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Short communication

# Simultaneous determination of primaquine and carboxyprimaquine in plasma using solid phase extraction and LC–MS assay

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ABSTRACT

Sensitive bioanalytical methods are required for pharmacokinetic studies in children, due to the small volume and modest number of samples that can be obtained. We sought to develop a LC–MS assay for primaquine and its active metabolite, carboxyprimaquine, following simultaneous, solid phase extraction of both analytes from human plasma. The analysis was conducted on a single-quad LC–MS system (Shimadzu Model 2020) in ESI+ mode, with quantitation by selected ion monitoring. Primaquine, carboxyprimaquine and 8-aminoquinoline (internal standard) were separated using a mobile phase of 80:20 methanol:water with 0.1% (v/v) formic acid and a Luna $C_{18}$ HPLC column, at ambient temperature. Solid phase extraction of the analytes from plasma (0.5 mL) was achieved with Oasis® HLB cartridges. The retention times for primaquine, 8-aminoquinoline and carboxyprimaquine were 3.3, 5.7 and 8.5 min, respectively. The calibration curve range (2–1500 μg/L) was appropriate for the limits of quantification and detection for primaquine (2 μg/L and 1 μg/L, respectively) and carboxyprimaquine (2.5 μg/L and 1 μg/L) and the anticipated plasma concentrations of the analytes. Intra- and inter-day precision for both primaquine and carboxyprimaquine was <10% across the concentration range 5–1000 μg/L. Accuracy for both analytes was <15% (5–500 μg/L). This validated LC–MS method with solid phase extraction facilitates the simultaneous analysis of primaquine and carboxyprimaquine from small volumes of human plasma, with run time <10 min, recovery >85% and sensitivity of 1–2 μg/L.



## 1. Introduction

Primaquine is an essential therapeutic agent in the treatment of vivax malaria, specifically to eradicate dormant parasites (hypnozoites) and prevent relapses [1–3]. It is also used to clear the sexual forms of *Plasmodia* (gametocytes) and can thus reduce transmission of malaria [4]. Recommended conventional hypnozoiticidal doses of primaquine are 0.25–0.5 mg/kg/day for 14 days, although successful therapy with doses up to 1 mg/kg/day for 7 days has recently been reported [1,2,5]. The principal metabolite of primaquine, carboxyprimaquine, has limited efficacy but also limited toxicity and other minor metabolites with antimalarial activity appear to be labile and are poorly characterised [6,7].

Despite the well-established therapeutic role of primaquine, a limited range of pharmacokinetic studies in healthy adults [8–12] and patients with vivax malaria [13,14] have been published and there is a paucity of studies in children [3]. Pharmacokinetic studies in children have ethical and practical challenges that preclude conventional study design, including the volume and number of samples that can be obtained [15,16]. Population modelling can be applied to the sparse data obtained from a modest number of samples for each subject, to ensure robust pharmacokinetic data, however sensitive analytical techniques are required for measurement of the drug concentration in small volumes [15,16].

Several pharmacokinetic studies of primaquine and its principal metabolite, carboxyprimaquine, have utilised a high-performance liquid chromatography (HPLC) method with liquid–liquid extraction and UV detection [8–12,17]. In the most recent studies, the analytes were extracted separately, requiring >750 μL of plasma, and the limits of quantitation (LOQs) for primaquine and carboxyprimaquine were 5 μg/L and 25 μg/L, respectively [9,10].

Two other studies have been conducted using HPLC with electrochemical detection of both analytes simultaneously, following protein precipitation of 500 μL plasma with acetonitrile [13,18]. Recovery and LOQ of primaquine and carboxyprimaquine were

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75% and 90%, and 5 μg/L and 20 μg/L, respectively, with a run time >30 min [18].

