Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Journal of Chromatography B, 803 (2004) 371–374
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Short communication

Liquid chromatographic determination of amodiaquine in human plasma

Virendra K. Dua [a,*], N.C. Gupta [a], V.P. Sharma [b], S.K. Subbarao [b]

[a] Malaria Research Centre, Field Station, Sector III, BHEL, Hardwar 249403, India
[b] Malaria Research Centre, 22 Sham Nath Marg, Delhi 110054, India

Received 27 March 2003; received in revised form 30 December 2003; accepted 8 January 2004

Abstract

A normal-phase high-performance liquid chromatographic method using dichloromethane– methanol–1 M perchloric acid (100:10:0.9, v/v/v) at a flow rate of 1.0 ml min^{-1} on a LiChrospher Si column with UV (254 nm) detection has been developed for the determination of amodiaquine and its metabolites desethyl amodiaquine and bisdesethyl amodiaquine in plasma. The limit of quantification was 5 ng ml^{-1}. Mean within-day and day-to-day coefficients of variation (CV) were 4.10 and 6.27% for amodiaquine, 3.43 and 4.80% for desethyl amodiaquine and 3.53 and 5.23% for bisdesethyl amodiaquine, respectively. Mean extraction recovery of amodiaquine, desethyl amodiaquine and bisdesethyl amodiaquine from plasma were 82.48, 74.50 and 69.65%, respectively. Chloroquine and its metabolite desethyl chloroquine, quinine, sulfadoxine and primaquine do not interfere in the detection of amodiaquine, desethyl amodiaquine and bisdesethyl amodiaquine in plasma.
© 2004 Elsevier B.V. All rights reserved.

Keyword: Amodiaquine

1. Introduction

The substituted 4-aminoquinoline amodiaquine has been used in the treatment and prophylaxis of *P. falciparum* malaria for over 40 years. Although there are concerns over the potential toxicity of amodiaquine when used prophylactically [1]. The drug has invariantly shown greater antimalarial activity against *P. falciparum* and most importantly retains therapeutic activity against chloroquine resistant parasites [2]. Amodiaquine had been initially recommended for chloroquine resistant RI level areas from India, however, it is withdrawn now due to toxicity factors. Recently, amodiaquine assumed significance because of its possible role in combination therapy with artimisnin derivatives [3]. HPLC methods have been developed to determine the concentration of amodiaquine in body fluids [4–7]. Bergqvist and Churchill [8] reviewed the determination of levels of different antimalarials in biological fluids. The most sensitive and complete HPLC assay of amodiaquine and its three known metabolites used oxidative electrochemical detection and has a limit of determination of 3 nmol l^{-1} for amodiaquine, desethyl amodiaquine and bisdesethyl amodiaquine in whole blood [7]. The method of Pussard et al. [4] determines the quantity of amodiaquine and desethyl amodiaquine simultaneously in body fluids with a limit of determination of 10 nmol l^{-1}. However, most methods lack baseline resolution between amodiaquine and its metabolites and the interference due to endogenous plasma peaks do not permit accuracy and sensitivity required for therapeutic monitoring of amodiaquine in plasma. This paper describes a suitable HPLC for the determination of amodiaquine and its metabolites in human plasma.

2. Materials and methods

2.1. Chemicals and standards

HPLC grade dichloromethane and methanol were obtained from Ranbaxy Fine Chemicals, Delhi, while diethylether, perchloric acid, K_2HPO_4 and other chemicals were of analytical reagent grade and were used without further purification. Amodiaquine, desethyl amodiaquine

* Corresponding author.
E-mail address: virendradua@hotmail.com (V.K. Dua).

1570-0232/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2004.01.011

and bisdesethyl amodiaquine were supplied by Parke Davis (Pontypool UK). *N*-Acetyl primaquine was obtained from CDC Atlanta and used as an internal standard (IS). Stock solutions of amodiaquine, desethyl amodiaquine and bisdesethyl amodiaquine ($0.02\,mg\,ml^{-1}$) were prepared separately in mobile phase. The concentration range of $25–500\,ng\,ml^{-1}$ were used for the calibration curves of amodiaquine, desethyl amodiaquine and bisdesethyl amodiaquine while the internal standard ($2\,\mu g\,ml^{-1}$) was used throughout the study. All solutions of drug, its metabolites and internal standard were prepared in mobile phase and stored at 4 °C.

2.2. Instrumentation and chromatographic conditions

A Waters HPLC system (Waters Assoc., Milford, MA, USA) consisting of a 510 pump, 486 multi-wavelength UV detector operated at 254 nm, a Rheodyne injector and a Shimadzu CR8 integrator was used for the analysis. LiChrospher Si (E. Merck)-based normal-phase column (4 mm × 250 mm; particle size 5 μm) was used for the study.

