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Determination of artemether and its metabolite, dihydroartemisinin, in plasma by high-performance liquid chromatography and electrochemical detection in the reductive mode

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Abstract

An analytical method for the determination of artemether (A) and its metabolite dihydroartemisinin (DHA) in human plasma has been developed and validated. The method is based on high-performance liquid chromatography (HPLC) and electrochemical detection in the reductive mode. A, DHA and artemisinin, the internal standard (I.S.), were extracted from plasma (1 ml) with 1-chlorobutane–isooctane (55:45, v/v). The solvent was transferred, evaporated to dryness under nitrogen and the residue dissolved in 600 μl of water–ethyl alcohol (50:50, v/v). Chromatography was performed on a Nova-Pak CN, 4 μm analytical column (150 mm×3.9 mm I.D.) at 35°C. The mobile phase consisted of pH 5 acetate–acetonitrile (85:15, v/v) at a flow-rate of 1 ml/min. The analytes were detected by electrochemical detection in the reductive mode at a potential of −1.0 V. Intra-day accuracy and precision were assessed from the relative recoveries (found concentration in % of the nominal value) of spiked samples analysed on the same day (concentration range 10.9 to 202 ng/ml of A and 11.2 to 206 ng/ml of DHA in plasma). The mean recoveries over the entire concentration range were from 96 to 100% for A with C.V. from 6 to 13%, from 92% to 100% for DHA (α-tautomer) with C.V. from 4 to 16%. For A, the mean recovery was 96% at the limit of quantitation (LOQ) of 10.9 ng/ml with a C.V. of 13%. For DHA, the mean recovery was 100% at the LOQ of 11.2 ng/ml with a C.V. of 16%.

Keywords: Artemether; Dihydroartemisinin

1. Introduction

Artemether (A) is a potent antimalarial compound under development as a combination with benflumetol. The measurement of artemisinin (antimalarial drug) and its derivatives (as A) in biological fluids has been reviewed [1]. HPLC methods with electrochemical detection in the reductive mode were described for arteether and its metabolite, dihydroartemisinin (DHA) [2], and also for A and its metabolite, DHA [3], in plasma. The first one [2] including an automated deoxygenation/injection step, was validated between 25 and 1000 ng/ml for each compound. The most recent method [3], without automated injection, was validated between 15 and 240 ng/ml for both compounds, A and DHA.

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The objective was to develop and validate a method offering a better sensitivity for A and DHA, including an automated sample deoxygenation and injection system allowing to greatly increase sample throughput.

2. Experimental

2.1. Chemicals and reagents

The following reference compounds were supplied by Ciba-Geigy (Basle, Switzerland): artemether (A), $C_{16}H_{26}O_5$, mol. wt.=298.38; dihydroartemisinin (DHA), $C_{15}H_{24}O_5$, mol. wt.=284.35, a mixture of α and β tautomers with unknown ratio of α versus β; and artemisinin (I.S.), $C_{15}H_{22}O_5$, mol. wt.=282.34.

All solvents and reagents were of analytical grade and used without further purification: 1-chlorobutane from Merck (Darmstadt, Germany), isooctane from Merck, ethyl alcohol from Carlo-Erba (Milan, Italy), acetonitrile from Carlo-Erba, anhydrous sodium acetate from Merck, acetic acid from Merck, sodium chloride from Merck, HPLC water from Baker (Deventer, Netherlands).

All glass tubes were pre-treated to prevent adsorption as follows: they were immersed in toluene containing hexamethyldisilazane (Fluka, Buchs, Switzerland), trimethylchlorosilane (Fluka) and pyridine (Fluka) (1%, v/v, each) for 30 min and rinsed with methanol. Between treatments, the tubes were cleaned as usual and rinsed with methanol. Silanized vials (1.5 ml) were used (Alltech, Deerfield, USA) for injection.

