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Determination of *N*-desethylamodiaquine by hydrophilic interaction liquid chromatography with tandem mass spectrometry: Application to *in vitro* drug metabolism studies

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Abstract

The antimalarial drug amodiaquine is extensively metabolized to *N*-desethylamodiaquine (DEAQ) by cytochrome P450 2C8 (CYP2C8). DEAQ formation is an enzyme specific reaction that is used to quantify *in vitro* CYP2C8 activity. A rapid and sensitive method for the determination of DEAQ in human liver microsomes was developed using hydrophilic interaction liquid chromatography/tandem mass spectrometry (HILIC–MS/MS). Microsomal incubation samples were processed by protein precipitation with acetonitrile. The analytes were separated on a BETASIL Silica-100 (50 mm × 2.1 mm, 5 μm) column by isocratic elution at a flow rate of 220 μl/min with a mobile phase consisting of 85% acetonitrile containing 5 mM ammonium acetate and 0.1% formic acid. Detection was by positive electrospray ionization on a TSQ Quantum Discovery triple quadrupole mass spectrometer operated in the selective reaction monitoring mode. The precursor–product ion pair was *m/z* 328 → 283 for DEAQ and *m/z* 331 → 283 for DEAQ-d_3. The lower limit of quantification was 10 nM for DEAQ and linearity was observed over the concentration range of 10–1500 nM. Intra- and inter-day accuracy and precision were within 3.4 and 7.0%, respectively. The method was successfully applied to CYP2C8 drug metabolism studies in pooled human liver microsomes.
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Keywords: Desethylamodiaquine; Cytochrome P450; CYP2C8; HILIC; Drug metabolism

1. Introduction

Amodiaquine (AQ), a 4-aminoquinolone antimalarial drug, is clinically effective against certain chloroquine resistant strains of *Plasmodium falciparum*. Although the global use of AQ has declined due to the high risk of agranulocytosis and hepatitis caused by the reactive quinine–imine metabolite [1], it is still being used as a first-line drug in the treatment of uncomplicated malaria, especially in African countries [2–5]. After oral administration, AQ undergoes rapid and extensive metabolism in the liver to form the pharmacologically active metabolite *N*-desethylamodiaquine (DEAQ), which is primarily responsible for the antimalarial effects [6]. In humans, desethylation of AQ is the major pathway of elimination with other minor metabolites being 2-hydroxyl DEAQ and *N*-bisdesethylAQ [1,7–9]. Studies in pooled human liver microsomes (HLMs) and recombinant human cytochrome P450 (CYP) enzymes show that AQ desethylation is almost exclusively catalyzed by CYP2C8 (Fig. 1) [10]. Therefore, AQ is used as an enzyme-selective probe substrate to quantify CYP2C8 enzyme activity *in vitro* [10].

Several analytical methods have been reported for quantification of DEAQ in various biological fluids (e.g., blood, plasma and urine) and subcellular fractions (e.g., HLM) [6,8–19]. Early reverse phase chromatographic methods suffered from poor retention of AQ and DEAQ, long run times and high mobile phase flow rates [11,12]. Analytical methods based on UV detection did not have the accuracy and sensitivity required for the quantification of the analytes due to endogenous interferences as a result of poor baseline resolution between AQ and its metabolites. Additionally, all of these methods involved tedious, multi-step extractions and large volumes of organic solvents [6,8,9,12–15]. Higher sensitivity was achieved by Trenholme et al. through conversion of AQ to a fluorescent product by refluxing it with borate buffer. Although this normal phase chromatographic method improved sensitivity and retention, it was found to be non-specific because the concentration of AQ was

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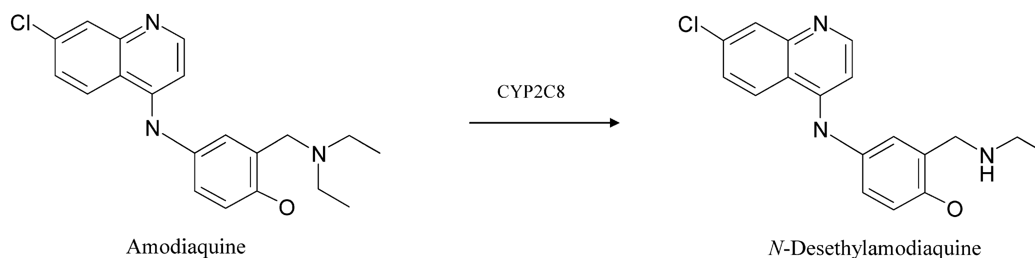

