AN ALTERNATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR DETERMINATION OF CLINDAMYCIN IN PLASMA

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Abstract. A simple, sensitive, selective and reproducible method based on a reversed-phase chromatography was developed for the determination of clindamycin in human plasma. Clindamycin was separated from the internal standard (phenobarbital) on a Luna C18 column (250 x 4.6 mm, 5 mm particle size: Phenomenex™, USA), with retention times of 5.6 and 14.2 minutes, respectively. Ultraviolet detection was set at 210 nm. The mobile phase consisted of a solution of 0.02 M disodiumhydrogenphosphate (pH 2.8) and acetonitrile (76:24 v/v), running through the column at a flow rate of 1.0 ml/min. The chromatographic analysis was operated at 25ºC. Sample preparation (1 ml plasma) was done by a single step liquid-liquid extraction with water saturated ethylacetate. Calibration curves in plasma at concentrations of 0.25, 0.5, 1.0, 2.0, 4.0, 8.0 and 16.0 μg/ml were all linear with correlation coefficients better than 0.999. The precision of the method based on within-day repeatability and reproducibility (day-to-day variation) was below 15% (% coefficient of variations: %CV) Good accuracy was observed for both the intra-day and inter-day assays, as indicated by the minimal deviation of mean values found with measured samples from that of the theoretical values (below ±15%). Limit of quantification was accepted as 0.07 μg using 1 ml plasma sample. The mean recovery for clindamycin and the internal standard were greater than 95%. The method was free from interference from fosmidomycin, including commonly used drugs, antimalarials and antihelminthics. The method appears to be robust and has been applied to a pharmacokinetic study of clindamycin in a patient with malaria following oral doses of clindamycin at 10 mg/kg body weight given twice daily for 7 days.

INTRODUCTION

Clindamycin [7(S)-chloro-7-deoxylincomycin] is a lincosamide antibiotic (Fig 1) with primarily bacteriostatic activity against gram positive organisms and a wide range of anaerobic pathogens as well as some antiprotozoal efficacy. This effect is exerted by its binding to the 50S ribosomal subunit and the consequent inhibition of bacterial protein synthesis (Booth, 2001). Additionally, clindamycin is active against different apicoplexan parasites, including *Plasmodium falciparum.* It has been demonstrated that clindamycin targets the prokaryote-like ribosomes of the apicoplast and by this means inhibits self-replication of the organelle (Fichera and Boos, 1997; Kohler *et al,* 1997). As a consequence of this mechanism, clindamycin displays a typical delayed kill kinetic effect, the growth of parasites being unaffected until the second replication after drug exposure. Under such conditions, the *in vitro* growth of *P. falciparum* is inhibited with an IC_{50} and IC_{90} of approximately 25 and 50 nM, respectively.

A number of analytical methods have been reported for measuring clindamycin in bulk drugs and formulations as well as in biological fluids and tissue or cell homogenates or organ extracts. These methods involve microbiological

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assay (Metzler *et al,* 1973; Brown *et al,* 1981), spectrophotometric assay (El-Yazbi and Blaih, 1993), radio-immuno assay (RIA) (Duckworth *et al,* 1993), gas-liquid chromatography (GLC) (Oesterling and Rowe, 1970; Brown, 1974; Gatti *et al,* 1993), and high performance liquid chromatography (HPLC) (Brown, 1978; Landis *et al,* 1980; La Follette *et al,* 1988; Hornedo-Nunes *et al,* 1990; Liu *et al,* 1997; Fieger-Buschges *et al,* 1999; Orwa *et al,* 1999; Sin *et al,* 2004). Microbiological, spectrophotometric assays, RIA and GLC are either non-specific, or time-and-reagent consuming. The HPLC technique is considered to be most appropriate method for application to pharmacokinetic investigation as it is sensitive, accurate, reproducible and relatively simple. Refractive index (Brown, 1978; Landis *et al,* 1980), electrochemical (Hornedo-Nunes *et al,* 1990), mass spectrometry (Yu *et al,* 1999; Martens-Lobenhoffer and Banditt, 2001; Cherlet *et al,* 2002; Rechberger *et al,* 2003; Sin *et al,* 2004), and UV (Landis *et al,* 1980; Munson and Kubiak, 1985; La Follette *et al,* 1988; Liu *et al,* 1997; Fieger-Buschges *et al,* 1999; Orwa *et al,* 1999) detection have been used in HPLC. HPLC/MS methods (Yu *et al,* 1999; Martens-Lobenhoffer and Banditt, 2001; Cherlet *et al,* 2002; Rechberger *et al,* 2003) display the highest performance; however, the LC/MS instruments are not yet readily obtainable in most laboratories.

