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A high-throughput spectroscopic-based bioassay method for determination of fosmidomycin in plasma: application for pharmacokinetic study

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Abstract

A simple, sensitive, selective and reproducible bioassay based on spectroscopic method was developed for the determination of fosmidomycin in human plasma. Enterobacter cloacae ATCC 23355 strain was used as a test organism. Inhibition of bacterial growth was assessed using MTT assay. Calibration curves were prepared from concentration response curves in plasma (0, 0.5, 1, 2.5, 5, 12.5, 25, 50 ng/μl) and were all linear with correlation coefficients better than 0.995. The precision of the method based on within-day repeatability and reproducibility (day-to-day variation) was below 10% (% coefficient of variations: % C.V.) Good accuracy was observed for both the intra-day and inter-day assays, as indicated by the minimal deviation of mean values found between the measured samples and the theoretical values (below $\pm$10%). Limit of quantification (L.O.Q.) was accepted as 0.5 ng using 20 μl plasma or 10 μl urine sample. The mean recovery for fosmidomycin was greater than 99%. The method was free from interference from other commonly used antibiotics including azithromycin. The method appears to be robust and has been applied to a pharmacokinetic study to determine the plasma of fosmidomycin in a Thai patient with acute uncomplicated falciparum malaria following oral doses of 1,800 mg fosmidomycin given every twelve hours for three days, in combination with azithromycin at the dose of 750 mg given every twelve hours for three days.

Keywords: *Plasmodium falciparum*, fosmidomycin, bioassay, spectroscopy.

Introduction

Fosmidomycin [3-(formylhydroxy-amino)-propylphosphonic acid mono-sodium salt,3-(*N*-formyl-*N*-hydroxy-amino)-propylphosphonic acid mono-sodium salt, FR-31564) is a phosphonic acid derivative originally isolated as a natural antibiotic from *Streptomyces lavendulae*. It acts as a potent inhibitor of 1-deoxy-*D*-xylulose 5-phosphate (DOXP) reductoisomerase, an essential enzyme of the non-mevalonate pathway and therefore, selectively blocks the biosynthesis of isopentenyl diphosphate and the subsequent development of isoprenoids in *Plasmodium falciparum* (1). The drug has been shown *in vitro* and *in vivo* in animal studies to be a potential antimalarial, but the development of recrudescence found in early phase of clinical trial precludes its use as monotherapy (2). Recently pharmacokinetics and pharmacodynamics of fosmidomycin monotherapy and combination therapy with clindamycin have been evaluated in Thai patients with multi-drug resistance falciparum malaria (3-4). Pharmacokinetic investigation to determine the minimum inhibitory concentration (MIC) is essential for optimal dose adjustment particularly in the event of multidrug resistant *P.falciparum*. Recently, we have reported a bioassay method based on agar diffusion disk assay (5). The method is sensitive with limit of quantification (L.O.Q.) of 1 ng using 40-μl plasma. Nevertheless, it requires tedious and long procedure of 3-4 days for bacterial culture and assessment of growth inhibition. In the present study, we develop a high throughput bioassay method for determination of fosmidomycin in plasma and urine based on spectrophotometrric assessment of growth by MTT assay (6).

Methods

The test organism used was *Enterobacter cloacae* ATCC 23355 strain. For each assay, plasma (20 µl) containing different concentrations of fosmidomycin (0, 0.5, 1, 2.5, 5, 12.5, 25, 50 and 100 ng/µl (triplicate wells each) was added into each well of the 96-well microtiter plate containing 150 µl of the prepared bacterial suspension in LB broth (2.1×10^5 CFU) per ml). The plate was then incubated at 37 °C for 18-24 h, and thereafter, 5 µl of the 5-mg/ml stock solution of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution (20 µl of 5 mg/ml) was added to each well of the plate. The pate was left at room temperature for 5 min and optical density reflecting bacterial growth (formation of formazan crystals) was measured at 595 nm using a microplate reader. The blank well consisted of LB Both and MTT. A nonlinear regression analysis of sigmoidal dose-response (variable slope) was performed using automated curve fitting software (GraphPad Prism 5.0™, CA, USA.). The developed method was validated for linearity, recovery, sensitivity, specificity, precision, and accuracy (7). The validated assay method was applied to the investigation of the pharmacokinetics of fosmidomycin in plasma and urine in a Thai patient with acute uncomplicated falciparum malaria (aged 22 years and weighing 50 kg) who received treatment with fosmidomycin (Jomaa Pharm Co. Ltd., Germany) at a dose of 1,800 mg given every twelve hours for 3 days, in combination with azithromycin (Zithromax™, Pfzer Pharm Co. Ltd.) at a dose of 750 mg given every twelve hours for 3 days. Venous blood samples (3 ml) were collected into heparinised-coated plastic tubes at the following time points: 0, 1, 2, 3, 4, 6, 8, 12, 14, 18, 24, 26, 30, 36, 38, 42, 48, 50, 54, 60, 62, 66, 72, 78 and 84 hours after the first dose of fosmidomycin.