2.2. Equipment

The chromatographic system consisted of a pump, Model LC 10 AD from Shimadzu (Tokyo, Japan) and a Gilson autoinjector Model 231 XL (autosampler), equipped with a Gilson Model 401C syringe dilutor (Villiers-le-Bel, France). The system was modified to automatically deoxygenate each sample with argon before injection, mainly as follows: all tubings were made of stainless steel, in order to prevent from any entry of oxygen into the system; two electromagnetic valves were installed on the hydraulic line, which respectively interrupted (cut-off valve) and diverted (3-way valve) automatically the argon flow; the sample injector needle was directly connected to the Rheodyne injection valve port, in place of the standard injection port. The automatic injection protocol included two steps: one deoxygenation sequence (Fig. 1), while injection valve is switched in "load" position, cut-off valve and 3-way valve are switched in "open" position: argon is backflushed through the Rheodyne injection valve towards the autoinjector needle, in order to deoxygenate both sample and hydraulic line and one injection sequence, while injection valve remains in position "load", 3-way valve is activated in "close" position, deoxygenated sample is withdrawn from the vial through the injection loop by the dilutor syringe (Fig. 1), and then the injection valve is normally switched in "inject" position (Fig. 1).

Argon was chosen in preference to helium because oxygen is better moved by argon. But helium, less expansive, could be used.

An electrochemical detector, Model Decade from Antec Leyden (Leiden, Netherlands) was used to quantify the compounds of interest. The instrument, equipped with a VT-03 flowcell with glassy carbon working electrode, operated in the reductive mode and the working potential was set at −1 V vs. Ag/AgCl. The detector range used was either 10 or 20 nA/V and the rise time filter was set at 1 s for DHA and at 2 s for the I.S. and A by making use of an internal time file, which was triggered by the autosampler. The integrated oven was set at +35°C and accommodated the VT-03 flowcell, the column and high-efficiency pulse dampener (SSI, State College, PA, USA). Since oxygen is a notorious interfering compound in LC combined with reductive electrochemical detection at the present setting, rigorous measures were taken to eliminate any dissolved oxygen from entering the flowcell. Therefore, both the mobile phase and the samples were thoroughly degassed with argon. To prevent any back-diffusion of this gas through the fluid lines, all Teflon tubing was replaced by stainless steel (from mobile phase reservoir to LC column) and by poly ethee ether ketone (PEEK) between column and flowcell. For the three compounds (A, DHA and I.S.), the highest sensitivities (I) were obtained at −1 V working potential (E). No problem of selectivities were met, so it was possible to work at this highest