With respect to HPLC/UV methods, a straight forward HPLC/UV method (La Follette *et al,* 1988) using direct injection of plasma after a precipitation step with acetonitrile has been reported to be non-reproducible (Liu *et al,* 1997; Fieger-Buschges *et al,* 1999) due to interferences from plasma components. The method described by Liu *et al* (1997) is rather sophisticated, using a couple of columns and two mobile phases to extract clindamycin from human plasma samples. Fieger-Buschges *et al* (1999) reported an automated method using couple column HPLC after the precipitation of plasma proteins with saturated ammonium sulfate solution. Recently, Batzias *et al* (2005) reported a new HPLC/UV method for the quantitative determination of clindamycin in dog serum. The method was based on deproteinisation of samples with acetonitrile followed by extraction with dichloromethane. In the present report, we describe an alternative method, which is relatively simple, sensitive, accurate and reproducible for the determination of clindamycin in biological fluids. The total run time was less than 18 minutes. The method was based on reversed-phase chromatography with ultraviolet detection. The method has been applied successfully to pharmacokinetic studies of clindamycin when used in combination with fosmidomycin in a patient with acute uncomplicated falciparum malaria.

MATERIALS AND METHODS

Chemicals

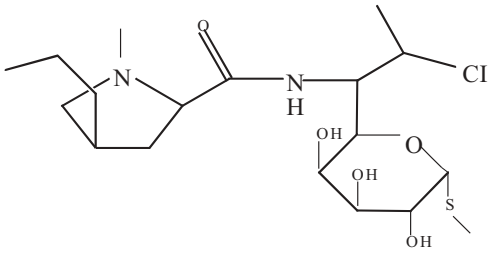
All solvents were HPLC grade. Organic solvents were purchased from Fison Scientific Equipment (Bishop Meadow Road, Loughborough, UK). Disodium hydrogenphosphate was of analytical grade, which was obtained from Sigma Chemical (St Louis, MO, USA). Ultrapure analytical grade Type I water (r > 18 MΩ/cm) was produced by a Milli-Q Plus, water system (Millipore Corporation, Bedford, MA, USA). Clindamycin [7(S)-chloro-7-deoxylincomycin]; Fig 1a) and internal standard (phenobarbital; Fig 1b) were obtained from Sigma Chemical (St Louis, MO, USA).

Standard stock solutions

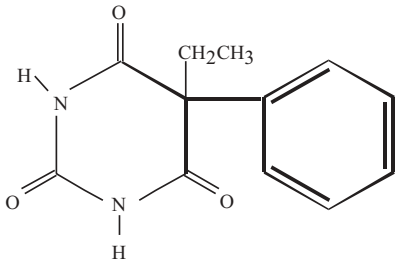
Stock solutions were made with clindamycin and the internal standard (phenobarbital). Appropriate amounts of chemicals were dissolved in distilled water in volumetric flasks. Stock solutions of clindamycin and internal standard were prepared at a concentration of 1,000 ng/μl. The stock solutions were further diluted to make working solutions at concentrations of 250 and 100 μg/ml, for clindamycin and the internal standard, respectively. Standard solutions were stored at -20ºC until use.

