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Comparison of the urinary excretion time profile of amodiaquine in albino rabbits by fluorometric and high-performance liquid chromatographic methods

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Summary

Fluorometric and high-performance liquid chromatographic (HPLC) methods have been used to study the urinary excretion time profile of amodiaquine in albino rabbits after single oral (18.5 mg) and i.v. (9 mg) administration. There was no significant difference between the total mean values obtained for the two methods ($P > 0.05$). Although the HPLC method is more selective, one can still rely on the fluorometric method to measure urine concentrations of amodiaquine for therapeutic drug monitoring where toxicological conditions are not taken into consideration.

Résumé

Des procédés fluorométriques et liquides chromatographiques à haute exécution (HPLC) ont été employés dans une étude du profile-excrétion-heure-urinaire d'amodiaquine des lapins blancs, après une seule administration par la bouche (18.5 mg) et intraveineuse (9 mg). Il n'y avait pas de différence signifiante entre les valeurs totales moyennes obtenues en utilisant les deux méthodes ($P > 0.05$). Bien que la méthode-HPLC soit plus sélective, on peut toujours compter sur la méthode fluorométrique pour mesurer les concentrations d'urine de l'amodiaquine d'une drogue thérapeutique, abstraction faite des conditions toxicologiques.

Introduction

Amodiaquine — 7-chloro-4-(3′-diethylaminomethyl-4′-hydroxyanilino)quinoline — has generally been considered equal in antimalarial activity to the more widely used chloroquine. However, it has been suggested by recent studies that amodiaquine is more effective than chloroquine for the treatment of drug resistant falciparum malaria [1,2]. Although amodiaquine has been shown to give rise to agranulocytosis in chronic users, it may still be useful in cases of acute malaria in endemic areas [3,4]. Knowledge of its disposition in man has only recently been re-evaluated as a result of the development of specific high-performance liquid chromatography (HPLC) analytical techniques [5–7]. On the other hand, the pharmacokinetics of chloroquine have been more extensively studied [8–13]. Amodiaquine (AMQ) is mostly used in the tropics where more advanced chromatographic methods are not readily available. Because of its toxicity [14] on the bone marrow, therapeutic drug monitoring may be advisable in a few instances. Therefore, there is the need to develop a simple, cheap and reliable method for quantifying amodiaquine in biological materials. In this investigation, urinary drug levels were used to estimate the kinetics of AMQ, on the assumption that renal clearance was constant and urinary excretion rate proportional to plasma concentration. Added to this, AMQ is rapidly converted to its metabolites. Albino rabbits were used as that strain was available in the central animal house, University of Ibadan, Ibadan, Nigeria at the time of analysis.

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Materials and methods

Fluorometry

All fluorometric analyses were carried out on a Perkin-Elmer fluorescence spectrophotometer

(Model 204), Orion ion-specific pH meter (Model 407A) and combination pH electrode. All reagents used were of analytical grade. 1,2-Dichloroethane was purified for fluorometric use, as employed by Trenholme *et al.* [15]. Trisodium orthophosphate ($Na_3PO_4 \cdot 12H_2O$), 50% dipotassium hydrogen phosphate (K_2HPO_4) were supplied by BDH. Amodiaquine tablets and amodiaquine dihydrochloride (pure) were from Parke-Davis.

HPLC

The equipment and reagents were similar to those employed by Winstanley *et al.* [6].

Treatment of animals and collection of urine

Twelve rabbits were divided into two groups (A and B) of six (male and female, mean weight 1.8 kg). They were collected from the animal house of the University of Ibadan, Nigeria. Rabbits from group A (oral) were given 10 mg/kg amodiaquine hydrochloride solution through a stomach tube, while rabbits from group B (i.v.) were given 5 mg/kg amodiaquine hydrochloride solution through their ear veins. All the rabbits in groups A and B were then housed separately in metabolic cages throughout the study period of 7 days, to facilitate complete urine collection. All urine samples were kept frozen at −20°C until time of analysis. Fluorometric determinations were made at the Department of Pharmacology and Therapeutics, University of Ibadan, Ibadan, Nigeria, while similar samples were later transported frozen in dry ice, packed in polyurethane insulation box to the Pharmacology Department, University of Liverpool, Liverpool, U.K. for the HPLC analysis. All samples were analysed within 3 months of collections.

Fluorometric assay of amodiaquine

The method described by Trenholme *et al.* [15] was modified; this modification involved mainly the use of phosphate buffer (pH 10.9) instead of borate buffer (pH 9.5). Other changes included the addition of 0.1 ml 50% w/v dipotassium hydrogen phosphate to 1 ml urine sample instead of 0.2 ml; the replacement of 6 ml for 10 ml 1,2-dichloroethane solution for extraction; and the transferral of 5 ml of the 1,2-dichloroethane layer into 2 ml 0.1 M hydrochloric acid instead of 8 ml and 3 ml respectively.

