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Simultaneous determination of quinine and chloroquine anti-malarial agents in pharmaceuticals and biological fluids by HPLC and fluorescence detection

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Abstract

Even nowadays millions of people suffer and even die each year from malaria and hundreds of millions of people especially in tropical countries.

Quinine (Q) a natural occurring alkaloid and chloroquine (CQ) a synthetic drug are widely used as anti-malarial agents. Herein an isocratic reversed-phase high performance liquid chromatographic (RP-HPLC) method is described for the simultaneous determination of quinine and chloroquine, at low concentrations, in pharmaceuticals and biological fluids. The present method is characterized by higher sensitivity and analytes are separated in less time than the already published methods. The analytical column, an MZ Kromasil, C_{18}, 5 μm, 250 × 4 mm, was operated at ambient temperature with backpressure values of 230 kg/cm^2. Mobile phase consisted of methanol–acetonitrile–0.1 mol/L ammonium acetate, (45:15:40 v/v) at a flow rate of 1.0 mL/min.

Fluorescence detection was performed at excitation 325 nm and emission 375 nm, respectively. Salicylic acid was used as internal standard at a concentration of 0.5 ng/μL, resulting in a detection limit of 0.3 ng, while upper limit of linear range was 0.7 ng/μL for quinine and 0.5 ng/μL for chloroquine. Separation was completed within 5 min.

The statistical evaluation of the method was examined performing intra-day ($n = 8$) and inter-day calibration ($n = 8$) and was found to be satisfactory, with high accuracy and precision results. Solid phase extraction provided high relative extraction recoveries from biological matrices: 92.1% for quinine and 105.4% for chloroquine from blood serum and 101.8% for quinine and 90.7% for chloroquine from urine.
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Keywords: Quinine; Chloroquine; Anti-malarial agents; Pharmaceuticals; Biological fluids; Blood serum; Urine; HPLC; SPE

1. Introduction

The cinchona alkaloids are an important subgroup of the polycyclic β-carboline alkaloids. Quinine (6′-methoxycinchonan-9-ol) a natural occurring alkaloid is a bitter tasting powder extracted from the bark of the cinchona tree of South America. It has been used in medicine for ages, as it has recognized anti-malaria properties. Quinine is also used for treatment of muscle cramps and it is commonly prescribed in the UK for leg cramps. Additionally, it is used as additive in soft drinks such as tonic water due to its bitter taste [1–7].

Quinine has been widely used for the treatment of malaria, a parasitical disease spread by mosquitoes, since 1633. However, quinine is also a potentially toxic drug and its overuse has been determined to cause and complicate other health conditions. It was linked to several cases of sudden death, as it relaxes muscles like the heart. The typical syndrome of quinine side effects is called cinchonism and it can be mild in usual therapeutic dosage or severe in larger doses. The toxic effects of quinine appear to be related to plasma con-

Abbreviations: CQ, Chloroquine; FL, Fluorescence detection; Q, Quinine; HPTLC, High performance thin layer chromatography; ICH, International Conference on Harmonization; IS, Internal standard; LOD, Limit of detection; LOQ, Limit of quantitation; SPE, Solid phase extraction; RP-HPLC, Reversed-phase high performance liquid chromatography; RSD, Relative standard deviation; UV, Ultra-violet detection

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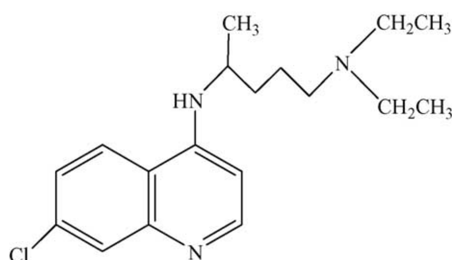
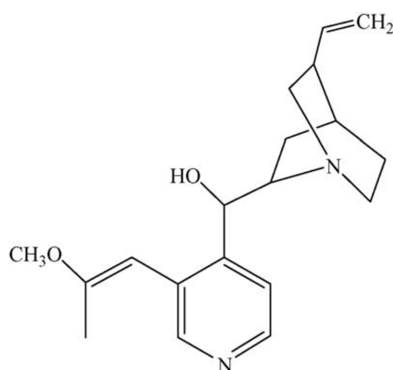
centrations. Quinine side effects include ringing in the ears, vertigo, nausea, blurred vision, diarrhea, abdominal pain, headache and fever, disturbed vision, renal failure, chest pain and asthma. It should not be prescribed during pregnancy as it can cause birth defects and miscarriages [8].