Chromatography

The method was developed on a chromatographic system consisting of a Waters 600 HPLC solvent Delivery/Controller, equipped with a Rheodye 7125 injector with a 100-ml loop (Rheodyne, Berkeley, CA, USA), an ultraviolet detector (Waters 996 Photodiode Array Detec-



(a)



(b)

Fig 1–Chemical structures of (a) clindamycin and (b) internal standard (phenobarbital).

tor; Milford, MA, USA), and Millenium 32 Software for data integrator. The wavelength was set at 210 nm. The separation was carried out on a reversed phase column Luna C18 (250 x 4.6 mm, 5 μm particle size: Phenomenex™, MA, USA). The elution solvent consisted of a solution of 0.02 M disodium hydrogenphosphate (pH 2.8) and acetonitrile (46:24, v/v). The chromatographic analysis was operated at 25ºC. Aliquots of 100 μl samples or standard solutions were injected onto the column with a mobile phase at a flow rate of 1.0 ml/minute. All buffers were vacuum filtered and degassed through 0.2 μm pore size polymeric PTFE filters.

Sample preparation

This procedure was validated on specimens using 1 ml of spiked human plasma. Human plasma was obtained from healthy subjects, and stored frozen in aliquots at -20ºC. To 1 ml plasma, 40 ml internal standard working solution (400 μg/ml) was added. After thoroughly mixing, the sample was extracted twice by mechanical tumbling (speed 7, 15 minutes) with 2 x 5 ml water saturated ethylacetate. The resulting clear organic layer was separated through centrifugation at 3,500*g* for 15 minutes, and evaporated to dryness under a gentle nitrogen stream at 40ºC. Samples were protected from light and stored at 4ºC until injection. For HPLC injection, samples were reconstituted with 200 μl mobile phase, and an aliquot of 100 μl was injected onto the chromatographic system.

Calibration curves

Detector linearity. Solutions of clindamycin in distilled water at concentrations ranging from 0 to 16.0 μg/ml were injected into the HPLC system in order to assess detector linearity. Peak height was plotted against the quantity of clindamycin injected. Clindamycin was linear (r^2 > 0.999) over the concentration range observed.

Plasma. Calibration curves were prepared by replicate analysis of six plasma samples (1 ml each) spiked with varying concentrations of clindamycin (0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0 mg/ml) and a fixed concentration of the internal standard (400 μg/ml). Samples were analysed as described previously.

Data analysis. Peak height ratios of clindamycin/internal standard were calculated. Concentrations of clindamycin were determined by matching peak height responses against a calibration curve of response ratio (height of clindamycin/height of internal standard) *vs* concentration, obtained from standard sample injection. The internal standard corrected for variation in the sample preparation step used. Peak detection, peak height integration, peak height ratio calculation, calibration curve fitting (least square regression without weighting) and calculation of sample concentrations were performed by Millenium 2000 Chromatograph® software.

Method validation

Precision. The precision of the method based on *within-day repeatability* was determined by replicate analysis of six sets of plasma samples (1 ml each) spiked with seven different concentrations of clindamycin (0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0 μg/ml). The *reproducibility (day-to-day variation*) of the method was validated using the same concentration range of plasma as described above, but only a single determination

of each concentration was made on six different days. The coefficient of variation (CV) was calculated from the ratios of standard deviation (SD) to the mean and expressed as a percentage.

Accuracy. Accuracy of the method was determined by replicate analysis of six sets of plasma samples (1 ml each) at seven different levels of clindamycin (0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0 μg/ml) and comparing the difference between spiked value and that of the actually found (theoretical value).

Recovery. The analytical recovery of sample preparation procedure for clindamycin was estimated by comparing the peak heights obtained from samples (1 ml plasma) prepared as described above, with those measured with equivalent amounts of clindamycin in distilled water. Triplicate analysis was performed at concentrations of 0.25, 2.0, and 8.0 μg/ml.

Selectivity. Blank (heparinised) plasma samples from healthy Thai volunteers were tested for interference by endogenous compounds. The selectivity of other method was verified by checking for interference by fosmidomycin and other commonly used drugs: antimalarials (fosmidomycin, chloroquine, quinine, mefloquine), anthelminthics (albendazole, praziquantel), paracetamol and dimenhydrinate after subjecting them to sample preparation procedures.

Limit of quantification. The limit of quantification (LOQ) of the assay procedure was determined from the lowest concentration of clindamycin (in the spiked plasma sample) that produced a peak height ten times the baseline noise at a sensitivity of -0.2 μA in a 1 ml sample.