HPLC assay of amodiaquine

Concentrations of AMQ and desethylamodiaquine (AMQm) were measured as described by Winstanley *et al.* [6].

Calculations

The concentration (fluorometry) of AMQ was calculated from its standard curves (0–5 μg/ml) in drug-free urine, using the relationship:

$$\text{Concentration} = \frac{A_u}{A_s} \times C_s,$$

where A_u = absorbance of the unknown, A_s = absorbance of the standard and C_s = concentration of AMQ in the standard, while concentrations (HPLC) of AMQ and AMQm in ng/ml were determined from the size of their chromatographic peaks in standard curves in the range of 0–500 ng/ml. Recoveries of AMQ, AMQm and the internal standard were estimated by comparing the peak-height ratio obtained from an extracted urine sample with that from aqueous solution containing the same amount of each compound. Replicate assays of the same sample were used to determine the intra- and inter-assay coefficient of variation for AMQ and AMQm.

The half-life ($t_{1/2}$) of AMQ for the intravenous or oral route was estimated from the calculated elimination rate constant (K_{el}) which is the slope of the log linear regression of the plot of log-excretion rate (ng h^{-1}) against mid-point interval of collection time. Assuming that renal clearance (Cl_r) is constant and the urinary excretion rate is proportional to plasma concentration [16], the areas under the concentration time curves from 0 to t (132 h) were calculated by linear trapezoidal summation [7] and from t to ∞ by the ratio of C_t/K_{el}, where C_t was the concentration at time t. The area under the curve — AUC (0, ∞) was then obtained by the summation of these two areas. The initial concentration of the drug, C_0 was calculated by the method of least squares [18]. This value, C_0,

was then used to estimate the urinary excretion rate, K_u [19]. The correlation coefficient (r) of individual paired rabbits for the two methods was determined to test for linearity. Similarly differences were evaluated using Student's t-test, and $P < 0.05$ taken as significant.

Results

The calibration curve for the concentration (0–5 μg/ml) of amodiaquine was linear ($r > 0.99$) by fluorometry, with a detection limit of 20 ng/ml. For the intra- and inter-assay of amodiaquine (100 ng/ml), the coefficients of variation were 7.3% and 10.0% respectively.

A plot of peak-height ratios of AMQ and AMQm to 6-methoxy-8-amino-quinoline as internal standard against the corresponding concentrations of drug-free rabbit urine is shown (Fig. 1). AMQm, AMQ and internal standard were resolved, with retention times of 3.6, 4.8 and 5.6 min respectively. Standard curves were linear ($r \geq 0.9994$) for both compounds in the ranges of 0–500 ng/ml. The minimum detectable concentrations of AMQ and AMQm in urine sample (a peak three times baseline noise at a maximum sensitivity of 0.005 AUFS) was about 5 ng/ml. Reproducibility of this analytical method was determined both intra- and inter-assay for each compound in urine. The coefficients of variation were 6.5% and 8.7% using 100 ng/ml AMQ ($n = 7$) for intra- and inter-assay respectively. Similarly the coefficients of variation were 7.7% and 9.4% for AMQm.

The total quinoline values of AMQ excreted over 7 days after a single oral dose of 18.5 mg amodiaquine (Table 1) range between 1.94 and 2.20 mg by fluorometry and 1.90–2.32 mg (AMQ + AMQm) for the same interval when assayed by HPLC. Similarly, the total quinoline values obtained were 1.02–1.27 by fluorometric assay and 1.00–1.20 mg by HPLC method when a single i.v. dose (9 mg) of amodiaquine was given. There was no statistically significant difference ($P > 0.05$) in the fluorometric assay of AMQ alone and AMQ + AMQm by HPLC for the two different routes of AMQ administration. The correlation coefficient ($r \geq 0.9807$) between individual paired rabbits for the two methods is good.

The pharmacokinetic parameters (Table 2) of amodiaquine were obtained from the plot of log-excretion rate (ng/h) of amodiaquine (total quinoline) *versus* mid-point interval of collection time in the urine of albino rabbits after