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) or liquid chromatography–mass spectrometry with time-of-flight detection (LC–MS/TOF) analysis have been reported for the determination of primaquine in spiked simian, murine and human plasma [19,20] and for in vitro studies of primaquine adducts [21]. In the simian study, liquid–liquid extraction of 100 μL plasma achieved a 60% recovery of primaquine (carboxyprimaquine not analysed) and the LOQ was 4 μg/L [19]. By comparison, protein precipitation with methanol was applied to 100 μL murine or human plasma, with approximately 90% recovery and LOQs for primaquine and carboxyprimaquine of 5 μg/L and 1 μg/L, respectively [20]. Enantiomeric separation of primaquine and carboxyprimaquine by LC–MS/TOF was applied to the pharmacokinetic study in mice [20], but the clinical importance of enantioselective analysis has not been elucidated.

These studies indicate the need for an efficient method of sample preparation and a sensitive assay for both primaquine and carboxyprimaquine that minimises the required volume of plasma. We report the development of a sensitive, validated LC–MS assay for primaquine and carboxyprimaquine following simultaneous, solid phase extraction of both analytes from human plasma. The clinical application of the method was demonstrated in healthy children participating in a pharmacokinetic study of primaquine as a prelude to rationalising paediatric dose regimens in vivax malaria.

## 2. Materials and methods

### 2.1. Materials and reagents

Primaquine diphosphate ($C_{15}H_{21}N_3O{\cdot}2H_3PO_4$; MW = 455; primaquine MW = 259) and 8-aminoquinoline ($C_9H_8N_2$; MW = 144) were from Sigma–Aldrich Pty Ltd., Castle Hill, NSW, Australia and carboxyprimaquine ($C_{15}H_{18}N_2O_3$; MW = 274) was from Epichem Pty Ltd., Murdoch, WA, Australia (Fig. 1). HPLC grade methanol was from Merck Pty Ltd., Kilsyth, Australia, and LC–MS grade formic acid was from Sigma–Aldrich Ltd., Gillingham, Dorset, UK. All other general laboratory chemicals were of analytical grade (Sigma–Aldrich Chemicals, St. Louis, MO, USA; Merck Chemicals, Darmstadt, Germany).

### 2.2. Instrumentation and chromatographic conditions

The single-quad LC–MS system (Model 2020, Shimadzu, Kyoto, Japan) comprised a binary pump (20AD), vacuum degasser, thermostatted autosampler (SIL 20ACHT), thermostatted column compartment (CTO 20A), photodiode detector (SPD M 20A) and mass analyser (MS 2020) with both electrospray ionisation (ESI) and atmospheric-pressure chemical ionisation (APCI) systems. Optimised mass spectra were acquired with an interface voltage of 4.5 kV, a detector voltage of 1.2 kV, a heat block temperature of 300 °C and a desolvation gas temperature of 200 °C. Nitrogen was used as the nebuliser gas at a flow rate of 1.5 L/min and dry gas flow of 10 L/min.

Primaquine, carboxyprimaquine and 8-aminoquinoline standard solutions were first scanned from $m/z$ 100 to 500 in ESI positive and APCI positive mode, as well as combined ESI+ and APCI+ (dual ion source; DUIS) mode to identify the abundance of ions corresponding to respective analytes. The base peak intensity of all three modes were compared and showed that ESI mode gave the highest signal intensity. Therefore, quantitation was performed by selected ion monitoring (SIM), using ESI mode for primaquine (protonated parent molecule $[M+H]^+$; $m/z$ = 260), carboxyprimaquine ($[M+H]^+$; $m/z$ = 275) and 8-aminoquinoline ($[M+H]^+$; $m/z$ = 145; Fig. 1).