Fig. 1. Structures of amodiaquine and *N*-desethylamodiaquine.

confounded by its metabolites [17]. Mount et al. developed the most sensitive method for assaying DEAQ in human blood and urine (limit of quantitation (LOQ) 1 ng/ml) by employing electrochemical detection [16]. However, it involved lengthy extraction steps and consumed high amounts of organic solvents making it unsuitable for high-throughput analyses. Mass spectrometric methods reported include an ion pair HPLC–MS/MS method for the determination of AQ and DEAQ in blood [19] and methods for *in vitro* applications. Considering the prospects of the use of AQ as a CYP2C8-specific probe substrate in drug metabolism studies, high-throughput LC–MS/MS-based methods were developed for analysis of DEAQ [10,18,20–23]. All of these methods use simple processing methods and are sensitive enough for the determination of DEAQ concentration in *in vitro* assays as well as clinical studies. However, DEAQ was separated on reverse phase columns resulting in the use of highly aqueous mobile phase gradients to prolong retention of DEAQ, which is not ideal for mass spectrometric detection. Additionally, they involved separation of DEAQ by gradient elution with long run times. Thus, a simple, sensitive and robust mass spectrometric method that could be easily applied to drug metabolism studies is needed.

The purpose of the present work was to develop a LC–MS/MS method using hydrophilic interaction liquid chromatography (HILIC) that involved minimal sample preparation. HILIC chromatography yielded excellent separation of AQ from DEAQ by prolonging DEAQ retention time while using high proportions of organic solvent in the mobile phase. The method was used to determine enzyme kinetic parameters for DEAQ formation in HLM.

2. Experimental

2.1. Chemicals and reagents

Amodiaquine, β-nicotinamide adenine dinucleotide phosphate (β-NADP), glucose-6-phosphate, glucose-6-phosphate dehydrogenase, magnesium chloride and ammonium acetate were purchased from Sigma (St. Louis, MO, USA). The DEAQ metabolite standard (99.3% purity) and deuterated internal standard, DEAQ-d_3 (>98% purity), were obtained from BD Biosciences (San Jose, CA, USA). Potassium phosphate, sodium citrate and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Glucose 6-phosphate dehydrogenase solution (40 U/ml) was prepared by dissolving lyophilized enzyme in 5 mM sodium citrate and stored at −20 °C until use. All chemicals used in the study were of analytical grade. HPLC grade acetonitrile was obtained from EMD Chemicals (Gibbstown, NJ, USA). Deionized water was prepared by using a Barnstead Nanopure Diamond UV Ultrapure Water System (Dubuque, IA, USA). Pooled HLMs were purchased from BD Biosciences (San Jose, CA, USA).

2.2. LC–MS/MS conditions

The LC system was comprised of a Thermo Scientific (San Jose, CA, USA) Surveyor HPLC autosampler and Surveyor MS quaternary pump. Chromatographic separation was achieved on a BETASIL Silica-100 (50 mm × 2.1 mm, 5 μm; Thermo Fisher Corp., Bellefonte, PA, USA) analytical column. Isocratic elution was performed at a flow rate of 220 μl/min for 4.7 min using a mobile phase consisting of 5 mM ammonium acetate and 0.1% (v/v) formic acid in water and 5 mM ammonium acetate and 0.1% (v/v) formic acid in acetonitrile (15:85, v/v). The autosampler was maintained at 10 °C and the injection volume was 10 μl. The mobile phase flow was diverted from the mass spectrometer to waste for the first 1.5 min of run time to remove nonvolatile salts. After each injection, the needle was washed and flushed with 1 ml of solution containing 0.1% formic acid in acetonitrile, 2-propanol, and water (35:35:30, v/v/v).