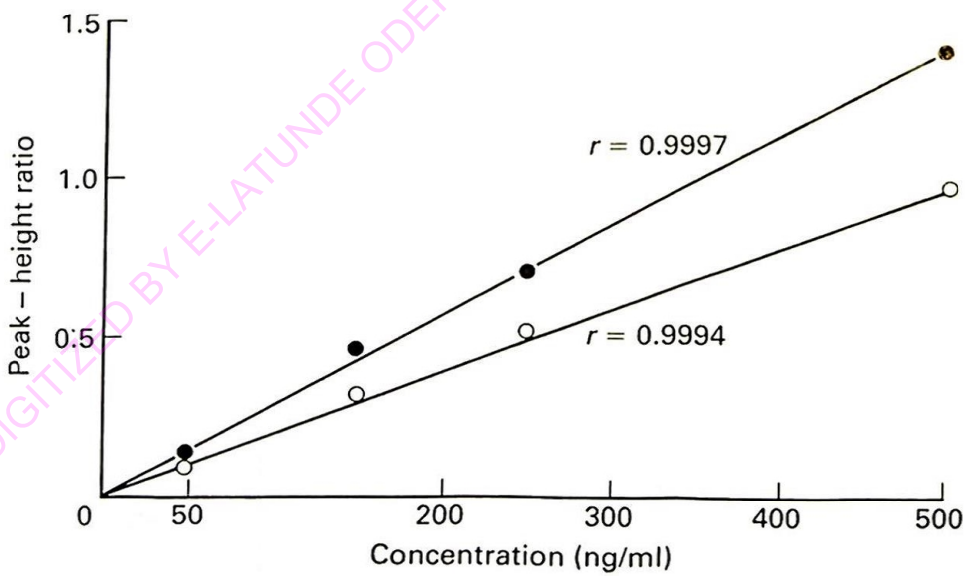

Fig. 1. Plot of peak-height ratios of amodiaquine (AMQ; ●) and desethylamodiaquine (AMQm; ○) to the internal standard *versus* concentrations of AMQ and AMQm, respectively, in rabbit urine.

Table 1. Fluorometric and HPLC analysis of AMQ (total quinoline, mg) in the urine of four albino rabbits after oral (10 mg/kg) and i.v. (5 mg/kg) administration; results are mean values of four determinations

	Oral								Intravenous							
	Fluorometry				HPLC				Fluorometry				HPLC			
Time of collection (h)	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
0–24	1.45	1.74	1.35	1.65	1.44	1.79	1.35	1.63	0.85	0.91	0.71	0.76	0.83	0.90	0.68	0.73
24–48	0.31	0.29	0.37	0.35	0.30	0.27	0.35	0.33	0.20	0.20	0.17	0.18	0.22	0.18	0.19	0.15
48–72	0.16	0.17	0.15	0.11	0.15	0.16	0.12	0.13	0.07	0.08	0.07	0.07	0.07	0.07	0.06	0.07
72–120	0.09	0.09	0.07	0.08	0.08	0.09	0.07	0.07	0.08	0.08	0.07	0.07	0.05	0.05	0.05	0.05
120–144	0.08	0.008	0.008	0.008	0.008	0.008	0.008	0.008	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003
144–168	0.004	0.004	0.003	0.003	0.004	0.004	0.003	0.003	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Total	2.02	2.01	1.94	2.20	1.98	2.32	1.90	2.17	1.22	1.27	1.02	1.08	1.17	1.20	1.98	1.00
*$r >$					0.999	0.99	0.999	0.999					0.98	0.999	0.999	0.999

*Correlation coefficient (r) of individual paired rabbits ranges between 0.98 and 1.00.

Table 2. Kinetics of AMQ in the urine of four rabbits per group after single oral and i.v. administration measured by fluorometric and HPLC methods; each result is the mean of four determinations

Route	Method of measurement	Rabbit	K_{el} (h^{-1})	$t_{1/2}$ (h)	C_0 (mg)	K_u (h^{-1})	$AUC_{0-\infty}$ (mg ml^{-1} h^{-1})
Oral	Fluorometry	1	0.046	15.2	2.0	0.009	0.75
		2	0.034	20.3	1.5	0.005	0.54
		3	0.050	14.0	2.2	0.011	0.83
		4	0.038	18.2	1.6	0.006	0.61
	HPLC	1	0.046	15.2	2.1	0.010	0.73
		2	0.035	19.8	1.6	0.006	0.53
		3	0.049	14.1	2.1	0.010	0.80
		4	0.038	18.3	1.5	0.006	0.60
i.v.	Fluorometry	1	0.051	13.5	1.7	0.017	0.39
		2	0.055	12.6	1.8	0.020	0.42
		3	0.041	16.7	1.4	0.011	0.32
		4	0.040	17.3	1.3	0.010	0.28
	HPLC	1	0.052	13.3	1.6	0.017	0.38
		2	0.055	12.6	1.9	0.021	0.41
		3	0.042	16.5	1.4	0.012	0.31
		4	0.039	18.0	1.3	0.010	0.28