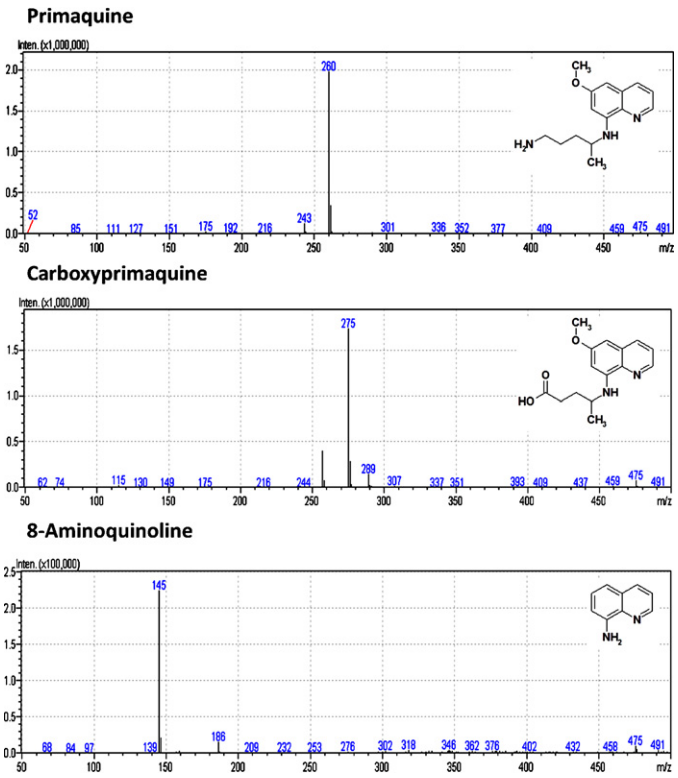


**Fig. 1.** Mass spectra showing $m/z$ for selected ion monitoring of primaquine ($m/z$ = 260), carboxyprimaquine ($m/z$ = 275) and 8-aminoquinoline ($m/z$ = 145). The chemical structures of primaquine, carboxyprimaquine and 8-aminoquinoline (internal standard) are shown as insets.

Analytes were separated in isocratic mode using methanol:water (80:20, v/v) with 0.1% (v/v) formic acid at a flow rate of 0.25 mL/min. Chromatographic separation was performed on a Luna $C_{18}$ 3 μm HPLC column (100 mm × 4.6 mm i.d.) in series with an octadecyl $C_{18}$ guard column (4 mm × 3 mm i.d.; Phenomenex, Lane Cove, Australia) at ambient temperature of approximately 22 °C.

### 2.3. Sample preparation

Plasma (0.5 mL) was spiked with internal standard (8-aminoquinoline; 100 ng) and concentrated $H_3PO_4$ (20 μL; 85%, w/w) and briefly vortex mixed. The solid phase extraction cartridges (Oasis® HLB 30 μm, 1 $cm^3$/10 mg; Waters Corporation, Milford, MA, USA) were pre-conditioned with 1 mL methanol (100%) followed by 1 mL water. The plasma sample was loaded and washed with 3 mL of water, followed by 1 mL of methanol:water (5:95, v/v). The column was dried under low vacuum for 30 min and the retained drugs were eluted with 150 μL of methanol:acetonitrile (50:50, v/v). The eluate was transferred to a vial with glass insert and 10 μL aliquots were injected into the LC–MS system.

### 2.4. Method validation

Stock solutions of primaquine, carboxyprimaquine and 8-aminoquinoline (internal standard) were prepared separately (1 mg/mL base in methanol) and stored protected from light at −80 °C. Working standard solutions were prepared from the primary stock at 1, 10 and 100 μg/mL, as required. Two 5-point calibration curves were constructed by spiking primaquine and carboxyprimaquine into blank plasma: 2–100 μg/L for lower concentrations and 100–1500 μg/L for higher concentrations.

Internal standard (8-aminoquinoline; 100 ng) was added to all samples. Chromatographic data (peak area ratio of primaquine:8-aminoquinoline and carboxyprimaquine:8-aminoquinoline) were processed using LAB Solution (Version 5, Shimadzu, Japan). Quality control (QC) samples were prepared in blank plasma at concentrations of 5, 25, 50, 200 and 500 μg/L, and stored at −80 °C prior to use.