Stability. The stability of clindamycin was determined by storing spiked plasma samples (1 ml each at concentrations of 0.25, 2.0, and 8.0 μg/ml; triplicate analysis for each concentration) in a -20°C freezer (Sanyo, Japan) for six months. Concentrations were measured periodically (1, 2, 3 and 6 months). For freeze and thaw stability, samples were frozen at -20°C for at least 24 hour and thawed unassisted at room temperature (25°C). When completely thawed, the samples were transferred back to the original freezer and refrozen for at least 24 hour. The process was repeated for three cycles.

Quality control. Quality control (QC) samples for clindamycin were made up in plasma (1 ml) using a stock solution separate from that used to prepare the calibration curve, at concentrations or 0.25, 2.0, and 8.0 μg/ml. Samples were aliquoted into cryovials, and stored frozen at -20°C for use with each analytical run. The results of the QC samples provided the basis of accepting or rejecting the run. A total of 6 quality control samples (2 for each concentration) were analysed during each run (at the beginning of each batch of samples and the standard curve). At least four of the six QC samples had to be within ±20% of their respective nominal values. Two of the six QC samples could be outside the ±20% of their respective nominal value, but not at the same concentration.

Application of the method to biological samples

The method was applied to the investigation of the pharmacokinetics of clindamycin in plasma in a patient (from MaeSot Hospital, Tak Province, Thailand) with acute uncomplicated falciparum malaria (aged 28 years, weighing 50 kg) who received treatment with clindamycin at a dose of 10 mg/kg body weight twice daily for 7 days, in combination with fosmidomycin at a dose of 15 mg/kg body weight twice daily for 7 days. Venous blood samples (3 ml) were collected in heparin-coated plastic tubes at the following time points: 0, 24 (pre-dose), 48 (pre-dose), 160 (pre-dose), 162, 164, 166, 172, 184, 208, 220, 232, 238, 244, and 250 hours after the last dose of clindamycin.

RESULTS

Chromatographic separation

A number of HPLC chromatographic systems were investigated to optimise the separation of clindamycin and the internal standard (phenobarbital). Retention maps were generated for both compounds as a function of stationary phase (Luna C18 reversed-phase column) and mobile phase. For, the mobile phase, composition of 0.02 M disodiumhydrogenphosphate (pH 2.8) and acetonitrile (76:24 v/v) running through the colum at a flow rate of 1.0 ml/min gave optimal separation of clindamycin and internal standard with a 20-minute run time. The retention

times (capacity factor) of clindamycin and the internal standard were approximately 5.6, and 14.2 minutes, respectively.

Sample preparation

The sample preparation step used in this study was very simple as it involved only one step liquid-liquid extraction. Extraction of plasma with water saturated ethylacetate was found to be the most optimal condition for sample preparation as it resulted in a clean chromatogram (Fig 2).

Chromatograms of blank plasma and plasma spiked with clindamycin at a concentration of 0.25 μg/ml (with a fixed concentration of internal standard of 400 μg) are shown in Fig 2a and 2b.

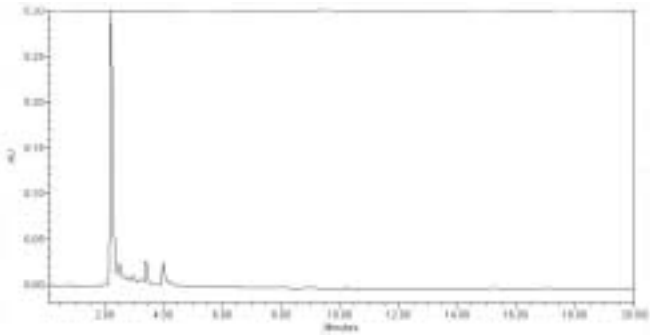
Calibration curves

Plasma analysis was calibrated using a concentration range of 0.25-16.0 μg/ml. All calibration ranges yielded linear relationships with correlation coefficients of 0.999 or better.