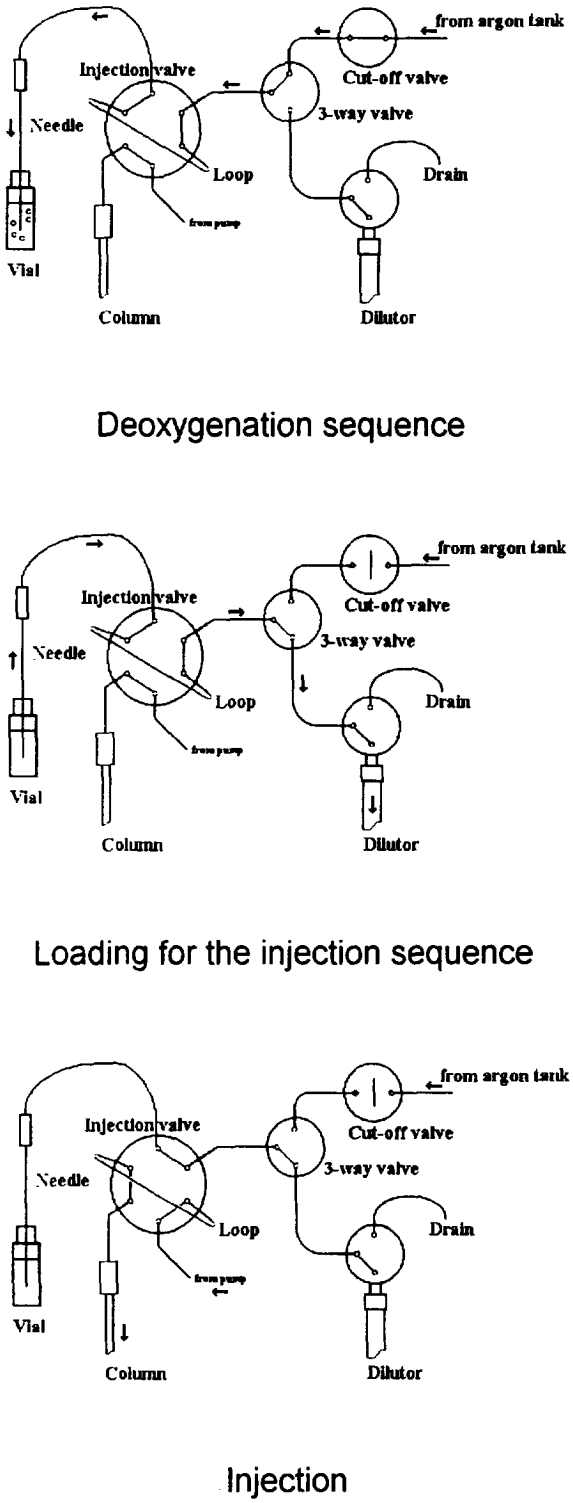

Fig. 1. Automated deoxygenation and injection device.

negative potential. It is the highest negative potential limit used with glassy carbon electrode and acidic mobile phase. A hydrodynamic voltammogram is shown in Fig. 2. A chromatography data acquisition workstation Model Millennium from Waters (Milford, USA) was used.

Chromatographic separations were performed at +35°C on a column packed with Nova-Pak CN, 4 μm (150×3.9 mm I.D.) supplied by Waters. The mobile phase used at a flow-rate of 1 ml/min consisted of pH 5 acetate buffer (0.1 *M* CH_3COONa solution adjusted to pH 5 with acetic acid)–acetonitrile (85:15, v/v).

2.3. Calibration and validation samples

The stock solutions of A and DHA were prepared by dissolving about 2 mg of substance in 100 ml of ethyl alcohol. Appropriate serial dilutions of the stock solutions with water–ethyl alcohol (50:50, v/v) were made subsequently in order to prepare the spiking solutions at concentrations ranging from about 400 to 2000 ng/ml. The spiking solutions were used for the preparation of the calibration samples. Other stock solutions of A and DHA in water–ethyl alcohol (50:50, v/v) at the same concentrations were prepared from a second stock and appropriately diluted to give spiking solutions for use in the preparation of validation (accuracy and precision assessments), quality control and stability samples. The I.S. stock solution was prepared by dissolving about 2 mg of I.S. in 100 ml of ethyl alcohol. Further dilution of the stock solution with water–ethyl alcohol (50:50, v/v) resulted in the internal standard

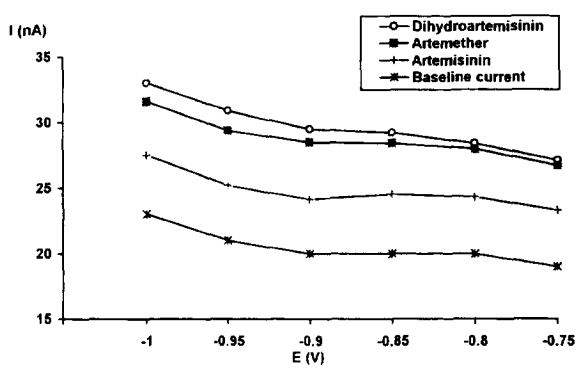

Fig. 2. Hydrodynamic voltammogram.

spiking solution (2.00 μg/ml). All solutions were prepared in polypropylene flasks and stored in darkness at about +4°C while not in use. Standard/calibration samples in the concentration range of 10 to 200 ng/ml were prepared for calibration, accuracy and precision, quality control and stability assessments by adding appropriate volume of spiking solutions to 1 ml of drug-free human plasma aliquots. The samples were analysed as described below (Section 2.4).