Responses from the analysis of four primaquine and carboxyprimaquine concentrations (5, 50, 200 and 1000 μg/L) spiked into five separate plasma samples were used to determine matrix effects (ion suppression/enhancement), absolute recovery and process efficiency [22,23]. Three sets of matrix were prepared: Set 1 comprised blank plasma spiked first and then extracted; Set 2 comprised blank plasma extracted first and then spiked post-extraction; and Set 3 comprised pure solutions of analyte in methanol using the same concentrations of primaquine, carboxyprimaquine and 8-aminoquinoline. The matrix effect (%) was determined from: [Set 2 response × 100]/[Set 3 response]. The process efficiency (%) was determined from: [Set 1 response × 100]/[Set 3 response]. The absolute recovery (%) was determined from: [Set 1 response × 100]/[Set 2 response].

Precision of the method was determined from the intra-day and inter-day relative standard deviation (RSD = SD/mean; %) at concentrations of 5, 50, 200, 500 and 1000 μg/L for both primaquine and carboxyprimaquine. Accuracy was determined from the QC samples at concentrations of 5, 25, 50, 200 and 500 μg/L that were analysed with each batch of samples.

The limit of quantification (LOQ) was based on a 10:1 signal to noise ratio and validated at a concentration measured with a RSD ≤ 20%. The limit of detection (LOD) was defined as the concentration with a signal to noise ratio ≥3:1.

### 2.5. Application of method

Plasma samples from two children recruited for a study of the pharmacokinetics of primaquine were analysed (Chief Investigators: Dr. I. Betuela and Dr. I. Mueller; PNG Institute of Medical Research). The study was approved by the Medical Research Advisory Committee of Papua New Guinea and the Human Research Ethics Committee at the University of Western Australia. Patients were recruited in Madang Province, Papua New Guinea in 2010.

Venous blood samples (2 mL) were collected at baseline (zero time) and 1, 2, 3, 4, 6, 8, 12, 18, 24, 36, 48, 72, 120 and 168 h after a single dose of primaquine diphosphate. Each sample was centrifuged for 5 min at 3000 × *g* and the plasma was separated from the red cell pellet and stored in a foil covered tube at −80 °C until HPLC analysis (within 6 months of sample collection). The principal pharmacokinetic parameters (area under the plasma concentration–time curve to infinity ($AUC_{0-\infty}$), half-life ($t_{½}$), apparent clearance (CL/*F* = dose/AUC, where *F* is bioavailability) and apparent volume of distribution at steady-state (Vss/*F* = CL × MRT, where MRT is mean residence time)) for primaquine and carboxyprimaquine were determined from the plasma concentration–time data using non-compartmental analysis (Kinetica Version 5.0; Thermo Fisher Scientific, Waltham, MA, USA).

## 3. Results and discussion

### 3.1. Optimisation of extraction and LC–MS conditions

The principal advantages of our method are the simultaneous extraction and analysis of primaquine and carboxyprimaquine from small volumes of human plasma, the high recovery of both analytes and the short run time using an isocratic system. These features ensure that the LC–MS assay is sensitive, robust and efficient, and could be adapted to more or less sophisticated apparatus. The target run time of <10 min was achieved, with approximate retention times for primaquine, 8-aminoquinoline and carboxyprimaquine of 3.3 min, 5.7 min and 8.5 min, and $k'$ values of 0.27, 1.2 and 2.3, respectively ($t_0$ = 2.6 min; Fig. 2).