Though quinine was the only effective treatment for malaria for 300 years, after World War II, it was supplanted by synthetic drugs such as chloroquine that were safer and easier to make. Chloroquine (7-chloro-4-[-4-diethylamino-l-methylbutyl-amino] quinoline) is the prototype synthetic anti-malarial drug most widely used to treat all types of malarial infections. It was also the cheapest time tested and safe anti-malarial agent. However, in recent years new outbreaks of synthetic drug-resistant strains of malaria are causing continuing high levels of the sometimes-fatal disease in parts of Africa, Asia, and Latin America. Recovered patients can relapse months or even years after the primary lung injury if the initial treatment is insufficient or if the parasite was resistant to the medication used [9–11].





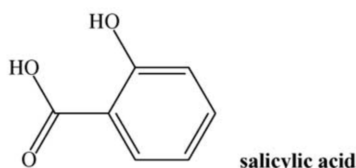


Fig. 1. Chemical structures of quinine, chloroquine and salicylic acid.

Strains of malaria resistant to the anti-malarials have begun to make their appearance and the value of quinine as an anti-malarial agent is once again on the rise.

The chemical structure of quinine and chloroquine are shown in Fig. 1.

Nowadays, drug combinations are increasingly used as the treatment of choice for malaria, so researchers are developing analytical tools that monitor more than one drug in a patient's blood, simultaneously. Scientists at the Central Drug Research Institute (CDRI) in Lucknow, India, have reported a technique that simultaneously monitors the blood levels of chloroquine and bulaquine as they are introduced in combination by a pharmaceutical company in India [11,12].

Several methods have been reported for the single determination of quinine and chloroquine concentration in human biological fluids. These methods include the use of normal [13] or reversed-phase columns [7,14–16] after liquid–liquid extraction of the drugs [10,13] and either UV [7,8,14,15,17] or FL [9,10,13,18] detection. The latter is better because of its greater sensitivity. Ion pairing methods have been also introduced [17,18]. Methods for the determination of quinine with its metabolites in biological fluids have been reported [19,20]. Only two methods have been reported for the simultaneous determination of quinine and chloroquine in biological fluids with satisfactory sensitivity but both required long analysis time, exceeding 15 min. [9,10]. An HPTLC method was reported for synthetic anti-malarial drugs that was only slightly less accurate than the HPLC technique, but had the advantage of saving time and reagents [12].

In the present paper a simple, rapid and sensitive reversed-phase method is developed and validated for the simultaneous determination of quinine and chloroquine in pharmaceutical preparations and biological fluids.

2. Experimental

2.1. Instrumentation

The chromatographic apparatus consisted of an SSI 222D pump (SSI, State College, PA, USA) and a Rheodyne 9125 injection valve (Rheodyne, Cotati California, USA) with a 50-μL loop, which were coupled to a fluorescence detector RF-551 Shimadzu (Kyoto, Japan). Data acquisition was performed using a Hewlett-Packard integrator HP3396A (Avondale, PA, USA).

A glass vacuum-filtration apparatus from Alltech Associates was employed for the filtration of the buffer solution, using 0.2 μm membrane filters obtained from Whatman (Maidstone, UK). Degassing of solvents was achieved by helium sparging before use. Dissolution of compounds was enhanced by sonication in a Transonic 460/H Ultrasonic bath (Elma, Germany). A Glass-col, Terre Haute 47802 small vortex mixer and a Hermle centrifuge, model Z 230 (B. Hermle, Gosheim, Germany) were employed for the sample pre-treatment.

All evaporations were performed with a 9-port Reacti-Vap evaporator (Pierce, Rocford, IL, USA).

2.2. Reagents and materials

Quinine hemisulfate salt (purity 94% by HPLC) and chloroquine diphosphate salt were supplied from Sigma (St. Louis, MO, USA). Methanol and acetonitrile gradient grade for liquid chromatography, ammonium acetate and salicylic acid of analytical reagent grade were supplied from Merck (Darmstadt, Germany).

Nexus Abselut SPE cartridges were purchased from Varian (Harbor City, USA), Lichrolut RP-select B by Merck, LC-18 Discovery by Supelco (Bellefonte, PA, USA), C_8 Bond Elut by Varian.

