Journal of Chromatography B, 877 (2009) 441–445

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Simultaneous liquid chromatographic analysis of ritonavir, quinine and 3-hydroxyquinine in human plasma

Julius O. Soyinka [a], Cyprian O. Onyeji [a,*], Sharon I. Omoruyi [b]

[a] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Nigeria
[b] Department of Clinical Pharmacy and Pharmacy Administration, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Nigeria

ARTICLE INFO

Article history:
Received 7 September 2008
Accepted 17 December 2008
Available online 25 December 2008

Keywords:
Ritonavir
Quinine
3-hydroxyquinine
Liquid chromatography

ABSTRACT

In regions with high prevalence of HIV and malaria, co-infection of both diseases is common; hence, there is a high possibility of concurrent administration of antiretroviral and antimalarial drugs. This study describes a new ion-pair reversed-phase high-performance liquid chromatographic (HPLC) method for simultaneous determinations of ritonavir (RTV), quinine (QN), and its major metabolite, 3-hydroxyquinine (3-HQN), in human plasma. Following a simple extraction with diethyl-ether under alkaline conditions, chromatographic separation was achieved on a 5-μm particle size C-18 column (200 mm × 4.6 mm I.D.) using a mobile phase consisting of methanol:acetonitrile:0.02 M potassium dihydrogen phosphate (15:10:75) containing 75 mmol/L perchloric acid (pH 2.8). Retention times for RTV, 3-HQN, QN and the internal standard were 2.8, 4.0, 7.0 and 12 min, respectively. The limits of detection and validated lower limits of quantitation were 10 and 12.5 ng/ml for RTV while the corresponding values were 5 and 70 ng/ml for both QN and 3-HQN, respectively. The new HPLC method is simple, rapid, selective, reproducible and cost-effective. As demonstrated in three volunteers, it will facilitate the conducting of simultaneous therapeutic monitoring of quinine and ritonavir in patients concurrently receiving both drugs.

1. Introduction

The prevalence of malaria and HIV as well as the extent of their geographical overlap varies widely within different regions. In countries with high prevalence of both infections, co-infection is common; hence, the possibility of a patient taking an antimalarial and an antiretroviral drug concurrently is very high [1]. The occurrence of resistance to chloroquine and sulphadoxine-pyrimethamine by the malaria parasite in Southern Asia, Africa and South America stimulated new interest in quinine as an alternative drug for treating multi-drug resistant *P. falciparum* malaria [2]. Quinine is available in oral and injectable formulations and it has tolerable side effects if it is used correctly and at the normal therapeutic doses [2]. It is the drug of choice for the management of severe malaria in most areas of the world, and is frequently deployed in conditions where intravenous infusions cannot be rapidly established or reliably monitored [3].

The emergence of non-nucleoside reverse transcriptase inhibitor (NNRTI) resistance has led to the increased use of protease inhibitors (PI) for the oral treatment of HIV infection. Thus, ritonavir and other drugs that inhibit HIV protease have become the most potent antiretroviral agents for the treatment of HIV-infected patients [4,5]. Ritonavir has also been used to optimize PI pharmacokinetics by combining low doses of the drug with a second PI [6].

From the foregoing, it is likely that quinine may be given to a malaria and HIV-infected patient receiving ritonavir. In order to successfully conduct any therapeutic drug monitoring, a suitable analytical procedure must be available. Several assay methods are available for determination of ritonavir and quinine separately in biological fluids [7–10] but there is no reported method for the simultaneous determination of both drugs. Several studies have established associations between plasma concentrations of protease inhibitors and their antiviral effects [11–13], suggesting a role for therapeutic monitoring of these drugs. Although the protease inhibitors have a great clinical impact as a drug class (with ritonavir as the most potent of the class), they have a narrow therapeutic index buttressing the need for therapeutic drug monitoring [14–16]. It was also suggested that quinine concentrations should be monitored [17], recommending a therapeutic peak plasma quinine concentrations of 10–15 μg/ml in severe falciparum malaria. These levels were reported as toxic in non-malaria patients. Monitoring the plasma levels of 3-hydroxyquinine along with quinine is also necessary since this major metabolite of quinine is reported to have a higher toxicity than quinine, but much less potent (10× lower) as an antimalarial [18]. Hence, 3-hydroxyquinine has toxicological implications, underscoring the need for its monitoring. Thus, therapeutic drug monitoring is a

* Corresponding author. Tel.: +234 8037058720.
E-mail address: conyeji@oauife.edu.ng (C.O. Onyeji).