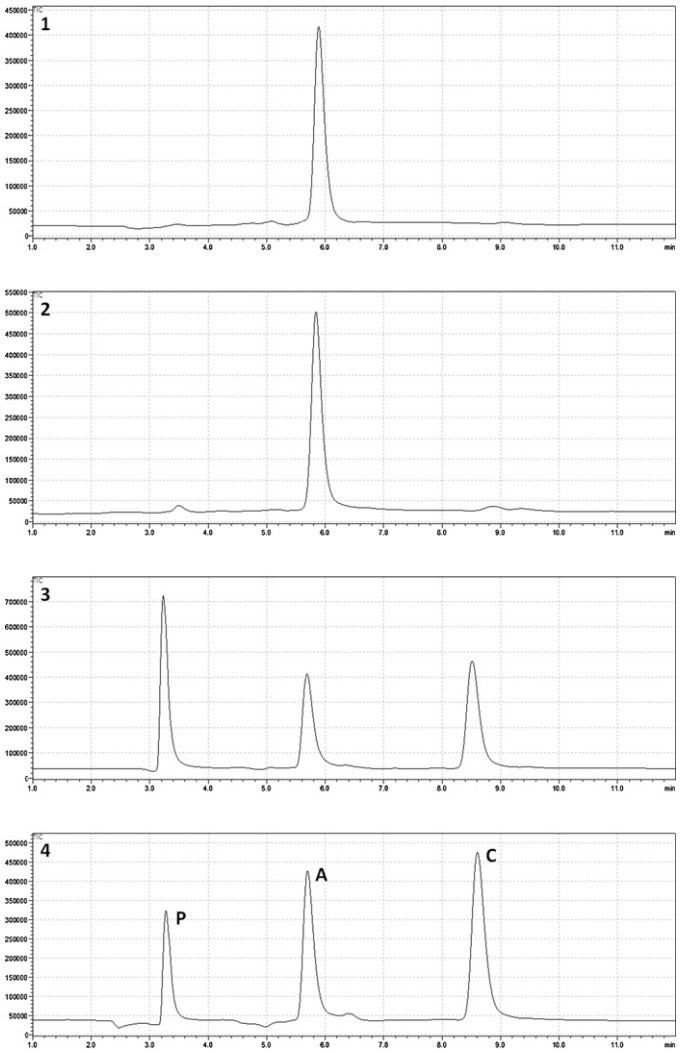


**Fig. 2.** Chromatograms showing primaquine (P; $t_R$ = 3.3 min), 8-aminoquinoline (A; $t_R$ = 5.7 min) and carboxyprimaquine (C; $t_R$ = 8.5 min). Panel 1 is blank plasma with internal standard (8-aminoquinoline); panel 2 is spiked plasma at LOQ for primaquine (2 μg/L each of primaquine and carboxyprimaquine); panel 3 is spiked plasma at 200 μg/L each of primaquine and carboxyprimaquine; panel 4 is a typical sample (Child YO3, 1 h after primaquine dose; 91 μg/L of primaquine and 265 μg/L carboxyprimaquine).

Important considerations in developing this LC–MS method were processing time, matrix volume and sensitivity, because the number and volume of plasma samples that can be obtained in paediatric pharmacokinetic studies are limited. A range of protein precipitation, liquid–liquid and solid-phase extraction techniques were trialled in order to achieve the goal of simultaneous extraction of both analytes and the internal standard at high recovery from plasma. Potential advantages of liquid–liquid and solid-phase extractions were concentration of analytes and a cleaner matrix. Recovery from protein precipitation trials using methanol and acetonitrile was <60% and dilution of the samples (2–4 fold; as in previous studies [18,20]) was counterproductive in regard to sensitivity of the assay. Liquid–liquid extraction strategies adapted from previous reports [9,11] were satisfactory for primaquine

**Table 1**
Matrix effect, process efficiency and absolute recovery for primaquine, carboxyprimaquine and 8-aminoquinoline. Data are mean ± SD ($n$ = 5 samples per concentration).

