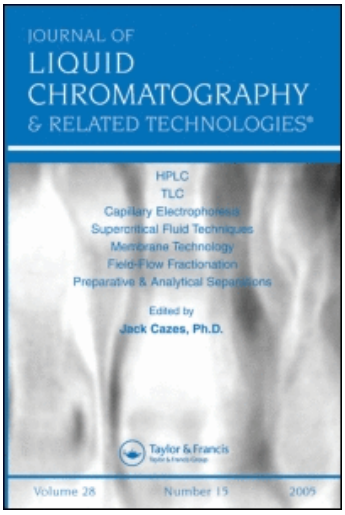

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SIMULTANEOUS DETERMINATION OF QUININE AND A MAJOR METABOLITE 3-HYDROXYQUININE IN BIOLOGICAL FLUIDS BY HPLC WITHOUT EXTRACTION

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ABSTRACT

A reverse phase, isocratic HPLC method has been developed for the quantitation of quinine and its major metabolite, 3-hydroxyquinine in human plasma, urine and hepatic microsomal samples. The method involves simple protein precipitation for sample treatment and ion-paired chromatography. The chromatographic separation is accomplished with a mobile phase comprising acetonitrile-aqueous phosphate buffer (40:60, v/v) containing 10 mM sodium dodecyl sulphate and 0.1 mM tetrabutylammonium bromide and adjusted to pH 2.1. The mobile phase is pumped at a flow rate of 0.5 mL/min. A microbore column is used (2 mm I.D. x 100 mm) packed with a C_{18} reverse phase material (5 μm ODS Hypersil). Biological samples (100-500 μL) were precipitated with two volumes of cold methanol, vortexed and then centrifuged at 1500 g for 10 min. The supernatant (30 μL) was injected into the HPLC column. The chromatograms were monitored using a fluorescence detector setting with excitation and emission wavelenths of 350 and 450 nm, respectively. Under

these conditions, the lower limit of detection was 0.1 μM (0.034 μg/mL) for the major metabolite 3-hydroxyquinine, and 0.5 μM (0.16 μg/mL) for quinine. The inter- and intra-assay coefficients of variation were found to be less than 7%. The assay procedure is applicable for studying the pharmacokinetics and metabolism of quinine.

INTRODUCTION

Quinine is one of the oldest drugs in the pharmacopoeia. While synthetic antimalarial drugs have largely replaced quinine, the emergence of strains of *Plasmodium falciparum* resistant to chloroquine in Southern Asia, South America and East Africa, has necessitated its continued therapeutic use.[1] Some 42% (2,117 million) of the world population live in endemic malaria area.[2] Quinine is also widely used for the prevention of night cramps in the elderly. Despite its long history in the treatment of malaria, the metabolism of quinine in man has not been fully elucidated. In man, quinine undergoes extensive oxidative metabolism with approximately 10-20% of an oral dose being excreted in the urine unchanged.[1,3,4]

In contrast, the metabolism of its diastereoisomer, quinidine, has been well investigated. Major metabolites of quinidine appear to be 3-hydroxyquinidine, quinidine-*N*-oxide and 2'-quinidinone.[5,6] The *in vitro* hepatic microsomal metabolism of quinidine to form 3-hydroxyquinidine and quinidine-*N*-oxide metabolites was shown to be catalysed by P450IIIA4.[5] A polar product of quinine was also detected after incubation with human liver microsomes. This had a similar retention time to 3-hydroxyquinidine, but this product was not identified.[5] Liddle et al.[7] identified 3-hydroxyquinine in human urine after administration of quinine. They also identified five other quinine metabolites which were 6'-hydroxycinchonidine (*O*-desmethylquinine), 6'-hydroxydihydrocinchonidine, 3-hydroxydihydroquinine, quinine-10,11-epoxide and quinine-10,11-dihydrodiol.

The lack of information on the pharmacokinetics and metabolism of quinine is largely due to non-availability of synthetic metabolites. In addition, there is no specific analytical method available for the determination of quinine metabolites in biological samples. A number of high performance liquid chromatography (HPLC) methods for determination of quinine have been published.[8-15] Most of these employ solvent extraction as a sample preparation, thus being time consuming, and none of these measured quinine metabolites. Therefore, this study was conducted to develop a simple and specific HPLC

assay for the simultaneous determination of quinine and its metabolites in biological fluids without solvent extraction.