1570-0232/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2008.12.045

valuable tool in improving the treatment of HIV patients that may be taking ritonavir and quinine concurrently. Despite some limitations, routine measurement of plasma concentrations not only provides practical information about pharmacokinetics and drug interactions, but may also protect patients from the occurrence of adverse effects and prevent therapeutic failure. Furthermore, since ritonavir is a potent inhibitors of CYP 3A4 [19], it can be projected that potentially significant interactions could occur with drugs that are extensively metabolized by CYP 3A4, and quinine is known to be mainly metabolized by this isoenzyme. It was therefore necessary to develop a rapid, selective and sensitive method for the simultaneous assay of quinine, 3-hydroxyquinine and ritonavir in human plasma. When compared with other methods used for the analysis of ritonavir, the present method is also less cumbersome and highly sensitive for the analysis of the drug.

2. Experimental

2.1. Chemicals and reagents

Quinine, [(9R)-6′-methoxycinchonan-9-ol] (Fig. 1) in its sulphate salt was purchased from British Drug House (BDH) (Poole, UK), while the major metabolite of quinine, 3-hydroxyquinine, [(9R)-6′-methoxycinchonan-3, 9-ol] (Fig. 1), was a generous gift from Prof James M Cook, University of Wisconsin, USA. Ritonavir capsules were obtained from Federal Medical Stores Lagos, Nigeria. Acetonitrile and methanol (HPLC grades), potassium dihydrogen phosphate, diethyl ether (analar grade) and other chemicals such as hydrochloric acid and sodium hydroxide were all purchased from Sigma–Aldrich Chemicals (Steinheim, Germany). Perchloric acid (Hopkins and Williams, Essex, UK) was of analar grade. Pyrimethamine, 2,4-diamino-5-4-chlorophenyl-6-ethylpyrimidine (Fig. 1), obtained from Swiss Pharma Nig Ltd. was used as the internal standard.

2.2. Preparation of stock solutions

Stock solutions containing 1 mg/ml quinine base and 3-hydroxyquinine were prepared in 0.1 M hydrochloric acid, while ritonavir and pyrimethamine were prepared in methanol and acetonitrile, respectively.

2.3. Chromatographic conditions and instrumentation

The HPLC equipment was an AKTA system (Amersham Pharmacia Biotech, Uppsala Sweden) consisting of binary pumps (P-900) fitted with a gradient mixer and a variable wavelength (200–800 nm) ultraviolet–visible detector (model UV-900). Sample injection was through a model INV-907 valve fitted with a 50 μL loop. The detector output was linked to a computer via a brain box interphase (AKTA instrument), which transforms signals from the detector to the computer that eventually records the chromatograms. Chromatographic separation was achieved at ambient temperature on Eclipse – XDB (C-18) (Agilent Technologies, Palo Alto, CA, USA), a 5-μm particle size C-18 column (200 mm × 4.6 mm I.D.). A mobile phase consisting of methanol: acetonitrile: 0.02 M potassium dihydrogen phosphate (15:10:75, v/v) containing 75 mmole/L perchloric acid (pH 2.8) was pumped through the column at a flow rate of 1.0 ml/min. The column effluent was monitored with the detector set at 254 nm. Vortex mixer (Gallenkamp, London, UK) and centrifuge (Gallenkamp) were used in the extraction procedure.