The mass spectrometer was a TSQ Quantum Discovery triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source (Thermo Corp., San Jose, CA, USA). The mass spectrometer was calibrated with a solution of polytyrosine-1,3,6 per manufacturer's instructions. The operating conditions were optimized by infusing DEAQ in the mobile phase in order to maximize the detector signal. The ESI source was operated in the positive mode and was set orthogonal to the ion transfer capillary tube. The TSQ Quantum was operated in selective reaction monitoring (SRM) mode and the precursor–product ion pair was m/z 328 → 283 for DEAQ and m/z 331 → 283 for DEAQ-d_3. The acquisition parameters were spray voltage 4.0 kV, source CID −10 V, heated capillary temperature 325 °C and capillary offset 35 V. Nitrogen was used as the sheath and auxiliary gas set to 35 and 10 (arbitrary units), respectively. The argon collision gas pressure was set to 1.5 mTorr. The collision energy was 24 eV for both the analyte and internal standard. The peak full width at half maximum (FWHM) was set at 0.2 and 0.7 Th for Q1 and Q3, respectively. Scan width was fixed to 0.1 Th for both SRM channels and scan time was set to 250 ms. Data were processed using Thermo Xcalibur^{TM} software (version 1.4).

2.3. Preparation of DEAQ standards and quality control samples

Two sets of stock solutions were prepared in acetonitrile and water (50:50, v/v) at concentrations of 0.1 and 0.5 mM. One set of stock solutions was used to spike standards and the other set was used to spike quality control (QC) samples. Standards were prepared by spiking phosphate buffer (50 mM, pH 7.4) at seven concentrations ranging from 10 to 1500 nM. For validation, QC samples were prepared by spiking phosphate buffer (50 mM, pH 7.4) at three concentration levels (50, 500 and 1200 nM). The standards and QC samples were stored at −20 °C until analysis. The internal standard solution was prepared by dissolving DEAQ-d_3 in acetonitrile to produce a final concentration of 200 nM and stored at 4 °C.

2.4. Sample preparation

The internal standard solution in acetonitrile (200 nM, 1400 μl) was added to DEAQ standard or QC sample (250 μl). After shaking for 2 min on a vortex shaker, samples were centrifuged at 20,817 × g for 8 min. An aliquot of clear supernatant was transferred to a 96-well plate and 10 μl was injected on the column. All samples were protected from light exposure during processing in order to avoid photodecomposition [24].

2.5. Method validation procedure

Calibration curves were constructed by linear regression of the peak area ratio of DEAQ to DEAQ-d_3 (Y-axis) and the nominal standard DEAQ concentration (X-axis) with a weighting factor of $1/y^2$. Concentrations of QCs and incubation samples were calculated by using the regression equation of the calibration curve. Standards at all concentrations were analyzed in duplicate except the limit of quantitation, which was run in triplicate.

The analytical method was validated with respect to selectivity, carry over, linearity, precision, accuracy and autosampler stability [25]. For selectivity, samples of blank incubation matrix were analyzed to check for lack of interference in the quantification of DEAQ. Carry over was evaluated by placing vials of blank mobile phase at several locations in the analysis set. The maximum allowable deviation of the back calculated concentration was set at 15% for all standards and at 20% for the LOQ. The accuracy and precision of the assay was determined by the analysis of QC samples at DEAQ concentrations of 50.0, 500.0 and 1200 nM. Six of each QC sample was analyzed on the same day to determine intra-day precision and accuracy, and on three different occasions to assess inter-day precision and accuracy. The stability of the DEAQ in the autosampler was tested after the processed samples were left in the autosampler for at least 36 h by reanalyzing the standards and QC samples. Stability was defined as less than 10% deviation in concentration from that determined on the day samples were processed. Ion suppression was investigated by a post-column infusion experiment as described by King et al. [26]. DEAQ was infused post-column while blank, processed incubation samples or mobile phase were injected. A decrease in signal from baseline would indicate ion suppression while an increase would indicate signal enhancement.

2.6. Incubation conditions

Preliminary experiments were conducted to optimize the microsomal protein concentration (0.01–0.2 mg/ml) and incubation time (5–20 min) in order to ensure the linearity of DEAQ formation. Amodiaquine and HLM (0.1 mg/ml) were mixed with phosphate buffer (50 mM, pH 7.4) and warmed at 37 °C for 5 min. Incubations were commenced by the addition of the NADPH regenerating system, which consisted of $MgCl_2$ (assay concentration, 3.3 mM), NADP+ (1.25 mM), glucose 6-phosphate (3.3 mM) and glucose 6-phosphate dehydrogenase (0.32 U/ml) in 5 mM sodium citrate solution. The final incubation volume was 250 μl. After incubating for 10 min at 37 °C, the reaction was terminated by the addition of 1400 μl of ice-cold acetonitrile containing DEAQ-d_3 (0.28 nmol). Samples were processed as described above.