The mobile phase consisting of dichloromethane–methanol–1 M perchloric acid (100:10:0.9, v/v/v) was pumped at a flow rate of $1.0\,ml\,min^{-1}$ at ambient temperature. The mobile phase was filtered and degassed by ultrasonication (Dacon FS 100, Hove, UK) before use.

2.3. Extraction procedure

All glasswares were pre-treated with silane (5% solution in toluene) in order to minimize adsorption of amodiaquine and its metabolites [5]. To a sample (standard or analysis) of 0.5 ml plasma, 100 μl of internal standard ($2\,\mu g\,ml^{-1}$), 1 ml of 50% aqueous K_2HPO_4 solution and 6 ml of diethylether were added. The tubes were shaken for 15 min on a orbital shaker (Denley, Billingshurst, UK). It is to note that the addition of K_2HPO_4 increased the recovery of desethyl amodiaquine during extraction process. The extracted sample was centrifuged at 1000 × *g* for 10 min and the ether layer (organic phase) was separated. The aqueous phase was re-extracted by adding 1 ml K_2HP04, 1 ml (1 M) NaOH and 6 ml diethyl ether. The extracted sample was again centrifuged at 1000 × *g* for 10 min and ether layer (organic phase) was separated. The pH of the solution before and after addition of NaOH were 8.5 and >10, respectively. Both organic phases were pooled together and evaporated to dryness under a stream of nitrogen at 25 °C.

The residue was reconstituted in mobile phase (200 μl) and injected (100 μl) to HPLC system for analysis.

2.4. Recovery and reproducibility

The recovery was determined at concentrations of 25, 100, 250, $500\,ng\,ml^{-1}$ of amodiaquine, desethyl amodiaquine and bisdesethyl amodiaquine in plasma by comparing peak-height/area ratios of spiked standard with the ratio obtained by direct injection of pure standards. Within-day and day-to-day reproducibility of the method were determined by repeated assay of different concentrations of amodiaquine, desethyl amodiaquine and bisdesethyl amodiaquine.

2.5. Stability of amodiaquine, desethyl amodiaquine and bisdesethyl amodiaquine

The stability of amodiaquine, desethyl amodiaquine and bisdesethyl amodiaquine were determined in the samples stored over a period of 3 months at 4 °C and their concentrations were determined at regular intervals.

3. Results and discussion

Various proportions of dichloromethane, methanol and perchloric acid as mobile phase were used to achieve the separation of amodiaquine, desethyl amodiaquine, bisdesethyl amodiaquine and internal standard. It was found that the separation was best achieved using dichloromethane–methanol–1 M perchloric acid (100:10:0.9, v/v/v) as mobile phase on LiChrospher Si-based normal-phase column with a flow of $1.0\,ml\,min^{-1}$. Amodiaquine, desethyl amodiaquine and bisdesethyl amodiaquine were resolved to baseline with retention times of 9.6, 12.4 and 21.2 min, respectively. Fig. 1 showed the chromatographic separation of amodiaquine, desethyl amodiaquine, bisdesethyl amodiaquine and *N*-acetyl primaquine (internal standard). The capacity factors (k') of most common antimalarial drugs detected by UV (254 nm) are given in Table 1, which clearly shows that chloroquine and its metabolites desethyl chloroquine, quinine, primaquine and sulfadoxine do not interfere in the determination of amodiaquine and its metabolites by this method.

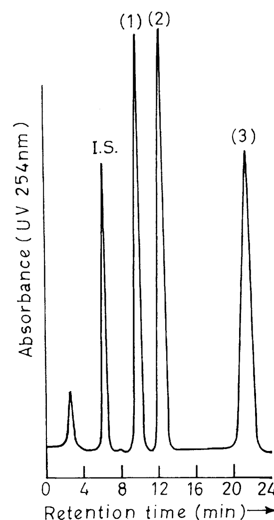

Fig. 1. Chromatographic separation of: (1) amodiaquine ($76\,ng\,ml^{-1}$), (2) desethyl amodiaquine ($75\,ng\,ml^{-1}$), (3) bisdesethyl amodiaquine ($62\,ng\,ml^{-1}$) and *N*-acetyl-primaquine (IS = internal standard).

Table 1
Capacity factors[a] of various antimalarials

Antimalarial	k'
Amodiaquine	3.00
Desethyl amodiaquine	4.17
Bisdesethyl amodiaquine	7.83
N-Acetyl primaquine (IS)	1.50
Chloroquine	2.67
Desethyl chloroquine	3.17
Quinine	2.40
Sulfadoxine	1.33
Primaquine	4.60

[a] Mobile phase: dichloromethane–methanol–1 M perchloric acid (100:10:0.9, v/v/v); flow rate, 1.0 ml min^{-1}; UV detection wavelength, 254 nm; column, LiChrospher Si normal-phase column.