2.4. Sample preparation and extraction procedure

To 1 ml of plasma placed in a 20-ml screw-capped extraction tube were added the internal standard (IS) (20 μl of 500 μg/ml) and 200 μl of perchloric acid (concentrated, 60% (w/w)) before vortex-mixing for 10 s. The plasma samples were rendered alkaline with 5 M NaOH (1 ml) and 4 ml of diethylether was added. This was vortex-mixed for 1 min and followed by centrifugation at 3000 g for 10 min. The ether layer was transferred into a tapered-end tube and 200 μL of 0.1 M HCl was added. After vortex-mixing (1 min) and centrifugation (3000 g for 5 min) the ether layer was discarded and a 50 μL aliquot of the aqueous layer was injected onto the HPLC column.

2.5. Calibration procedure

Standard curves based on peak-area ratios (drug to IS) were prepared by spiking drug-free plasma with standard solutions of quinine (QN), 3-hydroxyquinine (3-HQN) and ritonavir (RTV) to give a concentration range of 0.125–8 μg/ml for QN and 3-HQN, and 0.01–16 μg/ml for RTV. Samples were processed as described above and the peak area ratio of each compound was plotted against the corresponding concentration. Linear regression analysis of the peak-area ratio compared with the compound concentration was performed to obtain correlation coefficient for each standard curve.

2.6. Analytical recovery, precision and accuracy

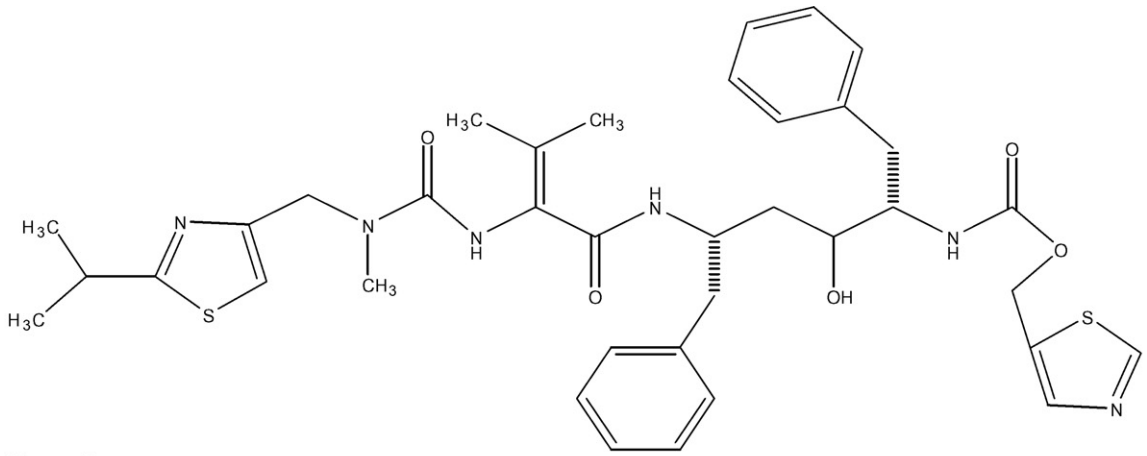
Replicate samples of blank plasma were spiked with standard solutions of the three compounds (QN, 3-HQN and RTV) to give concentrations at their limits of quantitation and upper ranges of their calibration curves (0.07, 0.5 and 4 μg/ml for QN and 3-HQN, and concentrations of 0.0125 and 8 μg/ml for RTV). Each sample was spiked with the IS (20 μL of 500 μg/ml) and taken through the extraction procedure as described above. The absolute recovery was determined by comparing the peak areas of the extracted compounds with those obtained from direct injection of known equivalent amounts of each compound. Peak-area ratios were converted to concentrations using the calibration curves, and the coefficients of variation of the estimated concentrations were determined and used for assessment of precision. Accuracy of the analytical method for each of the three compounds was evaluated from the percentage ratio of the experimentally determined drug concentration to that of the actual concentration. In these determinations, a standard curve based on plasma-extracted samples was used for extracted samples, while for unextracted samples, a corresponding standard curve was used.