Enzyme kinetic parameters were obtained by performing incubations at nine different concentrations of AQ ranging from 0.5 to 50 μM. AQ was dissolved in acetonitrile and water (50:50, v/v; final acetonitrile concentration was 0.4%, v/v). Microsomes were stored at −80 °C and thawed immediately before use. Polypropylene microcentrifuge tubes were used to store AQ stocks as well as to conduct the microsomal incubations. All incubations were performed in duplicate and were protected from light to avoid photodecomposition of AQ and the metabolite.

2.7. Data analysis

Enzyme kinetic parameters were obtained by nonlinear regression using GraphPad Prism version 4.03 (San Diego, CA, USA). Data were fit to the Michaelis–Menten equation:

$$V = \frac{V_{max}[S]}{K_m + [S]}$$

in which V is the initial velocity, V_{max} is the maximal velocity, [S] is the substrate concentration and K_m is the substrate concentration at half-maximal velocity.

3. Results and discussion

3.1. Method development

In order to improve the retention of DEAQ and to avoid the use of a highly aqueous mobile phase, we selected a BETASIL Silica column to separate the analytes based on the principles of HILIC. In HILIC, analytes are eluted by passing a hydrophobic or mostly organic mobile phase across a neutral hydrophilic stationary phase [27–29]. The analytes elute in order of increasing hydrophilicity resulting in better separation of highly polar compounds. Indeed, in contrast to reverse phase chromatography in which separation of DEAQ from AQ can be difficult, HILIC yielded excellent separation of DEAQ (retention time, 2.9 min) from AQ (retention time, 1.2 min) while still allowing

use of 85% acetonitrile in the mobile phase. The HILIC-based method also provided an approximately fivefold increase in sensitivity compared with a reversed-phase method developed in our laboratory. The increase in sensitivity is likely due to the higher percentage organic in the mobile phase (85% vs. 20%) and is similar in magnitude to that reported for other compounds [29].

The mobile phase of ammonium acetate buffer with 0.1% (v/v) formic acid was used as the addition of formic acid enhanced ionization and improved the peak shape of DEAQ. Electrospray ionization gave high signal intensity for DEAQ and full scan mass spectra of DEAQ were obtained in the positive and negative mode. The most abundant parent ion of DEAQ (*m/z* 328) was obtained in the positive mode, which was selected for SRM scanning. Further, the fragmentation pattern of the precursor ion was obtained and a highly specific ion pair (*m/z* 328 → 283) was selected based on the intensities of the most abundant product ions. Thus, for quantification purposes, the TSQ quantum was operated in SRM mode and the precursor–product ion pair of *m/z* 328 → 283 and 331 → 283 was followed for DEAQ and DEAQ-d_3, respectively. In order to minimize the sample preparation time, a one-step protein precipitation method was utilized by the addition of a solution of internal standard in acetonitrile followed by a short mixing and centrifugation step. Considering the simplicity of sample processing, the present method could potentially be applied to a high-throughput drug metabolism assay.

3.2. Method validation

Validation of the assay method was conducted according to the FDA guidelines with respect to selectivity, carry over,