Commercial Pharmaceuticals were purchased from a local drugstore. Plaquénil coated tablets containing 200 mg of hydrochloroquine were manufactured by Sanofi Winthrop, Gentilly, France and Lafran tablets containing 204 mg of quinine chlorohydrate were manufactured by Laboratoires Lafran (Bonneuil/Marine, France).

Serum samples were kindly provided from the Blood Donation Unity of a State Hospital. Urine samples for calibration curve construction and recovery assay were taken from healthy volunteers.

2.3. Chromatographic conditions

Chromatographic separation was performed on a Kromasil C_{18}, 5 μm, 250 × 4 mm, analytical column (MZ-Analytical, Mainz, Germany). The mobile phase consisted of methanol–acetonitrile–0.1 ml/L ammonium acetate (45:15:40 v/v). This was delivered at a flow rate of 1.0 mL/min. Backpressure observed was 230 kg/cm^2. Fluorescence detection was performed at 325 nm (excitation) and 375 nm (emission). HPLC analysis was conducted at ambient temperature. Retention time was 3.5 min for chloroquine and 5.1 min for quinine. Resolution was satisfactory as indicated by R_s values that were greater than 2.5.

2.4. Preparation of standard solutions

Aqueous stock standard solutions were prepared at a concentration of 100 ng/μL. Working standards were prepared from these stocks in the range of 0.001–0.67 ng/μL, by appropriate dilution. All working standards contained the internal standard salicylic acid at a concentration of 0.5 ng/μL. These were found to be stable for at least 3 months, when stored refrigerated.

2.5. Standardization, determination of sensitivity, validation and stability

Standardization was carried out by injecting a series of standard mixtures, ranging in concentration from 0.001 to 0.67 ng/μL, in order to determine sensitivity. The response factors for each concentration were calculated from the ratio of peak area of analyte to that of IS. Five determinations were performed at each concentration level. Validation of the procedure was determined by the consistency of relationship between each concentration and the corresponding factor. Stability was examined by repeated injections on the same day or different days. The detector response was checked daily at minimum three concentrations.

2.6. Assay of pharmaceutical preparations

Two anti-malarial pharmaceutical preparations were found in local market namely Plaquénil coated tablets containing 200 mg of hydrochloroquine and Lafran tablets containing 204 mg of quinine chlorohydrate.

Ten tablets of each preparation were weighed and finely pulverized. A portion of this powder corresponding to the average weight of the tablets was transferred in a 50 mL volumetric flask and diluted to volume with water. After sonication, a portion of this sample was filtered and dilute solutions were prepared at three concentrations: 0.5–1.0 and 2.0 ng/μL for quinine and 0.05–0.1 and 0.3 ng/μL for chloroquine. Internal standard was added in each solution. Aliquots of 50 μL were injected onto the chromatographic column. Triplicate analysis was executed for each sample.

2.7. Solid phase extraction

Four SPE cartridges namely Lichrolut RP-select B by Merck, LC-18 Discovery by Supelco, C_8 Bond Elut and Nexus Abselut by Varian were tested concerning their efficiency to retain quinine and chloroquine from biological fluids.

Cartridges were pre-conditioned with methanol. During the second conditioning step several solvents were tested. These included water, 0.1 mol/L ammonium acetate and acetic acid 1%. Elution efficiency of different solvents was tested for methanol–acetonitrile (50–50%, v/v), methanol, acetonitrile, acetic acid 1%, methanol–0.1 ml/L ammonium acetate–acetonitrile (45–40–15 v/v), 2-propanol, methanol–acetone (80–20%, v/v), 2-propanol–acetonitrile (50–50%, v/v), water, hydrochloric acid 1%. Sequential elution by 1.3 mL of methanol–0.1 ml/L ammonium acetate–acetonitrile (45–40–15 v/v) and 0.7 mL acetic acid 1% was also tested.

2.7.1. Biological fluids

Two hundred microlitres of Acetonitrile are added to 40 μL of blood serum samples that are placed in eppendorf vials. Two-hundred microlitres of water for blank samples or standard (at five concentration levels 0.2, 0.5, 0.75, 1 and 2 ng/μL) for spiked were added. After centrifugation for 10 min at 4000 rpm, organic solvent was evaporated under nitrogen stream and the remaining sample was applied to the preconditioned cartridge. Optimized SPE protocol was applied to isolate quinine and chloroquine from samples.