Correlation coefficient (r) of individual paired rabbits for the two methods > 0.99

single oral and i.v. administration by fluorometric (Fig. 2a) and HPLC (Fig. 2b) methods. Straight line curves were drawn for both routes by the process of least squares. For the fluorometry, the elimination rate constant (K_{el}), terminal half-life ($t_{1/2}$), area under the curve ($AUC_{0-\infty}$), and urinary excretion rate (K_u) were 0.04 h^{-1}, 16.9 h, 0.68 mg ml^{-1} h^{-1} and 0.01 h^{-1} for the oral route, and for the i.v. route were 0.05 h^{-1}, 15.0 h, 0.35 mg ml^{-1} h^{-1} and 0.02 h^{-1} respectively. Similarly, the kinetic values for the HPLC method were 0.04 h^{-1}, 16.9 h, 0.67 mg ml^{-1} h^{-1} and 0.01 h^{-1} for the oral route, while the values for the i.v. route were 0.05 h^{-1}, 15.1 h, 0.35 mg ml^{-1} h^{-1} and 0.02 h^{-1} respectively.

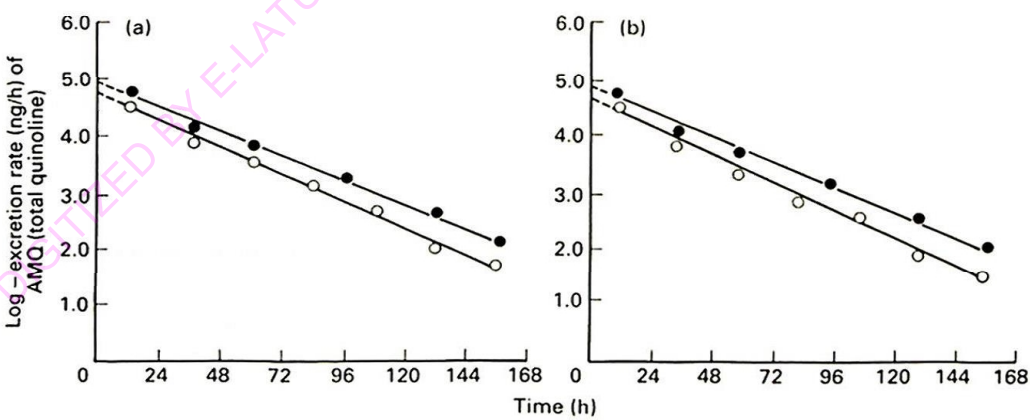

Fig. 2. Log-excretion rate of amodiaquine (AMQ) *versus* time after oral (●) and i.v. (○) administration, measured by (a) fluorometric and (b) HPLC methods.

Discussion

In this report, the use of phosphate buffer pH 10.9 instead of borate buffer pH 9.5 [15] resulted in a 60% increase in sensitivity of the systems to UV detection. This increases the limit of detection to 20 µg/l as opposed to 50 µg/l when borate buffer is used. The percentage recovery was between 98% and 100% while that of the previous method was between 80% and 100% using the same concentration of 500 µg/l amodiaquine in the urine of rabbit. This shows a substantial improvement in methodology. The recovery obtained using the HPLC method [6] was 60%. This was quite low. However, the use of internal standard ensures that errors of extraction are erased when variation is largely due to changes in the system. The limit of detection was between 5–10 µg/l, which makes this system more sensitive than the fluorometric method. One added advantage is the separation of the metabolites and other substances from the same extraction.

The fact that there was no statistical difference ($P > 0.05$) between the total mean values obtained for the two methods lends support to the hypothesis that fluorometry is not selective when quinoline moieties are assayed. This same problem was highlighted by Rombo *et al.* [20] when they compared the fluorometric assay of chloroquine to HPLC.

The total mean concentrations of AMQ excreted unchanged in the urine of rabbit up to 48 h for the oral and i.v. routes by HPLC (Table 1), represent only about 4.3% and 4.7% respectively of the administered doses. It seems probable that the remaining drug will be bound to tissues as metabolites [21,22] which are released slowly or excreted through other media (faeces, bile and skin).

The pharmacokinetic values (Table 2) support the findings of Barrow [22] and of Winstanley *et al.* [7] in rats that amodiaquine is quickly metabolized and eliminated from the body.

In summary, the fluorometric and HPLC methods have been used to estimate the urinary excretion time profile of amodiaquine in urine without any significant difference in total quinoline content ($P > 0.05$). There was a good correlation coefficient ($r > 0.98$) of individual paired rabbits for the two methods (Table 1). Nevertheless, there was inter-individual variation. Although the former method is not selective, it may have clinical applicability, especially where toxicological climate is not of importance. With HPLC, expensive reagents of a high level of purity are required. Added to this is the high cost of its maintenance. In contrast, fluorometric instruments do not require the same high level of attention and solvents need not be of the highest level of purity. As we have shown, when the properties of the compounds to be assayed are fairly well-known, not much is gained by using the more expensive chromatographic method.

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