2.7. Selectivity

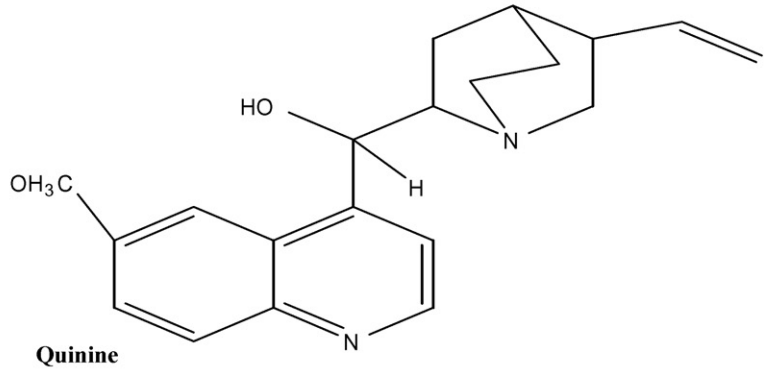
Various antimalarial drugs and other drugs commonly co-administered with antimalarials such as amodiaquine, chloroquine, proguanil, primaquine, cycloguanil, paracetamol, chlopheniramine and promethazine were evaluated for interference with the assay. Also, the selectivity of the method in relation to some other antiretroviral drugs (saquinavir, indinavir, zidovudine and abacavir) was evaluated. Drug-free plasma was spiked with therapeutic concentrations of the drugs followed by extraction and analysis as described.

2.8. Application of the analytic method

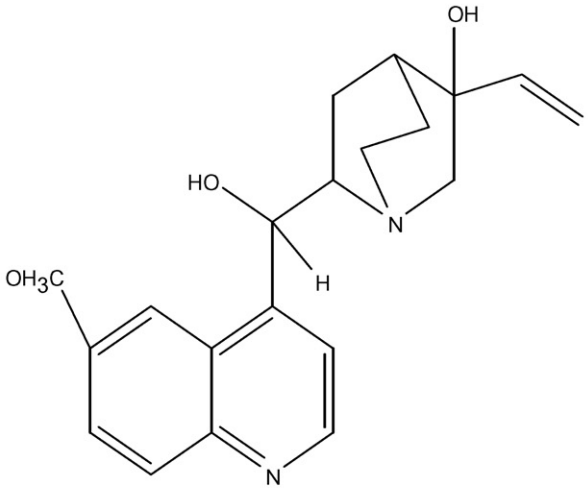
Three healthy volunteer who had not been taking any other drug, received 200 mg oral doses of ritonavir 12 hourly for 9 days (i.e. 18 doses). A single oral dose of 600 mg quinine sulphate was concurrently given with the 15th ritonavir dose (day 8). Thereafter, venous



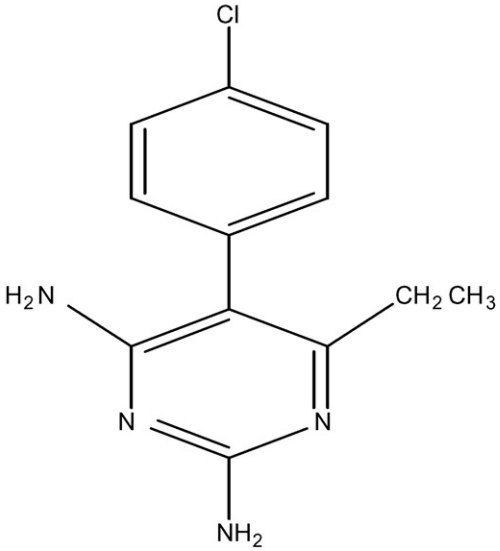
Ritonavir



Quinine



3-Hydroxyquinine



Pyrimethamine (Internal Standard)

Fig. 1. Chemical structures of quinine, 3-hydroxyquinine, pyrimethamine (internal standard) and ritonavir.

blood samples (5 ml) were collected into heparinised tubes just before the concurrent drug administration and at 1, 2, 4, 6, 8, 12, 24, 36 and 48 h after drug administration. The blood samples were centrifuged at 2000 × g for 15 min to obtain the plasma which was analyzed for QN, 3-HQN and RTV concentrations using 1 ml aliquots of plasma samples and following the procedures described.