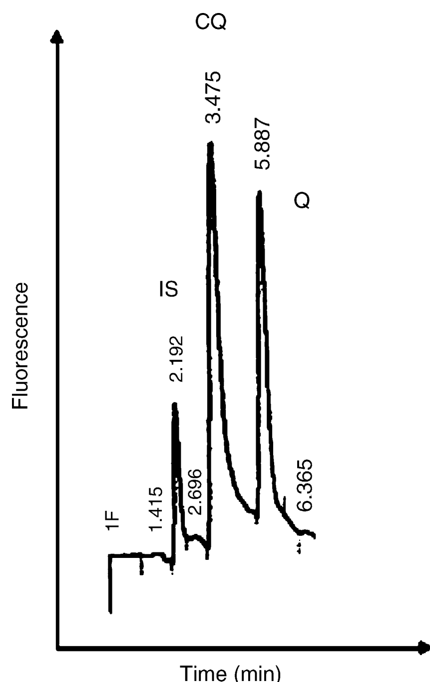


Fig. 2. Chromatogram of chloroquine (3.475 min) and quinine (5.087 min) in the presence of salicylic acid as internal standard (2.192 min).

Same procedure was followed for urine samples. Urine samples of 100 μL were spiked with 100 μL of standard solution at four concentration levels 0.1, 0.2, 0.3 and 0.5 ng/μL. These samples were treated by SPE as described above.

3. Results and discussion

3.1. Chromatography

A Chromatogram obtained using the developed methods conditions is illustrated in Fig. 2. Resolution factors calculated by the equation $R_s = 2(t_2 - t_1)/(W_1 + W_2)$ were greater than 2.0 indicating satisfactory separation of resolved analytes.

3.2. Method validation

3.2.1. Linearity and sensitivity

Eight points' calibration curves were constructed covering a concentration range from 0.001 to 0.67 ng/μL. Equations were obtained by least-squares linear regression analysis of the peak area ratio of analyte/internal standard versus analyte concentration. The method was linear up to 0.7 ng/μL for quinine and up to 0.5 ng/μL for chloroquine. Correlation coefficients ranged from 0.9926 and 0.9997 for quinine and chloroquine, respectively.

The LOD was defined as the compound concentration that produced a signal-to-noise ratio greater than three, while the limit of quantitation of the assay was evaluated as the concentration equal to 10 times the value of the signal to-noise ratio. LOD and LOQ values for each compound based upon these criteria are shown in Table 1. These values were observed for ten samples and were found to be 0.3 and 0.5 ng, respectively.

3.2.2. Precision and accuracy

The precision of the method based on within-day repeatability was performed, by replicate injections ($n = 8$) of three standard solutions covering different concentration levels: low, medium and high, where peak areas were measured, in comparison to the peak area of the internal standard. Statistical evaluation revealed relative standard deviations, at different values.

The reproducibility (day-to-day variation) of the method was established using the same concentration range as above. A triplicate determination of each concentration was conducted during routine operation of the system over a period of eight consecutive days.

Accuracy was determined by replicate analysis of three different levels (0.01, 0.07 and 0.5 ng/μL). Expressed as relative error can be calculated by the equation:

$$\text{Relative error (\%)} = \frac{\text{Mean determined value} \\ -\text{Theoretical (added amount)}}{\text{Theoretical}} \times 100$$

Table 2 summarizes the results of the method validation regarding accuracy, within-day and day-to-day preci-

Table 1
Performance parameters for the quantisation of quinine and chloroquine in standards, serum and urine samples (x = analyte's concentration ng/μL)

Analyte	Regression equationss	Correlation coefficient	Linear range (ng/μL)
Standards			
Quinine	$y = (4.6792 \pm 0.5708)x + (1.0366 \pm 0.7550)$	$R = 0.9926$	0.01–0.7
Chloroquine	$y = (7.1464 \pm 0.0951)x - (0.0758 \pm 0.0.0219)$	$R = 0.9997$	0.01–0.5
Serum			
Quinine	$y = (4.4452 \pm 0.7849)x - (1.0704 \pm 0.9353)$	$R = 0.9972$	
Chloroquine	$y = (7.0749 \pm 0.0726)x + (0.0819 \pm 0.0294)$	$R = 0.9917$	
Urine[a]			
Quinine	$y = (1.7 \times 10^9 \pm 7.8 \times 10^6)x + 1.4 \times 10^7 \pm 2.8 \times 10^6)$	$R = 0.9990$	
Chloroquine	$y = (1.8 \times 10^9 \pm 2.0 \times 10^6)x + (5.0 \times 10^7 \pm 0.6 \times 10^6)$	$R = 0.9999$	

[a] Peak areas of analytes were used since endogenous compounds interfered with the internal standard.

