of CQ ranges from 10 days to 2 months. The adverse effects of CQ have been noted, which are more likely to appear when used to treat inflammation due to higher dosage and longer duration. The most serious adverse effect is retinopathy which can cause blindness.³ Several studies reported a relationship between adverse effects and elevated blood concentrations of CQ and DCQ,⁴⁻⁶ thus monitoring their blood levels is an effective way for early detection of such adverse effects.

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A number of methods for determination of CQ or DCQ concentration in biological fluid using different techniques were reported including spectrophotometry,⁷ high performance thin layer chromatography (HPTLC),⁸ high performance liquid chromatography (HPLC)⁹⁻¹⁵ and liquid chromatography tandem mass spectrometry (LC-MS/MS).¹⁶⁻¹⁸ One study reported that the steady state blood concentration of CQ and DCQ were very low and variable which ranged from 36.6-3,895 ng/mL for CQ and 24.7-1,506 ng/mL for DCQ, respectively.⁴ Thus, highly specific and sensitive techniques like LC-MS/MS were required for accurate analysis. The previously reported LC-MS/MS method determined only CQ level in human plasma.¹⁸ DCQ was also suspected to play a role in adverse effects, therefore monitoring its level should have advantages. To date, no data for determination of DCQ in human plasma has been reported.

In this study, we aim to develop the LC-MS/MS method for determination of both CQ and DCQ concentrations in human plasma. We expect that our method will be useful for monitoring these drug levels in clinical practice.

MATERIALS AND METHODS

1. Chemicals

Chloroquine diphosphate (CQ) and internal standard (IS), hydroquinidine (HQD), were purchased from Sigma-Aldrich Ltd. (Steinheim, Germany). Desethylchloroquine (DCQ) was kindly supported by Dr. Michale D. Green from the Center for Disease Control and prevention (CDC), USA. Their structures have been presented in Fig 1. HPLC grade acetonitrile and methanol were purchased from Labscan Ltd. (Bangkok, Thailand). Milli-Q water from water purification system, Millipore Corporation (Massachusetts, USA) was used. Other chemicals were of analytical grade.

2. Instrumentations

AcquityTM Ultra Performance Liquid Chromatography from Waters Co. Ltd. (USA) was used for the separation module. Chromatographic separation was carried out on a Synergi polar RP column, 2.5 μ m (150 x 4.6 mm.i.d.) from Phenomenex Ltd. (CA, USA). The mobile phase was isocratic conditions of 0.3% formic acid/acetonitrile (70/30, v/v) which was pumped at a flow rate of 0.2 mL/min. Mass analyses were performed on QuattroPremier™, Micromass Technologies (UK). The mass spectrometry was operated with positive electro spray ionization mode and multiple reaction monitoring as acquisition mode. The software Masslynx version 4.1 was used for data management.

3. Preparation of standard stock solutions

Standard stock solutions of CQ, DCQ and HQD were prepared with 1% formic acid/methanol (50/50, v/v). The

dilution of stock solution to concentrations which ranged from 2 to 10,000 ng/mL were used as working solution. The quality control (QC) samples were prepared at 0.2, 0.6, 550, and 950 ng/mL for CQ and 0.4, 1.2, 550 and 950 ng/mL for DCQ.

4. Sample preparations

Ten μ L of IS, HDQ (50 ng/mL) was added to 100 μ L of plasma then 60 μ L of ammonium hydroxide solution was added for adjusting the pH to 12. Afterwards, 1 mL of methyl *t*-butyl ether (MTBE)/isooctane (90/10, v/v) was added, mixed for 10 minutes and centrifuged at 10,000 rpm for 15 minutes. Eight hundred μ L of organic layer was transferred into a conical polypropylene tube and evaporated under nitrogen stream. The residue was reconstituted by adding 200 μ L of 0.3% formic acid/acetonitrile (70/30, v/v), and 180 μ L of this solution was transferred into a vial and 10 μ L were injected.