2.4. Extraction procedure

About 57 ng of I.S., appropriate volume of standard solutions for standard samples or actual samples, 0.25 ml of NaCl saturated solution and 5 ml of 1-chlorobutane–isooctane (55:45, v/v) were added to 1 ml of plasma and placed in a silanized extraction tube. These tubes were placed on an horizontal shaker for 5 min at a velocity of 250 rpm. Following centrifugation for 10 min at 3000 *g* at room temperature, the organic layer was transferred into another silanized conical tube. The solvent was evaporated to dryness at room temperature under a stream of nitrogen. The residue was dissolved in 600 μl of water–ethyl alcohol (50:50, v/v) by vortex mixing and the solution was transferred into a 1.5 ml silanized injection vial. The residue was reconstituted in water–ethyl alcohol instead of the mobile phase or water because an important chromatographic interference with A was obtained with the two vehicles. The sample was thoroughly deoxygenated during 15 min with argon. Then, an equilibrium was obtained between concentrations of α and β tautomers of DHA with a stable ratio of around 4(α):1(β) (see Section 3.1). A 500-μl volume of the sample was aspirated and a 20-μl aliquot was injected into the HPLC system.

2.5. Calibration and sample quantification

Calibration standard samples at 5 different concentrations in single in the range 10 to 200 ng/ml were prepared and analysed. Calibration curves ($y = mx + b$), represented by the plots of the peak-height ratios (y) of A or DHA to I.S. versus the concentrations (x) of the calibration samples, were generated using weighted ($1/x^2$) linear least-squares regression as the mathematical model [4]. Concentrations in clinical/preclinical, quality control and stability samples were calculated from the resulting height ratios of A or DHA (only height ratio of α-tautomer was considered) and the regression equation of the calibration curve.

3. Results and discussion

3.1. Specificity

Representative chromatograms of extracts of drug-free human plasma and of the same plasma sample spiked with A, DHA and I.S. are shown in Fig. 3.

DHA, I.S. and A were eluted from the column with retention times of approximately 4.4 and 5.4 min (α:β tautomers), 7.1 min and 9.4 min, respectively. The k' values were 2.64 for α-DHA, 3.44 for β-DHA, 4.85 for I.S. and 6.74 for A. Only the α-tautomer (the predominant peak) of DHA was considered for quantification, the constant ratio α versus β tautomer being around 4. Peak assignment was established from samples spiked individually with each compound and treated as described in the sample preparation procedure (Section 2.3) in order to check precisely that either A, DHA or I.S. did not interfere reciprocally after extraction and chromatography. As shown, the compounds of interest were clearly separated from co-extracted endogenous plasma components. Similar chromatographic profiles were observed for 6 different plasma from volunteers receiving no medication and for plasma samples of dog, rat and rabbit. Two chromatographic columns of the same characteristics but with a different batch number demonstrated comparable chromatographic profiles.

3.2. Extraction efficiency

The extraction efficiencies of A and DHA in human plasma were assessed by comparison of the peak heights from extracted samples ($n = 4$) to those from non-extracted standards spiked at the same A and DHA concentrations (41.4 ng/ml and 208 ng/ml for A, 40.4 and 202 ng/ml for DHA) and at a concentration of 50 ng/ml of I.S. ($n = 8$). The mean±S.D. efficiencies of extraction were 83±2%