No degradation was detected for amodiaquine, desethyl amodiaquine and bisdesethyl amodiaquine in plasma at 4 °C over 3 months. The minimum detectable concentration for simultaneous determination of amodiaquine and its metabolites were 5 ng ml^{-1} by requiring a signal-to-noise ratio of greater than 5:1. Laurent et al. [9] have reported plasma quantification limits of 3.3 ng ml^{-1} for amodiaquine.

Precision of the HPLC method for amodiaquine and its metabolites is given in Table 2. Mean within-day coefficient of variation for amodiaquine desethyl amodiaquine and bisdesethyl amodiaquine were 4.10, 3.43 and 3.53%, respectively. Similarly day-to-day variation for amodiaquine, desethyl amodiaquine and bisdesethyl amodiaquine were 6.27, 4.80 and 5.23%, respectively (Table 2). Mean extraction recoveries of amodiaquine, desethyl amodiaquine and bisdesethyl amodiaquine in plasma were 82.48, 74.50 and 69.65%, respectively (Table 3).

During the study, calibration curves were obtained for the concentrations of 25, 100, 200 and 500 ng ml^{-1} for amodiaquine, desethyl amodiaquine and bisdesethyl amodiaquine, which showed linear relationships over the concentration used with the correlation coefficients $r > 0.99$.

Amodiaquine and its metabolites were detected by UV at 340 nm earlier [4,5] inspite of at 254 nm because endogenous components peaks of plasma extract produced

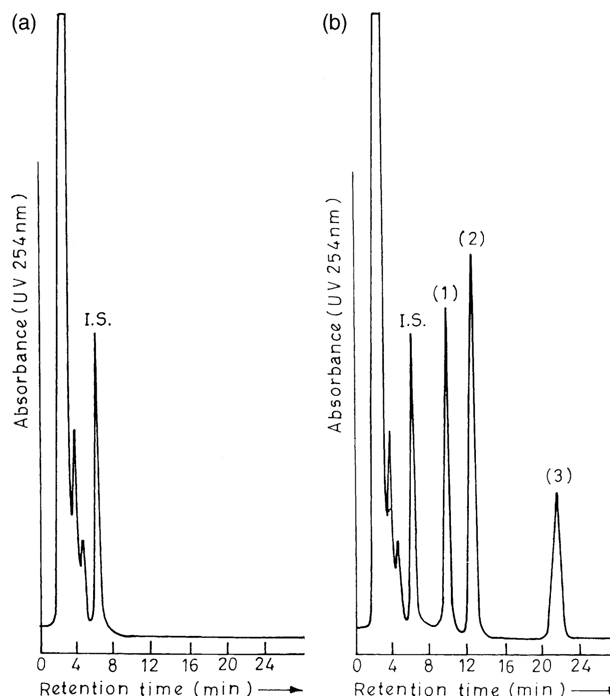

Fig. 2. Chromatographic behavior of a blank plasma extract spiked with internal standard (a) and a plasma extract spiked with: (1) amodiaquine (57 ng ml^{-1}), (2) desethyl amodiaquine (65 ng ml^{-1}), (3) bisdesethyl amodiaquine (30 ng ml^{-1}) and *N*-acetyl primaquine (IS).

chromatographic interference at 254 nm [4]. However, it is clearly stated that sensitivity at 340 nm was less than at 254 nm because of their differences of molar extinction coefficients at these wavelengths. It is to point out that no interference due to endogenous plasma peaks were observed by the present method for the detection of amodiaquine and its metabolites at 254 nm. Which appeared before the peaks of amodiaquine and its metabolites. A comparison of analytical sensitivity of amodiaquine, desethyl amodiaquine and bisdesethyl amodiaquine at 254 and 340 nm revealed that detection was 25, 27 and 24% higher, respectively, at 254 nm than at 340 nm. Fig. 2 showed the chromatographic behavior of a blank plasma extract spiked with internal

Table 2
Precision of the HPLC method for amodiaquine, desethyl amodiaquine and bisdesethyl amodiaquine in plasma (spiked samples)[a]

	Concentration (ng ml^{-1})	Coefficient of variation (cv%)		
		Amodiaquine	Desethyl amodiaquine	Bisdesethyl amodiaquine
Within-day	25	5.8	4.2	4.8
	100	2.6	3.6	3.2
	500	3.9	2.5	2.6
Mean ± S.D.	–	4.10 ± 1.61	3.43 ± 0.86	3.53 ± 1.14
Day-to-day	25	8.5	5.2	6.2
	100	6.5	6.3	5.6
	500	3.8	2.9	3.9
Mean ± S.D.		−6.27 ± 2.94	4.80 ± 1.73	5.23±1.19

[a] $n = 5$ in each case.