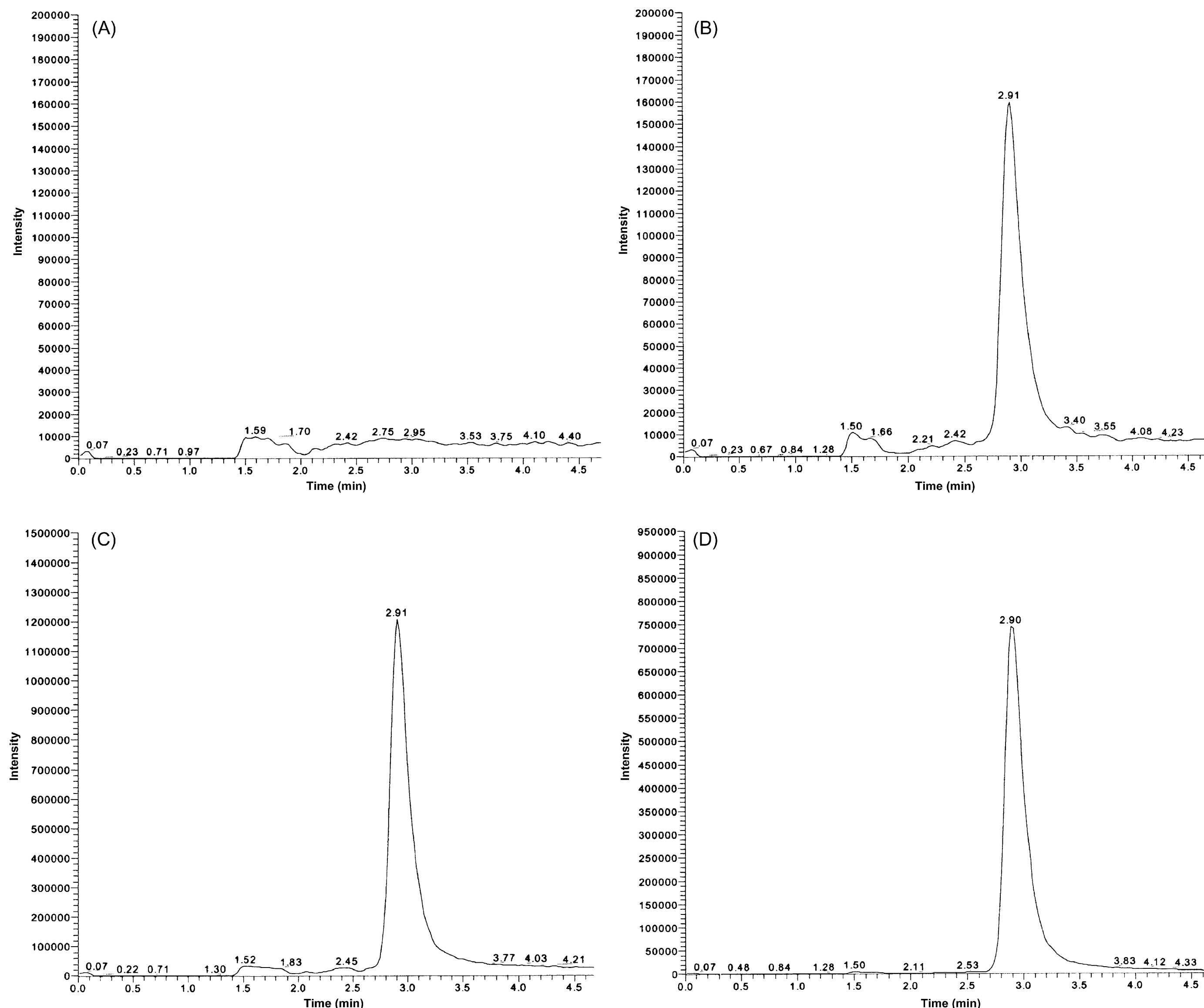

Fig. 2. The extracted HILIC–MS/MS chromatograms of DEAQ in (A) blank phosphate buffer (50 mM, pH 7.4), (B) spiked lowest standard (10 nM) and (C) study sample after 10-min incubation of AQ (0.5 μM) in HLM (DEAQ concentration = 110.6 nM); (D) representative HILIC–MS/MS chromatogram of DEAQ-d_3 (280 pmol) as the internal standard.

linearity, precision, accuracy and stability [25]. For validation purposes, QC samples at low, medium and high concentrations were prepared independently and six of each QC sample was analyzed on three occasions.

The calibration curve was linear over the concentration range of 10–1500 nM with a mean correlation coefficient (R^2) of 0.9969 ± 0.0012. To determine the selectivity of the method, blank microsomal incubation samples were used to investigate the potential interferences due to endogenous compounds in the matrix. There was no significant interference in the baseline at the retention times of DEAQ and DEAQ-d_3. No carry over was observed in any of the blank samples. Representative chromatograms of (A) blank buffer, (B) DEAQ standard at the LOQ (10 nM), (C) a microsomal incubation sample, and (D) the internal standard DEAQ-d_3 (280 pmol) are depicted in Fig. 2.