MATERIALS AND METHODS

Reagents and Chemicals

All chemicals were of analytical grade. Quinine bisulphate (M.W. of quinine free base = 324.4), was kindly supplied by Kimia Pharma, Indonesia. 3-Hydroxyquinine, M.W. of 340.4 (free base), was a gift from Dr. P Winstanley, Department of Pharmacology and Therapeutics, University of Liverpool, UK. Tetrabutylammonium bromide (TBA) was purchased from Sigma Chemical Co (St Louis, MO, USA). Sodium dodecyl sulphate (SDS), HPLC-grade methanol and acetonitrile were purchased from BDH Chemicals Ltd (Poole, UK). All water was double glass distilled and MilliQ® filtered.

Stock solutions of 1 mg/mL quinine free base and 0.5 mg/mL of 3-hydroxyquinine were prepared in 50% (v/v) methanol-water. The solutions were protected from light and stored at -20 °C until required. These solutions were found to be stable for at least 1 month. Standard solutions (in plasma, urine, or hepatic microsomal samples) with known concentrations of quinine and its metabolite were prepared by mixing stock solutions of quinine, 3-hydroxyquinine and drug-free samples.

Identification of Urinary Metabolites of Quinine

When this study was commenced, synthetic metabolites of quinine were not available. *The metabolites of its diastereoisomer quinidine were therefore used as reference compounds* to identify possible metabolites of quinine in the human urine, plasma samples and the samples from human liver microsomal studies. Quinidine metabolites, 2'-oxoquinidine (2'-oxoquinidinone), quinidine-*N*-oxide, 3-hydroxyquinidine and *O*-desmethylquinidine (6'-hydroxycinchonidine) were kindly provided by Professor I A Blair, Vanderbilt University School of Medicine, Tennessee, USA. Later, when synthetic 3-hydroxyquinine (kindly donated by Dr P Winstanley, University of Liverpool, UK) became available, it was used as a reference standard instead of using 3-hydroxyquinidine.

Stock solutions of 10 μg/mL of each metabolite of quinidine (2'-oxo-quinidinone, quinidine-*N*-oxide, 3-hydroxyquinidine and *O*-

desmethylquinidine) were prepared in 96:4 (v/v) methanol-water. The solutions were further diluted with distilled water and mixed to give a final concentration of 0.1 μg/mL of all the metabolites except for *O*-desmethylquinidine (7 μg/mL). The mixture of metabolites was injected into the HPLC column and used as a reference to identify the possible metabolites of quinine in the biological samples.

The concentration of quinine and its metabolite 3-hydroxyquinine were determined from calibration plots of the chromatographic peak heights versus drug or metabolite concentration.

Biological Samples

Samples for development and evaluation of the method were obtained both by spiking drug-free plasma and urine with known amounts of quinine and its metabolite 3-hydroxyquinine, and by collecting blood and urine samples from healthy volunteers participating in a pharmacokinetic study.[4] These volunteers took a single oral dose of 600 mg quinine sulphate. Venous blood was obtained in a 5 mL heparinised tubes and plasma was prepared by centrifuged blood at 1500g for 10 min. All samples collected were kept at -20°C until analysis. Microsomal samples were obtained after incubation of quinine (as a substrate) with human liver microsomes. Procedures for the microsomal incubation have been described previously.[16]

Sample Preparation

To 100 μL of plasma was added 200 μL of cold methanol. The samples were vortexed for 10 seconds, then centrifuged at 1500g for 10 minutes. After centrifugation, an aliquot (30 μL) of the supernatant was injected into the HPLC column.

It was found that the concentrations of quinine and its metabolites in urine samples (0-48 h) collected from healthy volunteers who took a single oral dose of 600 mg quinine sulphate, were too high. So it was necessary to dilute the urine samples 1 in 10 with HPLC water before the sample preparation. In brief, 100 μL of urine samples were diluted by adding 900 μL of HPLC water. Then, to 100 μL of the diluted urine samples was added 200 μL of cold

methanol. The samples were vortexed, then centrifuged and processed as above.

For hepatic microsomal samples, the samples were obtained after incubation of quinine (500 μM) with 1 mg/mL human liver microsomes and 1 mM NADPH, in a final 500 μL volume, for 30 minutes as described previously.[16] In order to terminate the metabolic reactions, two volumes of cold methanol (i.e. 1 mL) was added to the microsomal samples. The samples were then vortexed for 10 seconds, and centrifuged at 1500 g for 10 minutes. The resultant supernatant was diluted 1 in 4 with HPLC water and the aliquot (30 μL) of this diluted supernatant was injected into the HPLC column. Dilution was made to allow the detection of both quinine and its major metabolite 3-hydroxyquinine within the appropriate attenuation of the HPLC integrator.