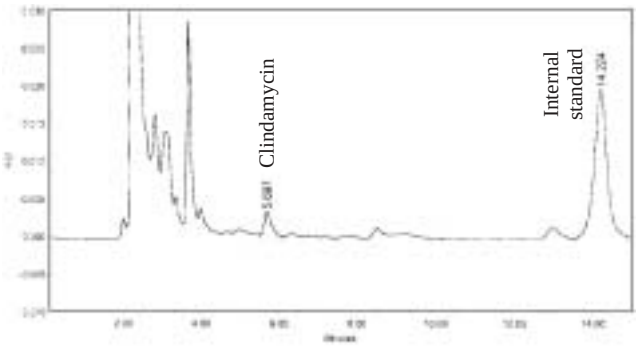
Method validation

Precision. Little variation in the clindamycin assays was observed. Coefficients of variation (CV) for six analyses at the concentration range observed were all below 15%. The intra-assay (within-day) and inter-assay (day-to-day) variation for the clindamycin assay at a concentration range of 0.25-16.0 μg/ml are summarised in Table 1. For intra-day assay validation in plasma, the coefficients of variation varied between 2.3 and 12.1%. The corresponding values for inter-day assay validation in plasma were 2.4 and 14.1%.

Accuracy. Good accuracy was observed from both the intra-day and the inter-day assays, as indicated by the minimal deviation of mean values found with measured samples from that of the theoretical values (actual amount added). The intra-assay (within-day) and inter-assay (day-to-day) accuracy for the clindamycin assays at a concentration range of 0.25-16.0 μg/ml are summarised in Table 1. For intra-day assay accuracy in plasma, the mean deviation from the theoretical values varied between -10.5 and +1.6%. The corresponding values for inter-day assay validation in plasma were -9.4 and +0.7.



(a)



(b)

Fig 2–Chromatogram of (a) blank plasma, (b) plasma spiked with 0.25 μg/ml clindamycin and 400 μg internal standard (retention times of 5.6 and 14.2 minutes, respectively).

Recovery. The mean recoveries for clindamycin in plasma at concentrations of 0.25, 4 and 8 μg/ml, and a internal standard at a concentration of 400 mg were greater than 88%, with a mean (SD) of 92.2 (2.5)%. The results reflect the lack of interference from the sample preparation procedure.

Selectivity. Selectivity of the chromatographic separation was demonstrated by the absence of interference from endogenous peaks, as well as those from commonly used drugs described above. Fig 2 (a, b) illustrates typical chromatograms for blank plasma, spiked plasma with clindamycin and the internal standard.

Limit of quantification. The limit of quantification (LOQ) in human plasma for clindamycin was accepted as 0.07 μg using 1 ml plasma.

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

Concentration added (μg/ml)	Precision (%CV)		Accuracy (%DMV)[a]	
	Intra-assay (n=6)	Inter-assay (n=6)	Inter-assay (n=6)	Inter-assay (n=6)
0.25	2.3	5.4	+1.6	-1.6
0.50	4.1	12.9	-2.1	-9.4
1.00	12.1	14.1	-6.8	-5.8
2.00	4.8	6.6	-1.8	-5.3
4.00	3	5.9	-3.7	-3.8
8.00	3.9	9.4	-10.5	-9.3
16.00	5.2	2.4	-2.5	+0.7

[a]%DMV = deviation of mean value from theoretical value (%)

Stability

Plasma samples containing clindamycin at concentrations of 0.25, 2.0, and 8.0 mg/ml were found to be stable when stored at -20ºC for a minimum of six months without significant decomposition of the drug. Freezing and thawing of the spiked samples did not appear to affect the quantification of the analytes (Table 2a). The mean deviation (%) of the measured concentrations after storage at the observed periods (1, 2, 3 and 6 months) varied between -4.1 and + 1.7%. Freezing and thawing for three successive cycles did not affect the measured concentrations. Mean deviation from the theoretical values varied between -1.0 and + 1.7% (Table 2b).

Quality control. Analytical values for all the quality control samples (6 samples for each run at concentrations of 0.25, 2 and 8 μg/ml were all within

Table 2
Storage stability data of clindamycin in plasma at concentrations 0.25, 2.0, and 8.0 μg/ml.