5. Bioanalytical method validation

The developed method was fully validated according to the USFDA guidelines.¹⁹

5.1 Selectivity and sensitivity

The selectivity was examined using six sources of free drug plasma which were extracted and analyzed by the developed method. The result should not have any interfering peak of CQ and DCQ. The sensitivity at the lower limit of quantification (LLOQ) was also examined by dilution of standard compound then quantification of the lowest detectable concentration using the developed method.

5.2 Accuracy and precision

Accuracy and precision were examined by six replicate analyses of QC samples at LLOQ, low, medium and high concentrations for three separate days. The percentage of relative error (%RE), indicating accuracy, was calculated as the experimental concentration divided by the nominal concentration. The percentage of coefficient of variation (%CV), indicating precision, was obtained by dividing standard deviation by the mean of the measured value. Both %RE and %CV should not deviate by more than 20% at LLOQ and 15% at other concentrations.

5.3 Linearity and calibration curve

A calibration curve was represented by a linear regression model, $y=mx+b$ and weighted by $1/x$, where y was the response of the peak area of analyte compared with the peak area of IS, x is the concentration of the interested drug at different levels including blank, zero, 0.2, 2, 20, 150, 300, 500, 750 and 1,000 ng/mL for CQ, and blank, zero, 0.4, 4, 40, 200, 400, 600, 800 and 1,000 ng/mL for DCQ, respectively. Lastly, the coefficient of determination (r^2) was calculated and its value had to exceed 0.995.

5.4 Recovery of extraction

The recovery of method was performed by comparing three different concentrations of the extracted QC samples with non-extracted standards. The percentage of absolute recovery (%RV) was calculated from the measured extracted concentration divided by non-extracted concentration. At least 80% was accepted.

5.5 Stability

The stability of analytes was tested by comparing results of three replicate QC samples analysis at low, medium and high concentrations under various conditions with freshly prepared samples. The first condition was freeze and thaw in which analytes were frozen at -70°C and thawed at room temperature for three cycles. For a short term stability test, analytes were stored at room tem-

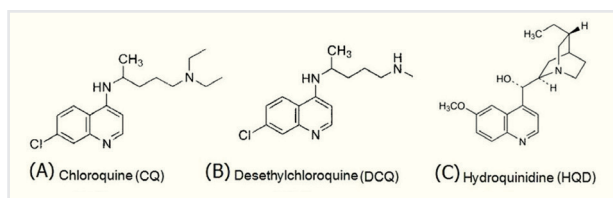


Fig 1. Chemical structure of (A) Chloroquine, (B) Desethylchloroquine and (C) Hydroquinidine.

perature for 6 hours. To test long term stability, samples were frozen at -70°C for 1 month. Lastly, post-preparative stability was done by placing the processed QC samples in the auto-sampler at 5°C for 10 hours, and another set of QC samples were processed and frozen at -70°C for 96 hours. The acceptable percentage of variation in each condition was within $\pm 15\%$.

5.6 Anticoagulant effect

Anticoagulant effect was examined by comparing the results of the analysis of six replicate QC samples at three different concentrations using two anticoagulants, lithium heparin which was used in patient's plasma and citrate phosphate dextrose which was used for method validation. Afterward, %CV and %RE were calculated. The accepted %CV was within $\pm 15\%$ and 85-115% for %RV.

5.7 Matrix effect

The matrix effect was determined by comparing results of the analysis of six replicate QC samples at three different concentrations in the presence and absence of plasma. The matrix factor (MF) was calculated as the ratio of the peak response area between absence and presence of matrix ions. The value within 0.8-1.2 indicates no matrix effect.

6. Bio-analysis

This method was used to determine the steady state concentration of CQ and DCQ in the plasma of 30 rheumatoid arthritis patients. The protocol was approved by Siriraj Institutional Review Board, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand.

RESULTS

The standard CQ, DCQ and IS were injected to the MS/MS. All parameters were tuned to optimize the most sensitive ion transitions. The main parameters have been summarized in Table 1 and the product ions spectra have been shown in Fig 2.