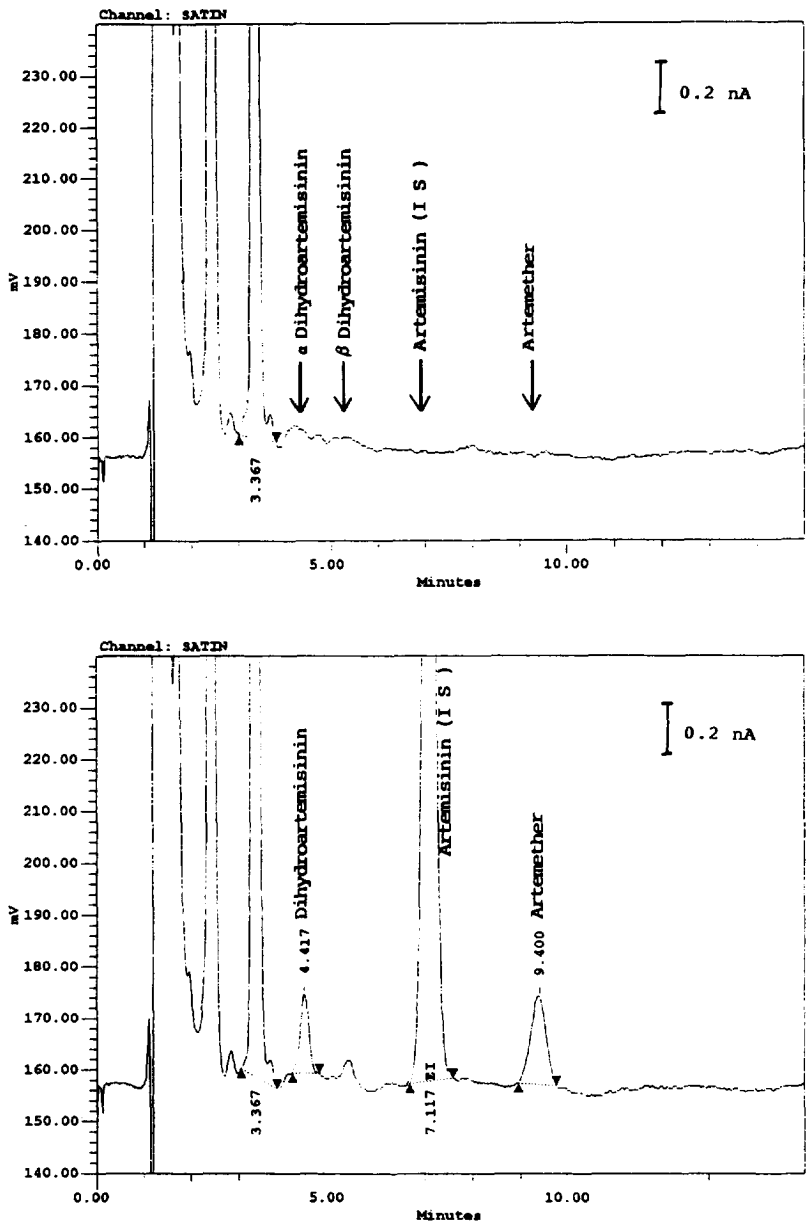

Fig. 3. Extract of drug-free human plasma (upper figure) and of spiked plasma standard sample (10 ng/ml for artemether, 10 ng/ml for dihydroartemisinin and 57.5 ng/ml for I.S.); arrows showing sites of artemether, dihydroartemisinin and artemisinin, the internal standard (I.S.).

(41.4 ng/ml), 86±2% (208 ng/ml) for A; 98±7% (40.4 ng/ml), 98±4% (202 ng/ml) for DHA (α-tautomer) and 90±3% for I.S..

The extraction efficiencies were also calculated in animal plasma at concentrations of 208 ng/ml for A, 202 ng/ml for DHA and 50 ng/ml for I.S. ($n=2$). The mean efficiencies of extraction were: 85% in rabbit, 88% in dog and 80% in rat for A; 100% in

rabbit, 96% in dog and 81% in rat for DHA (α-tautomer); 90% in rabbit, 87% in dog and 87% in rat for I.S..

Isooctane was selected instead of pentane to modify the polarity of 1-chlorobutane because the extraction efficiency was lower with pentane (less than 70% for A and DHA). Moreover, the chemical/biological chromatographic background was better with isooctane than with pentane.

Automated solid-phase extraction and injection using Aspec Gilson or other similar devices was not feasible due to a lack of an automated sample deoxygenation before injection. That is the reason why rapid liquid–liquid extraction was considered the method of choice, followed by an automated deoxygenation and injection using Gilson autosampler duly modified to automatically deoxygenate each sample before injection.

3.3. Calibration curves

Representative calibration curves of A and DHA gave relevant regression lines of $y=0.010232x-0.016078$ and $y=0.011181x-0.015218$, respectively. Correlation coefficients were 0.9991 for A and 0.999 for DHA. A new calibration curve is prepared every working day. Individual fit of the calibration standards to the curve was assessed from the relative error (R.E. in %): 100×[(back-calculated concentration from the regression line equation)−(nominal concentration)]/(nominal concentration). As shown in Table 1, the differences for back-calculated concentrations did not exceed 0.2% for A, 0.1% for DHA from theory and indicated a good fit of the weighted regression model over the range of the calibration curve.