Chromatographic Conditions

The HPLC system consisted of a Model 250 Perkin-Elmer LC pump (Perkin-Elmer Corporation, Norwalk, CT, USA) equipped with a WISP 712 autoinjector (Waters Assoc., Milford, MA, USA). A Hitachi fluorescence detector (Hitachi, Tokyo, Japan) was used and operated with exitation and emission wavelengths of 350 and 450 nm, respectively. The chromatographic response was recorded by a Hitachi D2500 integrator (Hitachi, Tokyo, Japan). A mocrobore HPLC column (2 mm I.D. x 100 mm) packed with a reverse phase C_{18} material, 5 μm ODS Hypersil (Shandon, London, UK) was used.

Analysis of the samples of quinine and its major metabolite 3-hydroxyquinine was performed using a mobile phase consisting of an acetonitrile-aqueous (40:60, v/v) containing 10 mM Na_2HPO_4, 10 mM sodium dodecyl sulphate (SDS) and 0.1 mM tetrabutylammonium bromide (TBA). The pH of mobile phase was finally adjusted to 2.1 with orthophosphoric acid. The flow rate was 0.5 mL/min. Chromatographic separations were performed at room temperature.

RESULTS AND DISCUSSION

An initial attempt had been made to identify possible metabolites of quinine in human urine by using the original HPLC mobile phase as previously described.[10] Initially, since the metabolites of quinine were not available,

metabolites of quinidine were used as reference compounds based on the assumption that there would be a similarity in the retention times of corresponding metabolites of quinine and quinidine. Admittedly, there was no evidence showing that the metabolites would necessarily have similar retention times. However, this assumption was supported by the evidence that quinine and quinidine had very similar retention times of 8.2 and 8.3 min, respectively, when the original mobile phase was used. Under the chromatographic conditions used by Zoest et al.,[10] i.e., using the original mobile phase consisting of an acetonitrile-aqueous phosphate buffer (10 mM) mixture (50:50, v/v) containing 25 mM SDS and 3 mM TBA, there was no resolution between the peaks of 3-hydroxyquinidine and quinidine-*N*-oxide. They had retention times of 3.9 and 4.4 min, respectively.

Therefore, the HPLC assay was further developed by modification of the original mobile phase. The mobile phase was selected on the basis of the retention behaviour of quinine and metabolites of quinidine (used as reference compounds) as a function of ion-pairing agent (SDS). Chromatographic separation of quinine and metabolites of quinidine was accomplished with a mobile phase of acetonitrile-aqueous phosphate buffer mixture (40:60, v/v) containing 10 mM disodium hydrogen phosphate, 10 mM SDS and 0.1 mM TBA, adjusted to pH 2.1. With these chromatographic conditions, a good resolution of 3-hydroxyquinidine and quinidine-*N*-oxide was achieved. Optimum separation of other quinidine metabolites (*O*-desmethylquinidine and 2'-oxoquinidine) was also obtained using this new modified mobile phase (Figure 1). Under these chromatographic conditions, 2'-oxoquinidine was eluted first, followed by 3-hydroxyquinidine, quinidine-*N*-oxide, *O*-desmethylquinidine and quinine. Their retention times were 4.0, 17.7, 21.0, 29.1 and 47.7 min, respectively. Under this modified mobile phase, 3-hydroxyquinine had a retention time of 17.8 min, and co-eluted with the 3-hydroxyquinidine peak. Quinine and quinidine also had very similar retention times of 47.7 and 47.9 min, respectively, when this modified mobile phase was used. These support the assumption that quinidine metabolites could be used as reference compounds.