The determination of CQ and DCQ in blank plasma without interfering peak revealed the method selectivity as shown in Fig 3. The appropriate retention time when spiked with plasma have also been shown in Fig 3 as 1.82, 1.68 and 2.20 minutes, for CQ, DCQ and IS, respectively.

High sensitivity of the method was demonstrated by the LLOQ at 0.2 ng/mL for CQ and 0.4 ng/mL for DCQ. The accuracy and precision were acceptable as summarized in Table 2. This method had good linearity ($r^2=0.997$) as illustrated in Fig 4. The samples were stable in various tested conditions as demonstrated in Table 3. The extraction efficiency was high as %RV ranged from 89.34-108.42%, 98.67-107.79% and 98.92% for CQ, DCQ and IS, respectively. There was no anticoagulant effect

as all values were acceptable (%CV=4.23-9.52, %RE=96.13-98.50 for CQ, %CV=4.89-7.41, %RE=97.85-104.85 for DCQ). The MFs were 0.98-1.25 for CQ and 0.86-1.12 for DCQ which indicated negligible matrix effect.

The measured drug concentrations of patients' plasma had high variability as shown by the wide range of 3.34-572.03 ng/mL for CQ and 3.00-230.36 ng/mL for DCQ. The chromatograms have been shown in Fig 3. The clearance of both drugs were calculated from their steady state concentrations and revealed high inter-individual variability which ranged from 7.28-1073.27 L/hr. However, this result was in agreement with other reports.

DISCUSSION

The LC-MS/MS technique used in our study was very specific since both CQ and DCQ were ionized to their two

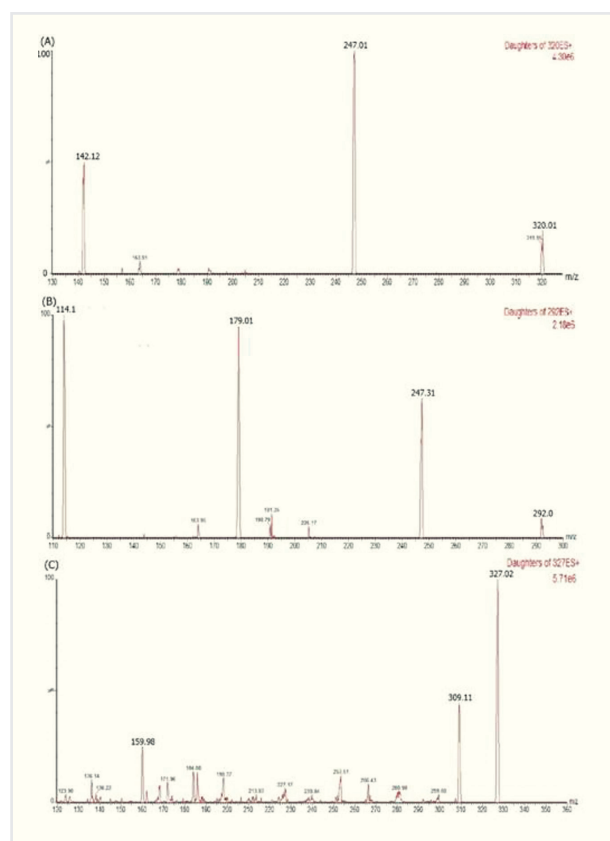


Fig 2. Production spectra on MS/MS of (A) Chloroquine at 320.01 to 247.01 and 142.12m/z, (B) Desethylchloroquine at 292.0 to 179.01 and 114.1 m/z and (C) Hydroquinidine at 327.02 to 309.11 and 159.98 m/z.

TABLE 1. The main parameters of MS/MS.