The maximum plasma drug concentration (C_{max}) was estimated by visual inspection of the concentration–time data. The total area under plasma concentration vs. time curve (AUC_T) was obtained from a sum of $AUC_{0\text{-}t}$ and Ct/β, where $AUC_{0\text{-}t}$ was derived using the linear trapezoid method up to the last time point concentration (Ct). β is the elimination rate constant obtained by linear regression analysis of the terminal phase of the curve.

3. Results

Typical chromatograms obtained from the described HPLC method are shown in Fig. 2. These demonstrate that the peak

Table 1
Precision, recovery and accuracy of the analytical method.

Drug	*n*	Concentration (μg/ml)	Intra-day precision (CV) (%)	Inter-day precision (CV) (%)	Recovery mean ± S.D. (%)	Accuracy mean ± S.D. (%)
QN	6	0.07	7.83	8.83	90.3 ± 5.6	93.3 ± 7.5
	6	0.5	3.57	3.53	92.0 ± 4.8	96.0 ± 8.0
	6	4.0	3.45	1.10	94.8 ± 3.3	92.2 ± 3.3
3-HQN	6	0.07	8.23	9.20	92.0 ± 3.5	92.3 ± 5.3
	6	0.5	2.75	3.26	95.4 ± 5.3	98.3 ± 5.0
	6	4.0	3.02	2.89	96.7 ± 4.5	94.8 ± 4.0
RTV	6	0.0125	2.20	1.86	96.5 ± 7.0	95.6 ± 3.2
	6	8.0	1.53	2.10	95.7 ± 3.2	97.7 ± 6.0

QN, quinine; 3-HQN, 3-hydroxyquinine; RTV, ritonavir.

of QN was well resolved from the internal standard as well as from 3-HQN and RTV. The retention times (t_R) of RTV, 3-HQN, QN, and IS were 2.8, 4, 7 and 12 min, respectively. There was no interference from endogenous compounds. Also no interference with the peaks of the compounds was found from chloroquine, primaquine, proguanil, cycloguanil, paracetamol, chlopheniramine, promethazine, saquinavir, indinavir, zidovudine and abacavir. The minimum detectable concentrations, taken as a concentration giving a peak three times the baseline noise was 5 ng/ml for QN and 3-HQN, and 10 ng/ml for RTV, using 1-ml sample volumes. The limit of quantitation was determined as the lowest concentration of the compound which can be quantitatively determined with an acceptable level of precision and accuracy [20]. The value of 70 ng/ml was obtained for QN and 3-HQN while that for RTV was 12.5 ng/ml. Linear curves were obtained for the compounds in plasma over the concentration ranges tested, with correlation coefficients of not less than 0.99 for each of the curves. The results of the precision, recovery and accuracy of the analytical method for the three compounds are shown in Table 1. The coefficients of variation (CV) for both the intra-day and inter-day analysis ranged from 1.10 to 8.42% for QN, 2.75 to 9.20% for 3-HQN, and 1.53 and 2.20% for ritonavir. The absolute recovery was over 90% for the three compounds.

The mean (±) plasma concentration vs. time profiles of QN, 3-HQN and RTV following concurrent administration of multiple doses of ritonavir (200 mg 12 hourly for 18 doses) and a single 600 mg oral dose of quinine sulphate to each of three volunteers are depicted in Fig. 3. The concentration of ritonavir did not fall below the limit of quantitation after 12 h of the drug administration. Ritonavir levels were only determined within a dosing interval of 12 h after the 15th ritonavir dose since such drug concentrations are more relevant for evaluation of pharmacokinetics of a drug when steady-state drug levels are approached in a multiple dose regimen. The pharmacokinetic parameters obtained for quinine for each of the three subjects were: 9.80, 10.10 and 10.96 mg/L for the Cmax, while the values for AUC_T were 208.81, 217.70, and 227.12 mg/L h, correspondingly. The plasma levels of 3-hydroxyquinine were low, with a C_{max} not exceeding 1 mg/L. The C_{max} values obtained for ritonavir were 10.08, 11.32 and 12.38 mg/L, for each of the subjects.