Possible interference 3-hydroxyquinine and quinine by other drugs was tested. None of the following drugs tested (at a concentration of 10 μg/mL) had a fluorescence response under the chromatographic conditions used, thus they do not interfere with the assay: chloroquinine, mefloquinine, primaquine, artemether, arteether, proguanil and its active metabolite cycloguanil, isoniazid, rifampicin, streptomycin, ethambutol, pyrazinamide, propranolol, debrisoquinine and its 4-hydroxy debrisoquinine. Piroxicam had a retention time of 2.3 minutes and did not interfere with the analytes of

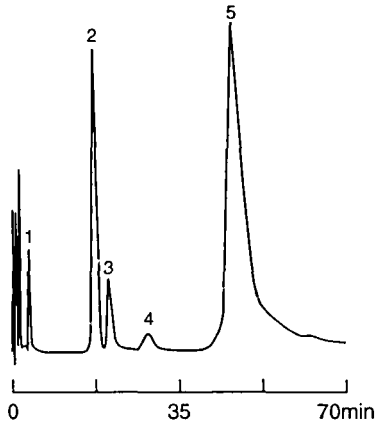

Figure 1. HPLC separation of quinine and metabolites of quinidine using a modified mobile phase consisting of an acetonitrile aqueous phosphate buffer (10 mM, pH 2.1) mixture (40:60 v/v) containing 10 mM SDS and 0.1 mM TBA. Other conditions are as described in the Methods section. Concentrations of 2'-oxoquinidine, quinidine-*N*-oxide, 3-hydroxyquinidine and quinine were 0.1 μg/mL and concentration of *O*-desmethylquinidine was 7 μg/mL. Peak identification: 1 = 2'-oxoquinidine; 2 = 3-hydroxyquinidine; 3 = quinidine-*N*-oxide; 4 = *O*-desmethylquinidine; and 5 = quinine.

interest. Two alkaloids, cinchonine and cinchocaine, had retention times of 45.1 and 52.3 minutes, respectively, which overlapped that of quinine. Consequently, the search for a suitable internal standard for the assay was not successful. Therefore, an internal standard was not used in the present method. Despite this, the reproducibility of the assay was good for both 3-hydroxyquinine and quinine (see Table 1 and further discussion), because the sample preparation only involved direct protein precipitation.

Plasma and urine, collected before administration of quinine tablets (i.e. blank samples), showed no endogenous sources of interference with the analytical assay (Figures 2A and 3A). More than 20 human blank plasma and urine samples were analysed in the study and no endogenous peaks with retention time similar to 3-hydroxyquinine and quinine were detected. Representative chromatograms of blank plasma, and a plasma sample obtained (2.5 h after dose) from a volunteer who took a single oral dose of quinine sulphate (600 mg) are shown in Figure 2. As can be seen, good

Table 1

Within-Day Reproducibility and Accuracy of Analysis for 3-Hydroxyquinine and Quinine in Human Urine Using the Method Described

Nominal Concentration (μM)	Observed Concentration (μM)[1]	C.V.[2] (%)	Accuracy[3] (%)
3-Hydroxyquinine			
0.1 μM (0.034 μg/mL)	0.091 ± 0.006	6.6	91
30 μM (10.2 μg/mL)	28.4 ± 1.3	4.6	94.7
Quinine			
0.5 μM (0.16 μg/mL)	0.48 ± 0.03	6.3	96
30 μM (9.6 μg/mL)	32.0 ± 1.5	4.7	106.7

[1] Results are given as mean ± S.D. (n = 5).
[2] C.V. is a coefficient of variation.
[3] Accuracy (%) = $\frac{\text{observed concentration}}{\text{nominal concentration}} \times 100$

separation of 3-hydroxyquinine, quinine and other possible unidentified metabolites with no interference from plasma was observed in the blank sample. There are at least 7 possible metabolites of quinine detected in urine samples from volunteers who were given a single oral dose of 600 mg quinine sulphate (Figure 3). A major peak identified as 3-hydroxyquinine was detected in all plasma and urine samples from the volunteers who took a single oral dose of quinine sulphate. This suggests that 3-hydroxyquinine is a major metabolite of quinine in human.

The assay procedure was also used to quantitate formation of 3-hydroxy-quinine in the *in vitro* metabolism of quinine by human liver microsomes. There was no endogenous interference from the blank liver microsomal samples (Figure 4A). After incubation of quinine with human liver microsomes in the presence of NADPH for 30 minutes, a major peak was formed, having a retention time of 18.0 minutes (Figure 4B) which was similar to that of the reference compound. This peak co-eluted with 3-hydroxyquinine when the samples were spiked with standard 3-hydroxyquinine solution,