Parameter	Chloroquine	Desethylchloroquine	Hydroquinidine
Source Temperature (°C)	120	120	120
Desolvation Temperature (°C)	350	350	350
Capillary (kV)	0.5	0.5	0.25
Cone (V)	30	25	35
Cone Gas Flow (L/Hr)	30	30	30
Desolvation Gas Flow (L/Hr)	750	750	750
Ion Energy 1(V)	0.5	0.8	0.5
Collision (eV)	20	20	25
Ion Energy 2 (V)	0.8	1	0.8
Syring Pump Flow (uL/min)	2	20	15

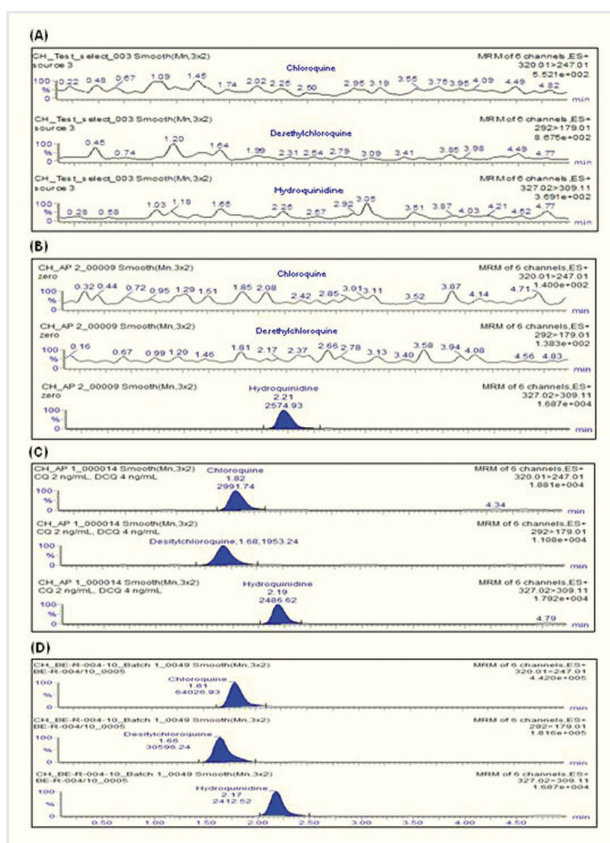


Fig 3. The chromatograms of extracted (A) blank plasma, (B) plasma spiked with IS (C) human plasma spiked with CQ, DCQ and IS (D) patient's plasma sample.

TABLE 2. The precision and accuracy (within and between days).

Expected concentration (ng/mL)	Nominal concentration (Mean±SD)	Precision (%)	Accuracy (%)
Chloroquine			
<i>Within day (n=6)</i>			
0.2	0.19 ± 0.03	13.76	99.32
0.6	0.58 ± 0.03	4.46	97.02
550	570.02 ± 38.97	6.84	103.64
950	946.98 ± 34.34	3.63	99.68
<i>Between days (n=18)</i>			
0.2	0.20 ± 0.02	11.82	98.51
0.6	0.61 ± 0.05	8.09	101.87
550	552.41 ± 41.50	7.51	100.44
950	902.18 ± 48.10	5.33	94.97
Desethylchloroquine			
<i>Within day (n=6)</i>			
0.4	0.45 ± 0.05	11.93	111.47
1.2	1.20 ± 0.01	1.03	100.85
550	536.71 ± 42.48	7.91	98.28
950	952.37 ± 21.34	2.24	98.93
<i>Between day (n=18)</i>			
0.4	0.46 ± 0.04	9.44	111.45
1.2	1.21 ± 0.04	2.91	100.83
550	540.52 ± 30.94	5.72	98.28
950	939.86 ± 32.10	3.41	98.93