3.4. Accuracy and precision

Accuracy and precision were studied from replicate sets of analyte samples of known concentrations at levels corresponding to the lowest, near the lowest, near the middle and the highest concentration values of the calibration range [5]. Accuracy was determined by calculating the mean recovery for the concentrations found in % of the nominal concentrations in standard samples. Precision was assessed from the coefficient of variation (C.V.) as % of the mean recoveries. The following validation criteria for accuracy and precision were used to assess the method suitability: mean recoveries must be within 85–115% except at the limit of quantitation (LOQ) where it should be within 80–120%; C.V. should not exceed 15%, except at the LOQ where it should not exceed 20% [6].

Human plasma samples were analysed on the

Table 1
Calibration curves

	C (ng/ml)	C' (ng/ml)	R.E. (%)
Artemether (A)	10.1	9.89	−2.1
	20.2	21.2	4.5
	40.4	39.7	−1.7
	101	102	1.0
	202	197	−2.5
			Mean |R.E.|=0.2
Dihydroartemisinin (DHA)			
	10.5	10.4	−1.0
	20.9	20.6	−1.4
	41.8	44.0	5.3
	105	105	0
	210	203	−3.3
			Mean |R.E.|=0.1

C=curve nominal concentration; C'=back-calculated concentration with the equation; R.E.(%)=relative error=$100\cdot(C'-C)/C$.

same day. Individual, mean recoveries and corresponding coefficients of variation are presented in Table 2 for A and in Table 3 for DHA. Mean recoveries for A over concentrations of 10.9 to 202 ng/ml ranged from 96% to 100% and the C.V. from 6 to 13%. Mean recoveries for DHA over concentrations of 11.2 to 206 ng/ml ranged from 92% to 100% and the C.V. from 4 to 16%. A and DHA are well extracted from dog, rat and rabbit plasma. For the respective plasma, accuracy for a concentration around 20 ng/ml of A and DHA was between 89 and 119%. Accuracy for around 200 ng/ml was between 93 and 115%. These data demonstrated that the method is also suitable for quantifying artemether and dihydroartemisinin in animal kinetic studies.

3.5. Limits of quantitation

The LOQ is defined as the lowest concentration on the standard curve that can be measured with acceptable accuracy, precision and variability. As indicated in Section 3.4, the mean recovery should be within 80–120% of the expected value with a C.V. not exceeding 20%. The lowest concentration values of 10.9 ng/ml for A and 11.2 ng/ml for DHA whose

Table 2
Artemether in plasma: intra-day accuracy and precision

Nominal concentration (ng/ml)	Recovery (%)[a]	Intra-day accuracy: mean recovery (%)	Precision: C.V.[b] (%)
10.9	81		
	105		
	113		
	113		
	95	96	13
	81		
	89		
	94		
	97		
40.2	96		
	102		
	106		
	95	100	6
	108		
	96		
	94		
101	105		
	82		
	93		
	102	97	8
	100		
	96		
	103		
202	105		
	96		
	107		
	96	100	7
	110		
	91		
	97		

[a] Recovery: concentration found expressed in % of the nominal concentration.
[b] Coefficient of variation on mean recovery.

Table 3
Dihydroartemisinin in plasma: intra-day accuracy and precision

Nominal concentration (ng/ml)	Recovery (%)[a]	Intra-day accuracy: mean recovery (%)	Precision: C.V.[b](%)
11.2	96		
	85		
	130		
	96		
	114	100	16
	113		
	79		
	92		
	92		
41.2	102		
	102		
	99		
	92	99	4
	99		
	97		
	102		
103	92		
	86		
	97		
	91	92	4
	93		
	92		
	96		
206	94		
	95		
	100		
	98	95	4
	97		
	90		
	93		

[a] Recovery: concentration found expressed in % of the nominal concentration.
[b] Coefficient of variation on mean recovery.

accuracies and precision (Tables 2 and 3) were within the proposed criteria are quoted as the LOQ.