Table 2
Intra-day and inter-day accuracy and precision data for quinine and chloroquine determination

Analyte	Added (ng)	Intra day ($n = 8$)			Inter day ($n = 8$)		
		Found ± S.D. (ng)	R.S.D.	Relative error (%)	Found ± S.D. (ng)	R.S.D.	Relative error (%)
Quinine	0.5	0.5 ± 0.04	8.0	0.0	0.5 ± 0.04	8.0	0.0
	3.5	3.6 ± 0.3	8.3	2.9	3.5 ± 0.2	5.7	0.0
	25	24.8 ± 2.0	8.1	−0.8	25.1 ± 2.3	9.2	0.4
Chloroquine	0.5	0.5 ± 0.05	10.0	0.0	0.5 ± 0.05	10.0	0.0
	3.5	3.6 ± 0.2	5.6	2.9	3.2 ± 0.3	9.4	−8.6
	25	25.8 ± 1.6	6.2	3.2	24.9 ± 1.3	5.2	−0.4

Table 3
Results of quinine and chloroquine determination in commercial pharmaceuticals

Analyte	Added (ng)	Measured ± S.D. (ng)	Found (mg per tablet)	Recovery (%)
Plaquénil				
Chloroquine (204 mg)	2.5	2.47 ± 0.04	192 ± 4.84	94.1
	5	4.78 ± 0.20		
	15	14.11 ± 0.36		
Lafran				
Quinine (200 mg)	25	24.24 ± 0.29	198 ± 1.34	99.0
	50	48.91 ± 1.49		
	100	96.82 ± 1.76		

sion assays. The measured concentrations had R.S.D values < 10%. Relative error (inaccuray) values were excellent for quinine ranging from −0.8 to 2.9% and satisfactory for chloroquine in the range from −8.6 to 3.2%.

3.3. Analysis of pharmaceuticals

High performance liquid chromatograms of anti-malarial agents in pharmaceutical formulations are shown in Fig. 3. The experimental results from these analyses are given