n = number of replicate

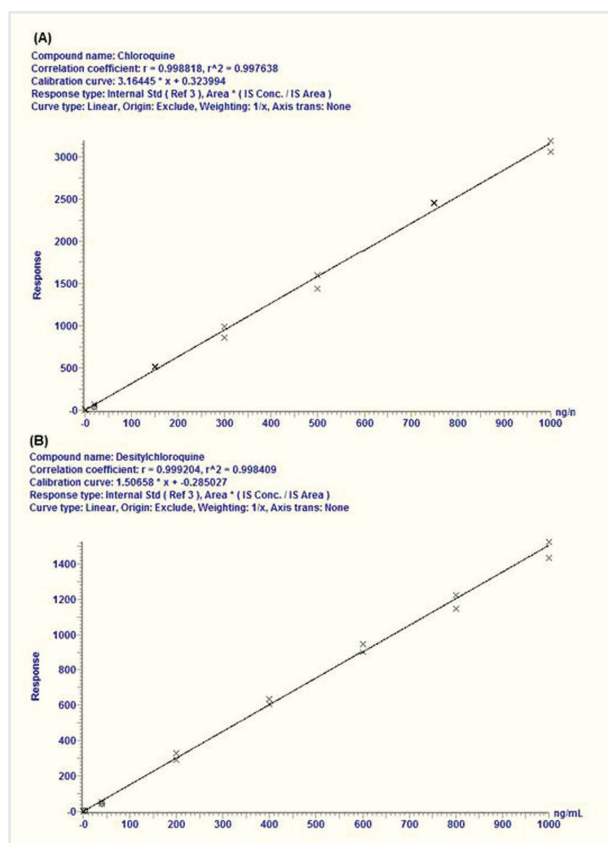


Fig 4. The calibration curve of (A) Chloroquine and (B) Desethylchloroquine.

daughter ions. The sensitivity of method for CQ detection was 6 to 20 times better than previously published studies.¹⁶⁻¹⁸ Moreover, it was extended to test for anticoagulant and matrix effects to ensure the lack of these effects when this method was applied. Anticoagulant effect should be tested when two different types of anticoagulant are used. A value within the acceptable range in our study suggested

TABLE 3. The stability of chloroquine and desethylchloroquine.

Concentration (ng/mL)(n=3)	Variation (%)	
	Chloroquine	Desethylchloroquine
Freeze and thaw		
0.2(CQ), 0.4(DCQ)	-1.32	-4.62
550	0.30	1.53
950	4.09	-2.56
Short term		
0.2(CQ), 0.4(DCQ)	-5.27	2.68
550	7.96	8.85
950	5.23	3.13
Long term		
0.2(CQ), 0.4(DCQ)	1.24	-1.67
550	2.65	4.66
950	7.03	9.3
Post-preparative set 1		
0.2(CQ), 0.4(DCQ)	-1.32	-4.71
550	0.3	6.17
950	4.09	2.9
Post-preparative set 2		
0.2(CQ), 0.4(DCQ)	-4.44	0.31
550	-0.43	0.35
950	4.85	-0.27

n = number of replicate

no anticoagulant effect. For matrix effect, the MF between 0.8-1.2 indicates that other interfering substances in the sample cannot significantly suppress or enhance ionization so both accuracy and precision were not altered.

CONCLUSION

The LC-MS/MS method for detection of CQ and DCQ in human plasma was developed and fully validated according to USFDA guidelines. This method was successfully applied to determine steady state plasma concentrations of these drugs in the clinical study. Both steady state plasma concentration and calculated drug clearance were dose independent with high inter-individual variability which were consistent with other reports.