| Analyte | Parameter | | |
|---|---|---|---|
| | Matrix effect (%) | Efficiency (%) | Recovery (%) |
| **Primaquine** | | | |
| 5 μg/L | 97 ± 14 | 98 ± 18 | 102 ± 11 |
| 50 μg/L | 117 ± 8 | 104 ± 15 | 89 ± 9 |
| 200 μg/L | 104 ± 8 | 107 ± 10 | 105 ± 12 |
| 1000 μg/L | 118 ± 6 | 106 ± 13 | 90 ± 11 |
| **Carboxyprimaquine** | | | |
| 5 μg/L | 95 ± 16 | 81 ± 6 | 86 ± 10 |
| 50 μg/L | 98 ± 14 | 93 ± 11 | 96 ± 14 |
| 200 μg/L | 96 ± 13 | 103 ± 12 | 108 ± 12 |
| 1000 μg/L | 98 ± 9 | 88 ± 7 | 90 ± 6 |
| **8-Aminoquinoline** | | | |
| 200 μg/L | 81 ± 9 | 86 ± 7 | 91 ± 15 |

(>75% recovery) but lower than desired for carboxyprimaquine (<65% recovery). Reversed-phase ($C_{18}$) solid phase extraction using a range of elution solvents, including methanol, acetonitrile and hexane:ethyl acetate (80:20, v/v), produced sub-optimal recovery of both primaquine (<70%) and carboxyprimaquine (<60%). Ion-exchange (weak cation-exchange) solid phase extraction, with conditioning and elution (e.g. methanol, acetonitrile) according to the manufacturer's specifications achieved 75% recovery for carboxyprimaquine but the recovery of primaquine was low (<60%) and variable. A satisfactory procedure with simultaneous elution of both analytes and internal standard at high recovery (>85% for primaquine, carboxyprimaquine and 8-aminoquinoline; Table 1) could only be achieved with the hydrophilic-lipophilic-balanced reversed-phase sorbent cartridges (Oasis® HLB). Evaporation of eluate from the extraction procedure and dissolution of the residue in mobile phase caused an increase in baseline noise and inferior LOD and LOQ, hence an aliquot of the extraction eluate was injected into the LC–MS.

### 3.2. Method validation

#### 3.2.1. Matrix effect and recovery

The matrix effect, process efficiency and recovery of the extraction process data are shown in Table 1. In general, the results are in the range of 90–110% and compare favourably with other bioanalytical methods [22,24]. Specificity of the assay was confirmed by processing blank plasma and the zero time plasma samples from the children in the pharmacokinetic study, all of which demonstrated no apparent interference with analyte specificity (data not shown). Storage stability of primaquine and carboxyprimaquine in plasma has been determined previously [20] and was not addressed in the present study.

#### 3.2.2. Linearity and calibration range

All standard curves were linear ($r^2 \geq 0.999$). The calibration curve range (2–1500 μg/L) and QC sample concentrations (5–500 μg/L) were based on previous reports of primaquine and carboxyprimaquine pharmacokinetics in adults [9,10,12,14,25]. These ranges were suitable for primaquine, however it was apparent that carboxyprimaquine concentrations may exceed 1500 μg/L in some samples for patients receiving higher doses of primaquine. Hence, for the present study, samples above the standard curve were re-analysed following appropriate dilution. In future studies, we recommend that the upper limit of the calibration range and the QC sample concentrations for carboxyprimaquine should be at least 2000 μg/L and 1500 μg/L, respectively.

**Table 2**
Accuracy and precision for primaquine and carboxyprimaquine. Data are mean ± SD.

| Accuracy (%; $n$ = 25) | 5 μg/L | 25 μg/L | 50 μg/L | 200 μg/L | 500 μg/L |
|---|---|---|---|---|---|
| Primaquine | 114 ± 10 | 109 ± 10 | 106 ± 8 | 104 ± 11 | 103 ± 10 |
| Carboxyprimaquine | 108 ± 9 | 113 ± 10 | 106 ± 10 | 105 ± 7 | 98 ± 6 |
| Precision (RSD; %) | 5 μg/L | 50 μg/L | 200 μg/L | 500 μg/L | 1000 μg/L |
| **Primaquine** | | | | | |
| Intra-day ($n$ = 5) | 9.7 | 6.1 | 4.8 | 9.2 | 5.8 |
| Inter-day ($n$ = 25) | 9.6 | 7.5 | 9.2 | 9.7 | 8.2 |
| **Carboxyprimaquine** | | | | | |
| Intra-day ($n$ = 5) | 9.6 | 6.3 | 6.1 | 6.3 | 4.8 |
| Inter-day ($n$ = 25) | 9.1 | 9.2 | 7.0 | 7.5 | 5.6 |

#### 3.2.3. Accuracy and precision

The precision (RSD < 10%) and accuracy (<15%) across the concentration range for both primaquine and carboxyprimaquine are shown in Table 2. These results were within accepted limits of 20% and 15% of nominal values, respectively [23,26].