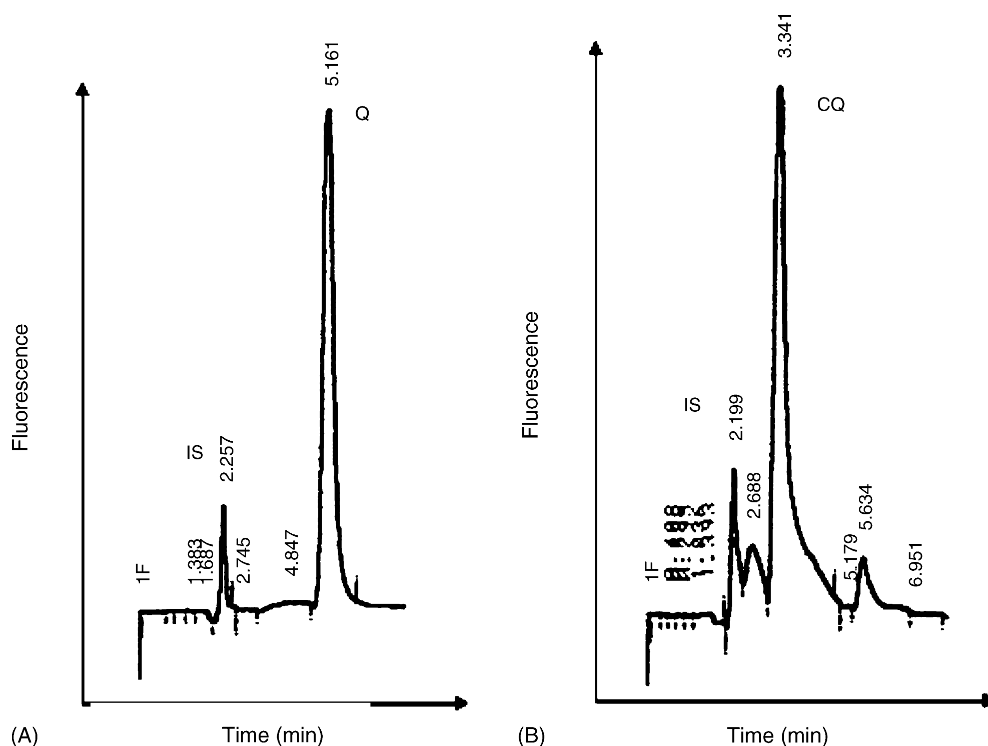


Fig. 3. (A) Chromatogram of quinine (5.161 min) determination in pharmaceutical tablets Lafran, in the presence of salicylic acid (2.257 min). (B) Chromatogram of chloroquine (3.341 min) determination in pharmaceutical tablets Plaquénil, in the presence of salicylic acid (2.199 min).

Table 4
Quinine and chloroquine recovery study of using various SPE protocols

SPE cartridge	Conditioning solvent A = CH_3OH (2 mL), conditioning solvent B (2 mL)	Elution solvent (2 mL)	Recovery (%)[a]	
			Quinine	Chloroquine
Lichrolut RP-select B Merck	H_2O	CH_3OH-CH_3CN (50/50%, v/v)	50.0	7.0
	$CH_3COONH_4$0.1 M	CH_3OH-CH_3CN (50/50%, v/v)	35.7	5.1
	CH_3COOH 1%	CH_3OH-CH_3CN (50/50%, v/v)	9.2	3.5
	H_2O	CH_3OH	46.3	6.7
	H_2O	CH_3CN	11.0	4.2
LC-18 Discovery	H_2O	CH_3OH-CH_3CN (50/50%, v/v)	42.0	10.3
C_8 Bond Elut Varian	H_2O	CH_3OH-CH_3CN (50/50%, v/v)	33.0	9.8
Nexus abselut Varian	H_2O	CH_3OH-CH_3CN (50/50%, v/v)	52.7	6.4
	CH_3COONH_4 0.1 M	CH_3OH-CH_3CN (50/50%, v/v)	66.4	80.2
	CH_3COOH 1%	CH_3OH-CH_3CN (50/50%, v/v)	62.9	58.6
	CH_3COONH_4 0.1 M	CH_3OH	75.4	7.3
	CH_3COONH_4 0.1 M	CH_3CN	58.0	4.9
	CH_3COONH_4 0.1 M	CH_3COOH 1%	25.6	37.0
	CH_3COONH_4 0.1 M	$CH_3CHOHCH_3$	71.8	65.6
	CH_3COONH_4 0.1 M	CH_3OH/CH_3COCH_3 (80/20%, v/v)	5.4	75.0
	CH_3COONH_4 0.1 M	$CH_3CHOHCH_3/CH_3CN$ (50/50%, v/v)	5.6	72.0
	CH_3COONH_4 0.1 M	CH_3COONH_4 0.1 M	30.0	7.2
	CH_3COONH_4 0.1 M	H_2O	<3.0	6.9
	CH_3COONH_4 0.1 M	$CH_3OH/CH_3COONH_4$0.1 M/CH_3CN (45/40/15 v/v)	28.0	8.1
	CH_3COONH_4 0.1 M	1.3 mL mobile phase 0.7 mL CH_3COOH 1%	>82.0	>90.0

[a] Mean values from three measurements at two concentration levels (0.5 and 1.0 ng/μL).

in Table 3. Recovery of chloroquine from the tablet is 94% while the respective recovery of quinine is 99%.

3.4. Solid phase extraction

Different protocols were tested to optimize retention and elution of quinine and chloroquine anti-malarial agents from biological fluids. Assays included various SPE sorbents, conditioning and elution solvents. Results are summarized in Table 4. As it can be seen, optimum recovery is achieved when Nexus cartridges are activated by methanol and 0.1 mol/L ammonium acetate (2 mL) and a sequential elution using 1.3 mL of mobile phase methanol/0.1 mol/L ammonium acetate/acetonitrile (45/40/15 v/v) and 0.7 mL of acetic acid 1% is used.