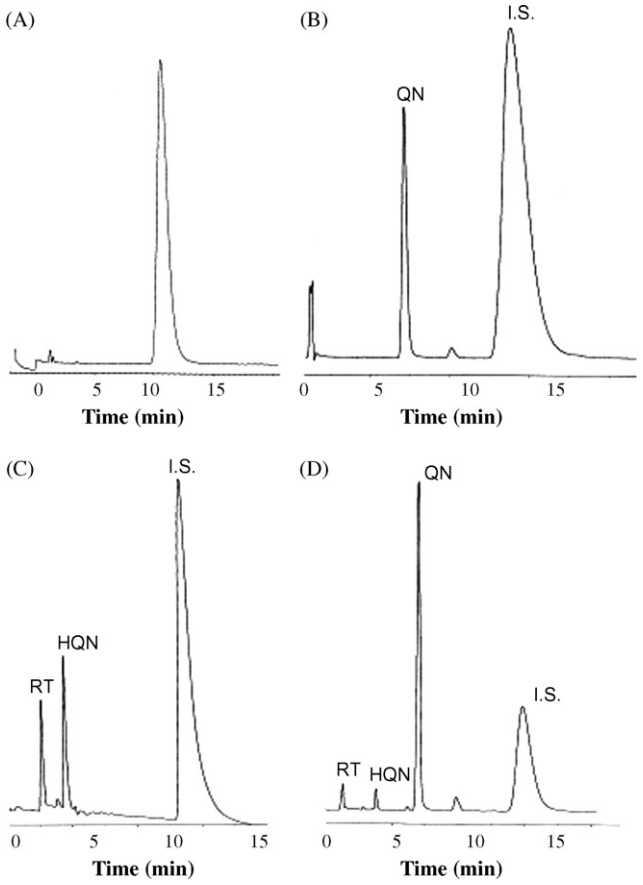


Fig. 2. High-performance liquid chromatograms of (A) an extracted blank plasma spiked with the internal standard (IS) (pyrimethamine 20 μl of 500 μg/ml), (B) extracted blank plasma spiked with 2 μg quinine (QN)/ml of plasma and IS, (C) extracted blank plasma spiked with 4 μg ritonavir (RTV) and 2 μg of 3-hydroxyquinine (3-HQN)/ml of plasma along with the IS, (D) extracted plasma sample obtained from a volunteer at 6 h following concurrent administration of a single 600 mg oral dose of quinine sulphate with multiple oral doses of RTV (200 mg 12 hourly for 18 doses). The quinine dose was co-administered with the 15th ritonavir dose. The concentrations of QN, 3-HQN and RTV were 4.52, 0.75 and 3.64 μg/ml, respectively.

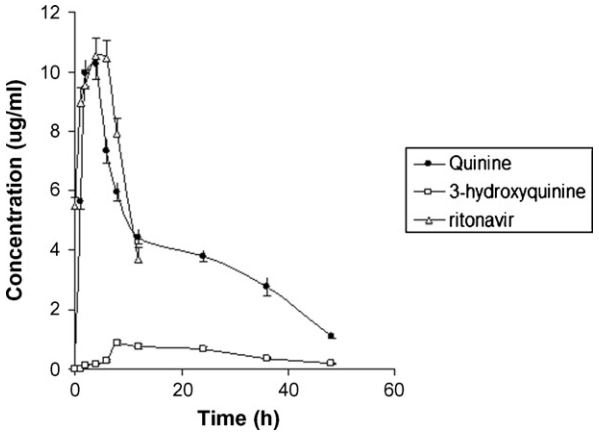


Fig. 3. Mean (±) plasma concentration vs. time profiles of quinine (QN), 3-hydroxyquinine (3-HQN) and ritonavir (RTV) following concurrent administrations of a single 600 mg oral dose of quinine sulphate with multiple oral doses of ritonavir (200 mg 12 hourly for 18 doses) to each of three healthy volunteers. The quinine dose was co-administered with the 15th ritonavir dose.