#### 3.2.4. Limit of quantification and limit of detection

The LOQ for primaquine and carboxyprimaquine were 2 μg/L and 2.5 μg/L, respectively, while the LOD for both primaquine and carboxyprimaquine was 1 μg/L.

### 3.3. Application of method

Child YO9 (female, 7.6 years old, 18.5 kg) was given 0.81 mg/kg primaquine, as primaquine diphosphate (1× 15 mg primaquine tablet comprising 26.4 mg primaquine diphosphate). Child YO3 (male, 7.4 years old, 16 kg) was given 0.47 mg/kg primaquine (½× 15 mg primaquine tablet). Plasma concentration–time profiles of primaquine and carboxyprimaquine are shown in Fig. 3. All plasma concentrations were above the LOQ and within the calibration range for the assay, with the exception of three carboxyprimaquine measurements (these concentrations ranged from 1590 to 1760 μg/L). Based on the volume of plasma available for analysis in the present study (0.5 mL), the volume of extraction eluate injected into the LC–MS (10 μL of 150 μL) and the LOQ for the assay, analysis of smaller plasma volumes for pharmacokinetic studies is feasible.

The $t_{½}$, CL/$F$ and Vss/$F$ for primaquine were 5.1 h, 0.36 L/h/kg and 3.0 L/kg for YO9 and 5.9 h, 0.45 L/h/kg and 3.5 L/kg for YO3, respectively. The $t_{½}$ for carboxyprimaquine was 18 h for YO9 and 17 h for YO3, respectively. These results are comparable to reported

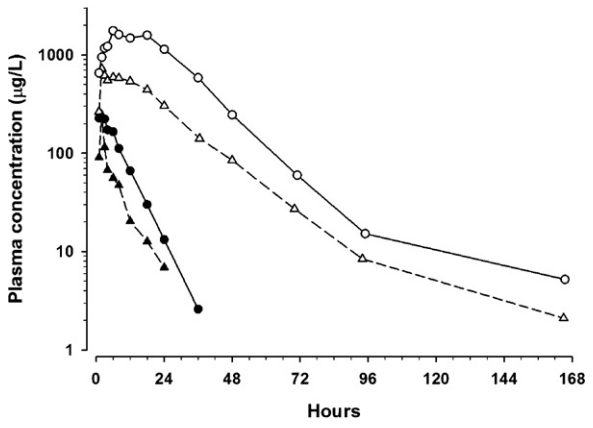


**Fig. 3.** Plasma concentration-time profiles of primaquine (▲; ●) and carboxyprimaquine (△; ○). Child YO3 (male, 7.4 years old, 16 kg; ▲: △) was given 0.47 mg/kg primaquine at zero time; Child YO9 (female, 7.6 years old, 18.5 kg; ●; ○) was given 0.81 mg/kg primaquine at zero time.

pharmacokinetic data for primaquine in healthy adult subjects and patients with vivax malaria [8–14].

## 4. Conclusion

Our novel, validated LC–MS method with solid phase extraction facilitates the simultaneous analysis of primaquine and carboxyprimaquine from small volumes of human plasma. The run time (<10 min), recovery (>85%) and sensitivity (1–2 μg/L) demonstrate that this procedure is suitable for pharmacokinetic studies of primaquine in patients and healthy volunteers.

## Conflict of interest statement

The authors declare that there are no conflicts of interest.

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