3.6. Stability

The standard solutions were found to be stable for at least 1 month at about +4°C.

The plasma sample extracts were found to be stable during at least 5 h at room temperature in the autosampler. The concentrations following this storage period were 100 and 102% of the nominal values of 40.4 and 202 ng/ml, respectively, for DHA; 104 and 101% of the nominal values of 41.4 and 208 ng/ml, respectively, for A.

The plasma sample extracts were found to be stable during at least 22 h at about +4°C, the concentrations following this storage period were 102 and 106% of the nominal values of 40.4 and 202 ng/ml, respectively, for DHA; 100 and 94% of the nominal values of 41.4 and 208 ng/ml, respectively, for A.

Drug-free 1 ml plasma samples spiked with 20 or 200 ng A and DHA/ml were stored at −80°C. Three months later, the recovery for A was 100 and 102% for 20 and 200 ng/ml, respectively. It was 109 and 98% for DHA. Thus, A and DHA spiked to plasma are stable at least 3 months at −80°C. Clinical samples ($n=6$) were determined on day 1 and 4

months later after storage at −80°C. For concentrations of A between 32.1 and 111 ng/ml on day 1, the recovery ranged between 92 and 112% 4 months later. For DHA concentrations between 22.8 and 94.3 ng/ml, the recovery ranged between 98 and 120%. These data support the stability of A and DHA in clinical samples for at least 4 months when stored at −80°C.

3.7. Automated deoxygenation and injection

The use of a modified Gilson autosampler (Model 231XL) equipped with syringe dilutor allows to automatically deoxygenate each sample before automated injection. This device is readily available from Gilson and the main features are described in Section 2.2 Fig. 1. With this device, a laboratory can greatly increase the sample throughput.

Using the rapid liquid–liquid extraction and this injection device, forty determinations could be run within-a-day and two hundred within a working week, the work being done by one technician. Moreover, the deoxygenation is really accurate and no oxygen leaking problem was observed in our laboratory. It is not the case with manual deoxygenation and injection. In this case, two technicians are needed (one for extracting, the other for injecting) to allow two hundred determinations by a week without obtaining the good accuracy at 10 ng A or DHA/ml concentration. It should be noticed that using manual deoxygenation and injection, the injector loop should be rinsed manually between each injection by a prealably deoxygenated rinsing solvent.

3.8. Validation of the analytical method according to GLP (Good Laboratory Practice)

The use of an automated device for deoxygenation and injection permits to obtain reproducible results, define with accuracy and precision the LOQ, run calibration curves and validation samples according to GLP. In these conditions, the increased sample throughput offers a distinct advantage over previous methods, allowing a reliable run of toxicokinetic, animal and human pharmacokinetic samples.

4. Application

This method was applied to plasma samples from rats given 143 mg artemether/kg body weight as a powder administered orally by gavage once daily. The plasma concentrations after first dosing are depicted in Fig. 4. DHA profile is parallel to that of A. Both compounds are rapidly eliminated from plasma.

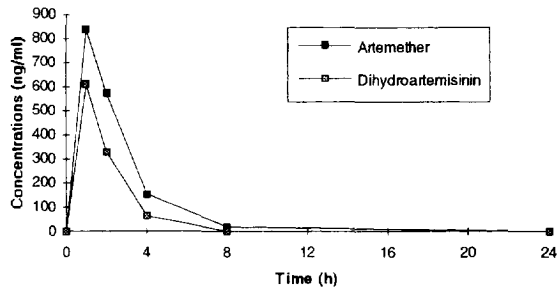

Fig. 4. Artemether and dihydroartemisinin plasma profiles in rats.

5. Conclusions

The present method has been developed and validated for quantification of artemether and dihydroartemisinin concentrations in human and animal plasma over the range of 10 to 200 ng/ml. The method appeared to be more sensitive than the ones described previously. In addition, it allowed an automated sample deoxygenation and injection system which greatly increased sample throughput.

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