Table 3
Extraction recovery of the HPLC method for amodiaquine, desethyl amodiaquine and bisdesethyl amodiaquine in plasma

Concentration (ng ml^{-1})	Recovery (%) (mean ± S.D.)[a]		
	Amodiaquine	Desethyl amodiaquine	Bisdesethyl amodiaquine
25	78.24 ± 3.53	71.34 ± 4.35	62.39 ± 4.35
100	81.34 ± 4.34	74.38 ± 2.45	73.24 ± 3.12
250	84.66 ± 5.12	73.95 ± 1.45	70.36 ± 1.25
500	85.68 ± 2.19	78.34 ± 3.46	72.62 ± 2.85
Mean ± S.D.	82.48 ± 3.38	74.50 ± 2.89	69.65 ± 4.99

[a] $n = 4$.

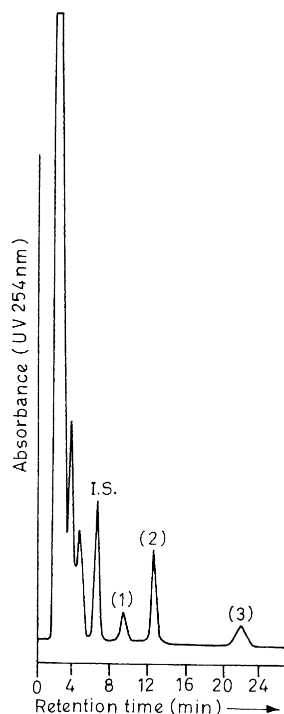

Fig. 3. The chromatographic behavior of a plasma extract taken at 2:30 h after the treatment with 600 mg amodiaquine base to a *P. falciparum* infected patient. Peaks: (1) amodiaquine (12 ng ml^{-1}), (2) desethyl amodiaquine (37 ng ml^{-1}) and (3) bisdesethyl amodiaquine (8 ng ml^{-1}).

standard (Fig. 2a) and a plasma extract spiked with amodiaquine, desethyl amodiaquine and bisdesethyl amodiaquine (Fig. 2b). The chromatographic behavior of a plasma extract taken at 2:30 h after the treatment with 600 mg amodiaquine base to a *P. falciparum* infected patient is given in Fig. 3.

A comparison of the retention times of amodiaquine, desethyl amodiaquine and bisdesethyl amodiaquine clearly revealed that the removal of ethyl group increases the retention time thus followed normal-phase phenomenon with ion-pair mode as stated earlier [10].

Although amodiaquine has been withdrawn for chloroquine resistant *P. falciparum* cases as monotherapy, its use in combination therapy with artemisinine derivatives or with sulfadoxine-pyrimethamine has regenerated interest as antimalarial. The present method may have application in therapeutic monitoring of amodiaquine during treatment of malaria cases with amodiaquine-based combinations.

References

[1] T.G. Geary, J. B Jensen, J. Parasitol. 69 (1983) 97.
[2] H.V. Scott, W.L. Tan, G.B. Barlin, Ann. Trop. Med. Parasitol. 81 (1987) 85.
[3] WHO, Antimalarial drug combination therapy WHO/CDS/RBM/2001.35.
[4] E. Pussard, F. Verdier, M. Blayo, J. Chromatogr. 374 (1986) 111.
[5] G.W. Mihaly, D.D. Nicholl, G. Edwards, S.A. Ward, M. Orme, D.A. Warrell, A.M. Breckenridge, J. Chromatogr. 337 (1985) 166.
[6] P. Winstanley, G. Edwards, M. Orme, A. Breckenridge, Br. J. Clin. Pharm. 23 (1987) 1.
[7] D.L. Mount, L. Patchen, P. Nguyen-Dinh, A.M. Barber, I. Schwartz, F. Churchill, J. Chromatogr. 383 (1986) 375.
[8] Y. Bergqvist, F.C. Churchill, J. Chromatogr. 434 (1988) 1.
[9] F. Laurent, S. Saivin, P. Chretien, J.F. Magnaval, F. Peyron, A. Sqalli, A.E. Tufenkji, Y. Coulais, H. Baba, G. Campistron, H. Regis, P. Ambroise-Thomas, A. Bryskier, G. Houin, Arzneim-Forsch./Drug Res. 43 (1993) 612.
[10] V.K. Dua, P.K. Kar, N.C. Gupta, V.P. Sharma, J. Pharm. Biomed. Anal. 21 (1999) 199.