(a) Long-term stability at 1, 2, 3 and 6 months

Time period (months)	Concentration (μg/ml)	Concentration measured (μg/ml)				
		Assay 1	Assay 2	Assay 3	Mean (SD)	% DEV[a]
1	0.25	0.245	0.247	0.244	0.245 (0.001)	+1.8
	2.0	2.125	2.145	1.980	2.083 (0.090)	-4.1
	8.0	7.025	8.025	8.125	7.725	+0.6 (3.4)
2	0.25	0.239	0.245	0.246	0.243 (0.003)	+2.6
	2.0	2.015	1.989	1.890	1.964 (0.065)	+1.7
	8.0	7.581	7.658	8.012	7.750 (0.229)	+3.1
3	0.25	0.247	0.254	0.248	0.249 (0.003)	+0.1
	2.0	2.015	1.989	1.897	1.967 (0.062)	+1.6
	8.0	8.569	8.125	7.898	8.197 (0.341)	-2.4
6	0.25	0.249	0.247	0.251	0.249 (0.002)	+0.4
	2.0	2.014	2.156	1.899	2.023 (0.128)	-1.1
	8.0	7.890	7.991	7.899	7.926 (0.055)	+0.9

[a] %DEV = deviation of single value from theoretical value (%)

(b) Freeze and thaw stability

Concentration added (μg/ml)	Concentration measured (μg/ml)				
	Assay 1	Assay 2	Assay 3	Mean (SD)	% DEV
0.25	0.258	0.249	0.251	0.252 (0.004)	-1
2.0	2.012	1.989	1.897	1.966 (0.06)	+1.7
8.0	7.890	8.123	8.012	8.008 (0.116)	-0.1

[a] %DMV = deviation of mean value from theoretical value (%)

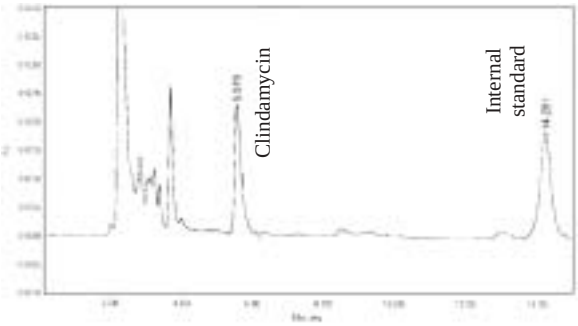


Fig 3–Chromatogram of plasma sample collected at 1 hr after the last dose of clindamycin on day-7 (spiked with 400 mg internal standard).

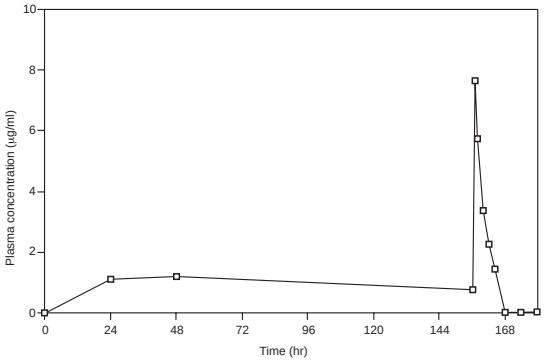


Fig 4–Plasma concentration-time profile of clindamycin in a patient following oral dose of clindamycin at 10 mg/kg body weight given twice daily for 7 days.

± 10% of their respective nominal values.

Application of assay and analysis of specimens

To demonstrate the clinical applicability of the method, plasma concentrations levels of clindamycin were determined in a patient following oral doses of clindamycin at 10 mg/kg body weight given twice daily for 7 days. A chromatogram of a plasma sample collected 1 hour after the last dose of clindamycin on day 7 of dosing (spiked with 400 µg internal standard) is shown in Fig 3.1. The plasma concentration-time profile for clindamycin is shown in Fig 4, which is in general agreement with those previously described.

DISCUSSION

We describe a HPLC assay procedure based on reversed-phase C18 chromatography with ultraviolet detection, for the selective, sensitive, accurate and reproducible quantitative analysis of clindamycin in human plasma samples. Total run time was within 20 minutes. The analytical method for the determination of clindamycin in plasma established in this study meets the criteria (simplicity, selectivity, accuracy, good recovery, and high sensitivity) for application to routine clinical drug level monitoring or pharmacokinetic studies. The advantage of the method over previously reported ones are its rapidity, simplicity and high sensitivity. In addition, the sample preparation procedure is simple, faster and less expensive.

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