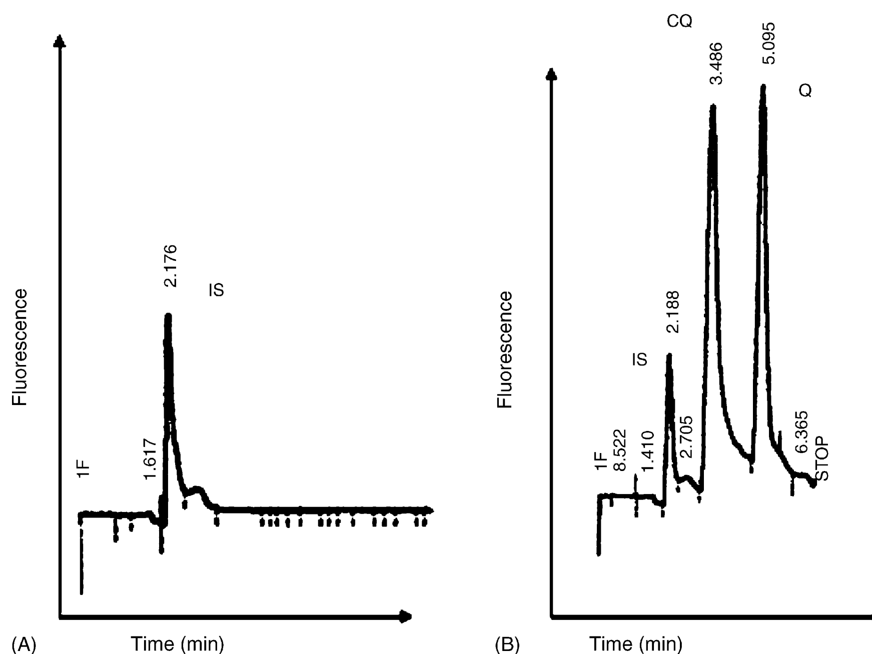


Fig. 4. (A) Blank chromatogram of serum sample. Salicylic acid (2.176 min) is used as internal standard. (B) Chromatogram of quinine (5.095 min) and chloroquine (3.487 min) determination in spiked serum sample in the presence of salicylic acid (2.188 min).

3.4.1. Biological fluids: human blood serum and urine.

Calibration graphs for serum and urine were constructed by transferring aliquots of the standard solutions and internal standard into blank pooled drug-free serum and urine sample to give final concentrations in the range of 0.2–2.0 ng/μL. These calibration standards were extracted as described under SPE paragraph. Calibration data are shown in Table 1. Regression equations for serum samples revealed correlation coefficients 0.9972 for quinine and 0.9917 for chloroquine. Endogenous compounds did not allow the application of internal standard method in urine samples. Regression equations for urine samples revealed correlation coefficients 0.9990 for quinine and 0.9999 for chloroquine. The precision and accuracy studies of SPE of anti-malarial agents from biological samples were conducted by spiking blood serum samples with known concentrations of the compounds. Results of recovery studies for serum samples are given in Table 5. Each value represents the mean of six measurements carried out. A blank chromatogram of human blood serum is illustrated in Fig. 4A. High performance liquid chromatogram of quinine and chloroquine extracted from human blood serum is shown in Fig. 4B. Relative recovery for serum was determined at three different concentrations (0.5, 0.75 and 1.0 ng/μL) by comparing the peak area ratios for extracted compounds from serum and the respective values derived from the serum calibration curve. Mean recovery of quinine was 92.1%, while the respective value for chloroquine was 105.4%. Urine samples were also extracted using SPE.

A blank chromatogram of urine sample is shown in Fig. 5A. High performance liquid chromatogram of quinine and chloroquine in spiked human urine is shown in Fig. 5B.

Table 5
Recovery of quinine and chloroquine from biological samples after SPE

Analyte	Added (ng)	Found (ng) ± S.D.	Recovery (%)
Serum			
Quinine	25	21.5 ± 0.06	86.0
	37.5	35.0 ± 0.02	93.3
	50	48.5 ± 0.08	97.0
Chloroquine	25	28.0 ± 0.06	112.0
	37.5	36.5 ± 0.05	97.3
	50	53.5 ± 0.13	107.0
Urine			
Quinine	10	11.0 ± 0.03	110.0
	15	14.0 ± 0.02	93.3
	25	25.5 ± 0.06	102.0
Chloroquine	10	9.0 ± 0.02	90.0
	15	13.5 ± 0.07	90.0
	25	23.0 ± 0.03	92.0