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REFERENCES

- Cooper RG, Magwere T. Chloroquine: novel uses & manifestations. *Indian J Med Res.* 2008 Apr;127(4):305-16.
- Projean D, Baune B, Farinotti R, Flinois JP, Beaune P, Taburet AM, et al. In vitro metabolism of chloroquine: identification of CYP2C8, CYP3A4, and CYP2D6 as the main isoforms catalyzing N-desethylchloroquine formation. *Drug Metab Dispos.* 2003 Jun;31(6):748-54.
- Tzekov R. Ocular toxicity due to chloroquine and hydroxychloroquine: electrophysiological and visual function correlates. *Doc Ophthalmol.* 2005 Jan;110(1):111-20.
- Augustijns P, Geusens P, Verbeke N. Chloroquine levels in blood during chronic treatment of patients with rheumatoid arthritis. *Eur J Clin Pharmacol.* 1992;42(4):429-33.
- Munster T, Gibbs JP, Shen D, Baethge BA, Botstein GR, Caldwell J, et al. Hydroxychloroquine concentration-response relationships in patients with rheumatoid arthritis. *Arthritis Rheum.* 2002 Jun;46(6):1460-9.
- Tett SE, Cutler DJ, Beck C, Day RO. Concentration-effect relationship of hydroxychloroquine in patients with rheumatoid arthritis: a prospective, dose ranging study. *J Rheumatol.* 2000 Jul;27(7):1656-60.
- Khalil SM, Mohamed GG, Zayed MA, Elqudaby HM. Spectrophotometric determination of chloroquine and pyrimethamine through ion-pair formation with molybdenum and thiocyanate. *Microchem J.* 2000;64(2):181-6.
- Dwivedi AK, Saxena D, Singh S. HPLC and HPTLC assays for the antimalarial agents chloroquine, primaquine and bulaquine. *J Pharm Biomed Anal.* 2003 Nov 24;33(4):851-8.
- Walker O, Ademowo OG. A rapid, cost-effective liquid chromatographic method for the determination of chloroquine and desethylchloroquine in biological fluids. *Ther Drug Monit.* 1996 Feb;18(1):92-6.
- Minzi OM, Rais M, Svensson JO, Gustafsson LL, Ericsson O. High-performance liquid chromatographic method for determination of amodiaquine, chloroquine and their monodesethyl metabolites in biological samples. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2003 Jan 15;783(2):473-80.
- Deng H, Liu H, Krogstad FM, Krogstad DJ. Sensitive fluorescence HPLC assay for AQ-13, a candidate aminoquinoline antimalarial, that also detects chloroquine and N-dealkylated metabolites. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2006 Apr 3;833(2):122-8.
- Lejeune D, Souletie I, Houze S, Le bricon T, Le bras J, Gourmel B, et al. Simultaneous determination of monodesethylchloroquine, chloroquine, cycloguanil and proguanil on dried blood spots by reverse-phase liquid chromatography. *J Pharm Biomed Anal.* 2007 Feb 19;43(3):1106-15.
- Dua VK, Kar PK, Gupta NC, Sharma VP. Determination of chloroquine and desethylchloroquine in plasma and blood cells of Plasmodium vivax malaria cases using liquid chromatography. *J Pharm Biomed Anal.* 1999 Oct;21(1):199-205.
- Samanidou VF, Evangelopoulou EN, Papadoyannis IN. Simultaneous determination of quinine and chloroquine anti-malarial agents in pharmaceuticals and biological fluids by HPLC and fluorescence detection. *J Pharm Biomed Anal.* 2005 Jun 1;38(1):21-8.
- Cheomung A, Na-Bangchang K. HPLC with ultraviolet detection for the determination of chloroquine and desethylchloroquine in whole blood and finger-prick capillary blood dried on filter paper. *J Pharm Biomed Anal.* 2011 Jul 15;55(5):1031-40.
- Dos Santos Magalhaes IR, Sueli Bonato P. Enantioselective determination of chloroquine and its n-dealkylated metabolites in plasma using liquid-phase microextraction and LC-MS. *J Sep Sci.* 2008 Sep;31(16-17):3106-16.
- Singhal P, Gaur A, Behl V, Gautam A, Varshney B, Paliwal J, et al. Sensitive and rapid liquid chromatography/tandem mass spectrometric assay for the quantification of chloroquine in dog plasma. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2007 Jun 1;852(1-2):293-9.
- Hodel EM, Zanolari B, Mercier T, Biollaz J, Keiser J, Oliaro P, et al. A single LC-tandem mass spectrometry method for the simultaneous determination of 14 antimalarial drugs and their metabolites in human plasma. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2009 Apr 1;877(10):867-86.
- U.S. Department of Health and Human Services, Food and Drug Administration. Guidance for Industry: Bioanalytical Method Validation. 2001.p.1-25.