4. Discussion

The extraction procedure employed in this study produced clean and clear supernatants from plasma as there was no interference from endogenous compounds. All the four compounds (ritonavir, 3-hydroxyquinine, quinine, and IS) were completely resolved to baseline and samples could be injected at 13-min intervals. Sulphadoxine–pyrimethamine is not used in combination with quinine in malaria chemotherapy; therefore, the possibility of interference from the internal standard is remote. Results of the assessments of precision, recovery and accuracy given in Table 1 show that the method has a high degree of precision as the intra-day and inter-day coefficients of variation were not greater than 9% at low and high concentrations of the three compounds. The recovery of over 90% for QN, 3-HQN and RTV by the analytical method shows that the sample preparation and extraction procedure was efficient for the compounds. Evidence of accuracy of the method is demonstrated in the results which ranged between 92 and 97% for the compounds at low and high concentrations. It was necessary to precipitate the protein so as to release more of the drugs since both quinine and ritonavir [21,22] are highly bound to plasma proteins. This was achieved by adding 200 μL of perchloric acid which was enough to denature the proteins before proceeding with the extraction of the drug.

Therapeutic drug monitoring is recommended for protease inhibitors including ritonavir [11–13], as well as for quinine [17]. The method reported here retains the sensitivity and precision of other previous methods for the separate analysis of the drugs but it also has the advantage of being simple and devoid of any cumbersome extraction procedure. The composition of the mobile phase also proves the simplicity of the method. The mobile phase consisted mainly (75%) of potassium dihydrogen phosphate solution and small amounts of methanol and acetonitrile which are commonly available, thus, making the method cost-effective and affordable. The rapidity of the method is underlined by the relatively short analysis time. The maximum time required for a sample treatment prior to injection was 10 min, which is much shorter than the analysis time of 62 and 40 min reported by other workers [23,24], for the analysis of quinine alone in plasma.

To evaluate the application of this method in therapeutic drug monitoring and pharmacokinetic studies, concentrations of QN, 3-HQN and RTV were measured in plasma of three volunteers after multiple oral doses of RTV (200 mg 12 hourly for 9 days) and a single 600 mg oral dose of quinine sulphate concurrently given with the 15th ritonavir dose. The pharmacokinetic parameters of quinine for the subjects indicate that the C_{max} were markedly higher than the range of C_{max} values (3–4.5 mg/L) [25,26] obtained in healthy volunteers following a single 600 mg oral dose of the drug administered alone. The AUC_T values are up to three times higher than the values (42–80 mg/L h) obtained for the drug when given alone to healthy volunteers [25,26]. These data, coupled with the low plasma exposure of the metabolite when compared to literature values obtained from volunteers that received quinine alone, are suggestive of significant interaction between quinine and ritonavir. This underscores the need for therapeutic monitoring of the antimalarial when co-administered with ritonavir. The therapeutic dose of ritonavir is 600 mg twice daily; however, the drug is frequently used in combination with other protease inhibitors as a booster of these compounds. The booster dose is 100 or 200 mg as used in this report and results in non-therapeutic plasma concentrations of ritonavir. The C_{max} of ritonavir obtained in the volunteers (10.08–12.38 mg/L) were understandably considerably below the C_{max} of 27 mg/L above which adverse effects of the drug have a tendency to occur [27]. Hence, therapeutic monitoring of ritonavir only becomes necessary when the drug is administered in therapeutic doses.

5. Conclusion

In conclusion, the HPLC method described is very simple, reproducible, sensitive and rapid. The method is also accurate, selective and cost-effective. It will facilitate the conducting of simultaneous therapeutic monitoring of quinine, based on its C_{max}, and ritonavir in patients concurrently receiving quinine and therapeutic doses of ritonavir. The method is also suitable for pharmacokinetic studies of quinine and 3-hydroquinine as well as for ritonavir.