Results

Selectivity of the bioassay system was demonstrated by the absence of interferences from endogenous substances and commonly used antibiotics. The calibration ranges yielded linear relationships with correlation coefficients of 0.995 or better (Fig 1). Good precision and accuracy for both the intra-assay (within-day) and inter-assay (day-to-day) was obtained (Table 1). The limit of quantification (L.O.Q.) in human plasma for fosmidomycin was accepted as 0.5 ng using 20 µl plasma. The pharmacokinetics of fosmidomycin accorded with those described in Thai patients with malaria following mono- or combination therapy of fosmidomycin with clindamycin (12-13). The mean (SD) peak plasma concentration of 4.59 (2.55) µg/ml was achieved at 3.0 h following the first dose. Trough concentrations at 12, 24, 36, 48, 60, 72 and 84 h were 2.18 (1.29), 1.62 (0.74), 1.62 (0.8), 2.20 (0.6), 2.09 (1.2) and 1.15 (0.43) µg/ml respectively.

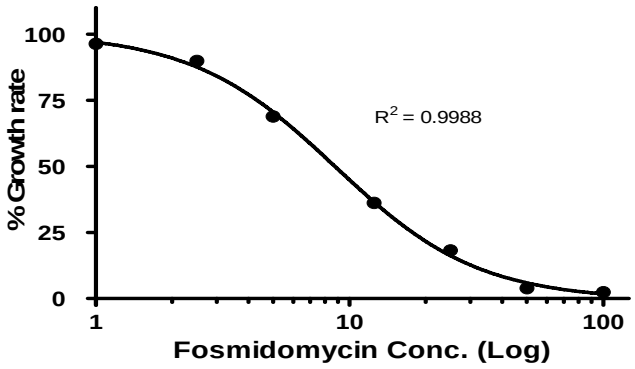

Figure 1 Calibration curves of fosmidomycin in plasma over the concentration ranges of 0-50 ng/µl.

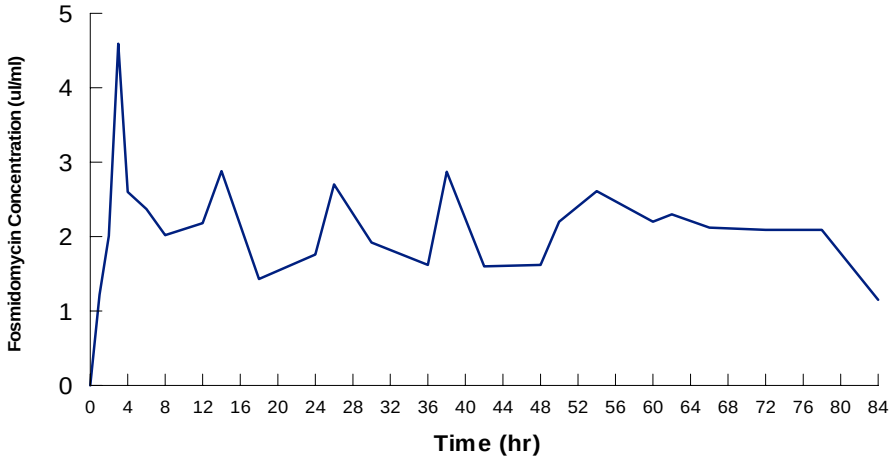

Figure 2 Plasma concentration-time profile of fosmidomycin in a Thai patient with acute uncomplicated falciparum malaria following treatment with 1,800 mg fosmidomycin given every 12 hours for 3 days, in combination with 750 azithromycin given every 12 hours for 3 days.

Table 1 Summary of assay precision and accuracy (intra- assay and inter-assay) for fosmidomycin assay in plasma.

Concentration added (ng/µl)	Precision (%C.V.)[b]		Accuracy (%D.M.V.)[a]	
	Intra-assay (n=6)	Inter-assay (n=6)	Intra-assay (n=6)	Inter-assay (n=6)
0.5	3.68	6.94	-8.00	+6.94
1	8.44	5.63	-6.00	+5.63
2.5	6.29	9.07	+4.40	+9.07
5	4.53	3.11	+2.60	+3.11
12.5	1.07	4.73	-1.76	+4.73
25.0	3.91	3.86	-5.56	+3.86
50.0	6.20	5.47	+7.76	+5.47

[a] %CV = coefficient of variation (%)
[b] %DMV = deviation of mean value from theoretical value (%)

Conclusion

The analytical method established in this study meets the criteria of simplicity, high sensitivity, accuracy and reproducibility for routine use in pharmacokinetic studies. The method has advantages over the recently developed bioassay (5) in its simplicity, accuracy

(end point of measurement was inhibition of bacterial growth assessed spectroscopically by MTT assay *vs* manual measurement clear zone diameter), short analysis time (1 day *vs* 3-4 days), good sensitivity (0.5 *vs* 1 ng), and the requirement for smaller volumes of samples (20 *vs* 40 μl).

Acknowledgement

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