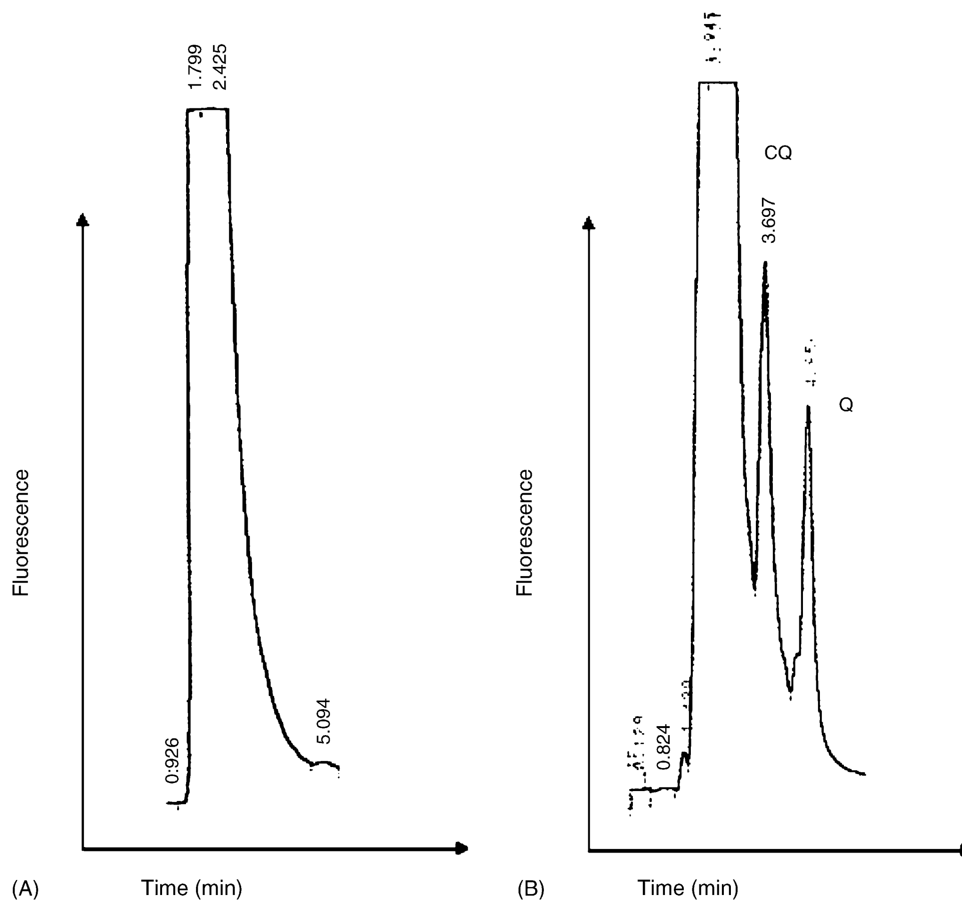


Fig. 5. (A) Blank chromatogram of urine sample. (B) Chromatogram of quinine (4.856 min) and chloroquine (3.607 min) determination in spiked urine sample.

Relative recovery for urine was determined at three different concentrations (0.2, 0.3 and 0.5 ng/μL) by comparing the peak area ratios for extracted compounds from urine and the respective values derived from the urine calibration curve. Mean recovery of quinine was 101.8%, while the respective value for chloroquine was 90.7%.

4. Conclusions

Malaria continues to be a major health problem in tropical countries. The major cure of this disease is quinine, the principal alkaloid of the cinchona tree from the tropical Andes Mountains. Mild toxic effects mainly visual and auditory disturbances can be found as complication of malaria therapy. Chloroquine is the prototype synthetic anti-malarial drug most widely used to treat all types of malarial infections. It is also the cheapest time tested and safe anti-malarial agent.

The monitoring of quinine and chloroquine concentrations in human biological fluids continues to be of great importance.

A rapid and sensitive isocratic reversed-phase HPLC method has been developed herein for the determination of two anti-malarial agents: quinine and chloroquine in pharmaceuticals and biological fluids is developed. The analysis techniques for the two anti-malarial drugs meet International Conference on Harmonization (ICH) standards for specificity and accuracy. Solid-phase extraction proved to be an adequate way, for the separation of the analytes from matrix interferences. The recovery of analytes from Pharmaceuticals was 94% for chloroquine and 99% for quinine. Recovery rates from biological fluids were in the range from 91 to 105% which means that their extraction efficiency is very satisfactory. These values are higher than those reported in literature.

Detection limits, the good sensitivity and resolution and the short analysis time (approximately 5 min) in comparison to the already published methods as well as the simplicity of the procedure should make this method a useful tool for the routine analysis of the examined compounds.

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