Acknowledgement

This work was supported by Obafemi Awolowo University, Ile-Ife, Nigeria, Research Grant No. 11813 AEC.

References

[1] World Health Organization, Malaria and HIV/AIDS interactions and implications: conclusions of a technical consultation convened by WHO, 23–24 June 2004.
[2] J.W. Tracy, L.T. Webster, Goodman and Gilman's The Pharmacological Basis of Therapeutics, McGraw-Hill, New York, 1996.
[3] K. Sanjeev, V. Nelamangala, P. Tim, A. Tsiri, W. Peter, Antimicrob. Agents Chemother. 45 (2001) 1803.
[4] D.D. Ho, A.U. Neumann, A.S. Perelson, Nature 373 (1995) 123.
[5] A.S. Perelson, A.U. Neumann, M. Markowitz, Science 271 (1996) 1582.
[6] R.L. Walmsley, B. Bernstein, M. King, N. Engl. J. Med. 346 (2002) 2039.
[7] D. Ouellet, A. Hsu, J. Qian, C.S. Locke, C.J. Eason, J.H. Cavanaugh, Br. J. Clin. Pharmacol. 48 (1998) 111.
[8] P. Leibenguth, C. Le Guellec, J.M. Besnier, F. Bastides, M. Mace, G. Paitaud, Ther. Drug. Monit. 16 (2001) 679.
[9] S. Wanwimolruk, S. Chalcroft, P.F. Coville, A.J. Campbell, Trans. R. Soc. Trop. Hyg. 84 (1991) 448.
[10] C.P. Babalola, O.O. Bolaji, P.A.F. Dixon, F.A. Ogunbona, J. Chromatogr. 616 (1993) 151.
[11] A. Molla, M. Korneyeva, Q. Gao, Nat. Med. 2 (1996) 760.
[12] E.P. Acosta, J.G. Gerber, AIDS Res. Hum. Retroviruses 18 (2002) 825.
[13] D.M. Burger, P.W.H. Hugen, R.E. Aanouste, Ther. Drug. Monit. 25 (2003) 73.
[14] A. Hsu, G.R. Granneman, G. Witt, Antimicrob. Agents Chemother. 41 (1997) 898.
[15] G.J. Moyle, Exp. Opin. Invest. Drugs 8 (1999) 473.
[16] R.E. Aarnoutse, J.M. Schapiro, C.A.B. Boucher, Drugs 63 (2003) 741.
[17] World Health Organization. WHO expert committee on malaria, 18th report. World Health Organization Technical Report Series, 735, 1986, 57.
[18] E.M. Geilling, F.E. Kelsey, Navnyn-Schmiedeberg's archives of Pharmacology 205 (2004) 167.
[19] G.N. Kumar, A.D. Rodrigues, A.M. Buko, J.F. Denissen, J. Pharmacol. Exp. Ther. 277 (1996) 423.
[20] Centre for Drug Evaluation and Research, FDA, Validation of chromatographic methods, 1994.
[21] K. Silamut, N.J. White, S. Looareesuwan, D.A. Warell, Am. J. Trop. Med. Hyg. 24 (1985) 681.
[22] S.H. Khoo, S.E. Gibbons, D.S. Back, AIDS 15 (2001) 5171.
[23] J. Karbwang, K. Nabagchang, P. Molunto, D. Bunnag, South East Asian J. Trop. Med. Pub. Health 20 (1989) 65.
[24] M.D. Edstein, A. Prasitthipayong, A. Sabchareon, T. Chongsuphajaisiddhi, H.K. Webster, Ther. Drug Monit. 12 (1990) 493.
[25] S. Wanwimolruk, M. Sunbhanich, M. Pongmarutai, Br. J. Clin. Pharmacol. 22 (1986) 346.
[26] A. Sowunmi, L.A. Salako, Eur. J. Clin. Pharmacol. 49 (1996) 383.
[27] G.G. Gatti, A.D. Biagu, R. Casazzo, AIDS 13 (1999) 2083.