Precision was represented as the relative standard deviation (%R.S.D.) whereas accuracy was calculated as the percent deviation (%bias) from the respective nominal concentration. The maximum acceptable limit for precision and accuracy was set at 15%. The intra-day and inter-day precision and accuracy were within 7.0 and 3.4%, respectively, for all standards and QC samples (Table 1). Thus, the present method was found to be highly reproducible and demonstrated a high degree of accuracy.

The autosampler was maintained at 10 °C during analyses and the stability of analytes at 10 °C was determined by reanalyzing the same standards and QC samples after 36 h. Both DEAQ and DEAQ-d_3 were found to be stable at 10 °C for at least 36 h. Ion suppression due to matrix was not observed as there was no change in signal from baseline in the region in which DEAQ eluted.

3.3. *Application to study metabolism of AQ in human liver microsomes*

AQ was incubated with HLM at nine different concentrations (0.5–50 μM). The linearity of AQ metabolism with respect to the microsomal protein content was studied at five protein concentrations (0.01–0.2 mg/ml). In order to avoid non-specific protein binding, the lowest protein concentration that produced quantifiable metabolite (0.1 mg/ml) was selected. Formation of DEAQ was linear up to 20 min. Considering the photosensitivity of AQ, samples were protected from light during incubations. Following the incubation of AQ in HLM, DEAQ was detected by using the validated method. The rate of formation of DEAQ was measured as the index of CYP2C8 enzyme activity [10,18,20–23]. The formation of DEAQ exhibited typical Michaelis–Menten kinetics (Fig. 3) with the maximal rate of formation of DEAQ (V_{max}) of 1914 ± 94 pmol/(min mg protein) and the concentration of AQ at half-maximal velocity (K_m) was 4.94 ± 0.76. Our results are in agreement with previous reports of DEAQ formation as a CYP2C8-specific probe reaction (Table 2) [10,18,30]. Thus, the present method was successfully applied to *in vitro* drug metabolism studies.

Table 1
Intra-day (n = 6) and inter-day (n = 18) precision (%R.S.D.) and accuracy (%bias) for analysis of DEAQ in 50 mM phosphate buffer, pH 7.4

Concentration (nM)		Precision R.S.D. (%)	Accuracy (%)
Nominal	Measured (mean ± S.D.)		
Intra-day			
50.00	51.70 ± 0.70	1.4	3.4
500.0	492.8 ± 9.1	1.8	−1.4
1200	1181 ± 28	2.4	−1.5
Inter-day			
50.00	51.20 ± 3.60	7.0	2.5
500.0	487.9 ± 13.0	2.6	−2.4
1200	1209 ± 83	6.9	0.8

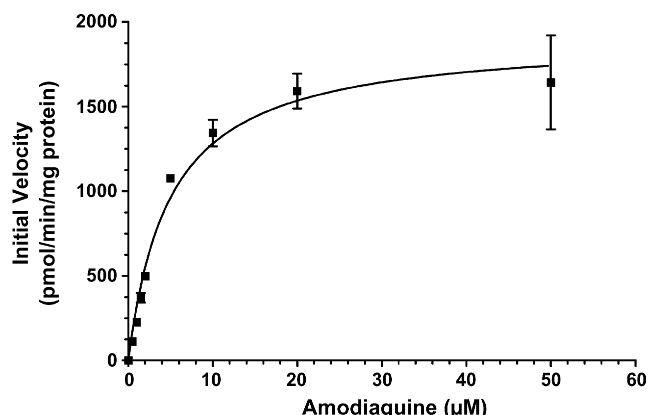

Fig. 3. Plot of initial velocity vs. amodiaquine concentration for the formation of *N*-desethylamodiaquine in HLM (n = 2).

Table 2
Enzyme kinetic parameters of DEAQ formation in HLM

	K_m ± S.E. (μM)	V_{max} (pmol/(min mg protein))
In house	4.94 ± 0.76	1914 ± 94
Walsky and Obach [18]	1.89 ± 0.06	1480 ± 20
Li et al. [30]	3.4	1696
Li et al. [10]	2.4	1462

4. Conclusions

The application of HILIC chromatography for separation and analysis of DEAQ resulted in a simple and robust LC–MS/MS-based method. The method was validated with respect to selectivity, carry over, linearity, precision, accuracy and autosampler stability. Enzyme kinetic parameters obtained by incubating AQ with HLM in the presence of NADPH regenerating system were in accordance with the available literature. Thus, the present method is applicable for CYP2C8 drug metabolism studies.

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