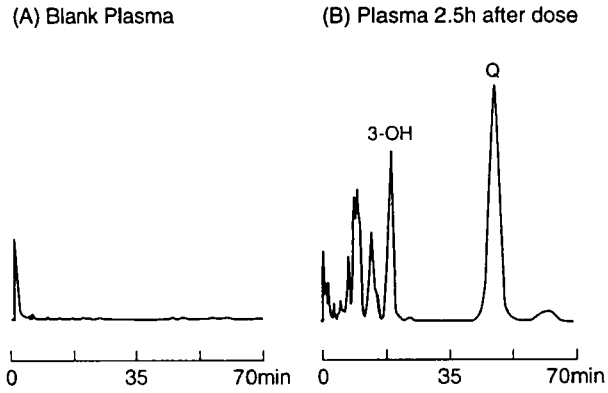

Figure 2. Typical chromatograms of blank plasma (A); and a plasma sample obtained from a volunteer, 2.5 hours after a single oral dose of quinine sulphate (600 mg). Chromatographic conditions used as described in Figure 1. Peaks : 3-OH = 3-hydroxyquinine (7.6 μM); Q = quinine (8.9 μM).

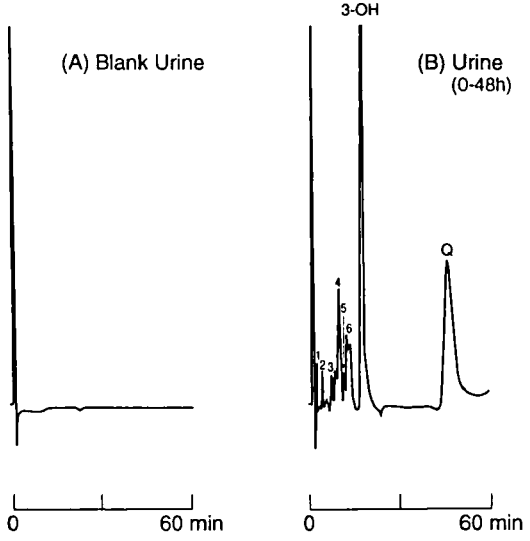

Figure 3. HPLC analysis of quinine and its metabolites in subject H's urine samples : (A) blank urine; and (B) urine sample collected from 0-48 hours after a single dose of 600 mg quinine sulphate, using the modified mobile phase. Chromatographic conditions used as described in Figure 1. Peaks : 1 = 2'-oxoquininone; 3 = quinine glucuronide; 3-OH = 3-hydroxyquinine (15.9 μM); 2, 4, 5 and 6 = unidentified metabolites of quinine; and Q = quinine (4.1 μM).

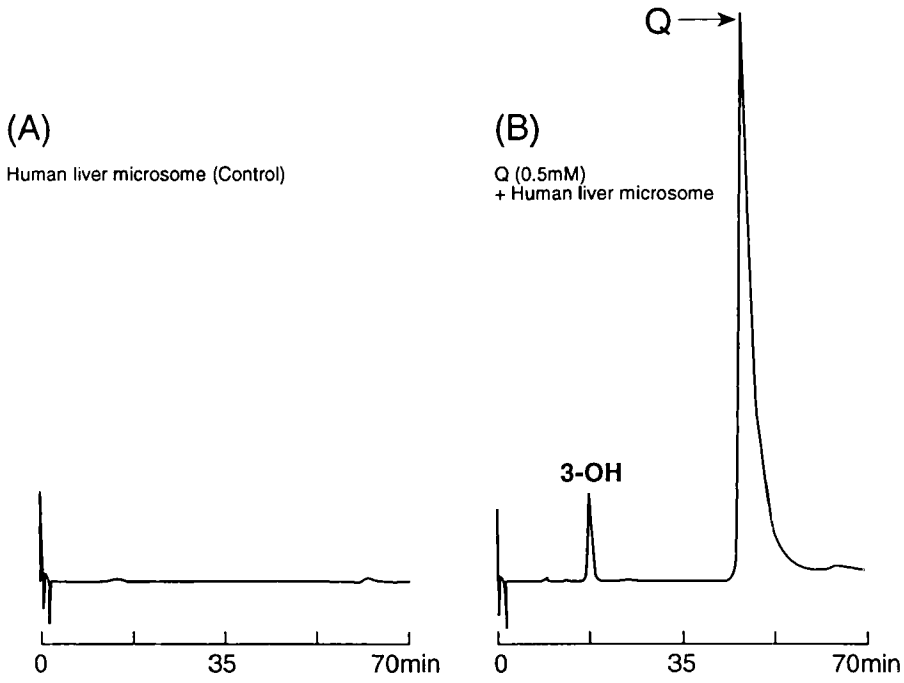

Figure 4. HPLC chromatograms showing the formation of the major metabolite (3-hydroxyquinine) by human liver microsomes : (A) blank liver microsomal sample; and (B) quinine (0.5 mM) was incubated with human liver microsomes (1 mg/mL) in the presence of 1 mM NADPH, at 37°C for 30 min. Chromatographic separation was performed by using the modified mobile phase as described in Figure 1. Peaks : 3-OH = 3-hydroxyquinine (19.5 μM); and Q = quinine (not determined

suggesting this metabolite is 3-hydroxyquinine. Evidence obtained from the inhibition study and enzyme activity correlation study also suggests that the formation of 3-hydroxyquinine is catalysed by P450IIIA.[16,17]

Detector response of 3-hydroxyquinine and quinine was found to be linear covering a concentration range of 0.1 to 30 μM (0.034 to 10.2 μg/mL) for 3-hydroxyquinine and 0.5 to 30 μM (0.16 to 9.6 μg/mL) for quinine. These were observed in plasma, urine and liver microsomal samples. The linear calibration curves for both 3-hydroxyquinine and quinine were obtained with square of the

correlation coefficient (r^2) greater than 0.99. The day-to-day coefficients of variation (C.V.) of the slope of the calibration curves for both compounds were less than 5% (n = 5).

The within-day reproducibility of the method was determined by replicate analyses (n = 5-6) of drug-free urine spiked with known concentrations of 3-hydroxyquinine and quinine. The results, expressed as mean values of the concentrations found, are given in Table 1. The within-day coefficients of variation (C.V.) for both 3-hydroxyquinine and quinine were less than 7%, illustrating the precision of a method suitable for metabolism and pharmacokinetic studies. The C.V. values of the assay at a concentration of 0.1 μM 3-hydroxyquinine was 6.6%, and for quinine at a concentration of 0.5 μM was 6.3%, which are lower than the generally accepted C.V. of 20% for minimum quantifiable concentration (MQC). Thus, the detection limit of sensitivity for this assay for 3-hydroxyquinine and quinine was assigned at 0.1 μM (0.034 μg/mL) and 0.5 μM (0.16 μg/mL), respectively. The results with respect to reproducibility and precision of the assay in plasma and liver microsomal samples were also similar to those of urine samples. Although the detection limit for quinine is much higher than that reported in previous methods[8-11, 13-15] this limit was found to be sufficient for determination of quinine concentrations in urine and hepatic micrsomal samples. If very low detection limits are desirable, e.g. for plasma quinine concentrations, the simple HPLC method by Zoest et al.[10] can be used.

The absolute recovery from biological samples of 3-hydroxyquinine and quinine was assessed by comparing the peaks of 3-hydroxyquinine and quinine with those obtained from direct injection of the pure standards of the drugs of equivalent quantities. The mean recovery for 3-hydroxyquinine (n = 4) from plasma samples was 90 ± 8% at 0.1 μM and 94 ± 5% at 10 μM. The mean recovery for 3-hydroxy-quinine (n = 4) from urine samples was 94 ± 4% at 0.1 μM and 97 ± 3% at 10 μM. The mean recovery for 3-hydroxyquinine (n = 4) from human liver microsomal samples was also similar, i.e. 89 ± 5% at 0.1 μM and 97 ± 4% at 10 μM.

Plasma and urine samples, and also the liver microsomal samples (after treatment with methanol) stored at -20°C for up to two months, showed no signs of decomposition and practically the same concentration values were obtained (n = 5). This suggests that 3-hydroxyquinine is stable under these storage conditions for at least two months.

This HPLC method is now intensively used in our laboratories to analyse the plasma and urine samples from healthy volunteers receiving oral doses of quinine in pharmacokinetic studies and to analyse the hepatic microsomal samples from metabolism studies. The method described here has proved to be simple as it does not involve solvent extraction. This specific HPLC assay is suitable for simultaneous quantitation of 3-hydroxyquinine and quinine concentrations in biological fluids. The limit of detection may be further improved by doubling the injection volume from 30 μL to 60 μL without overloading the column.

In summary, the method presented in this communication is simple. It is sufficiently reproducible and sensitive to be used both in metabolic and pharmacokinetic studies. With ease of analytical assay for 3-hydroxyquinine, it is suggested that quinine could be used as an alternative model drug for P450IIIA.

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