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DETERMINATION OF CLINDAMYCIN IN PLASMA OR SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ULTRAVIOLET DETECTION

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SUMMARY

A simple, sensitive high-performance liquid chromatographic assay for the determination of clindamycin in human plasma or serum has long been hampered by inability to separate it from endogenous compounds. We describe here such an assay. Proteins from a 200-μl sample were precipitated by addition of acetonitrile containing the internal standard, triazolam. The sample was then vortex-mixed and centrifuged at approximately 3000 *g* for 10 min. The supernatant was evaporated to about 250 μl under nitrogen, and 10–30 μl were analyzed using an autoinjector. An octadecylsilane column with acetonitrile–water–phosphoric acid–tetramethylammonium chloride as mobile phase and ultraviolet detection at 198 nm provided a reproducibly quantifiable peak corresponding to 0.17 μg/ml. Retention times for clindamycin and triazolam averaged 8 and 11.8 min, respectively. Replicate standard curves run over a 36-h period showed no loss of integrity; r^2 values generally exceeded 0.999. The method has successfully been applied to the analysis of samples taken up to 12 h after administration of intravenous infusions of 600–1200 mg in healthy volunteers.

INTRODUCTION

Clindamycin (CLD, I, Fig. 1) is an antibiotic highly effective against gram-positive and gram-negative anaerobic pathogens, as well as gram-positive aerobes [1, 2]. It is synthesized from microbially fermented lincomycin (II, Fig. 1) by

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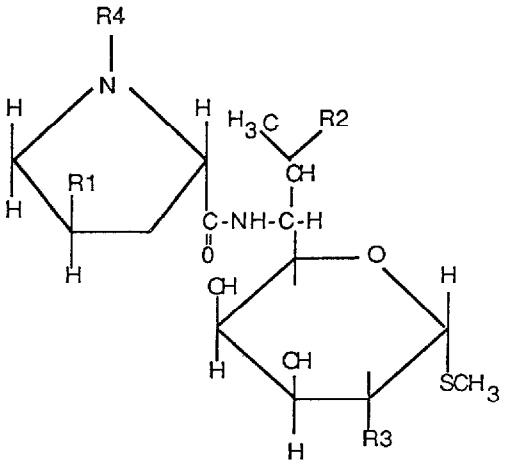

	R1	R2	R3	R4
I:	n-propyl	(S) -Cl	OH	methyl
II:	n-propyl	(R) -OH	OH	methyl
III:	n-propyl	(S) -Cl	phosphate	methyl
IV:	ethyl	(S) -Cl	OH	methyl
V:	n-pentyl	(S) -Cl	OH	H

Fig. 1. Structures of clindamycin and analogues.

substituting chlorine for a hydroxyl group, which concurrently increases its biological activity. Clindamycin 2-phosphate (III, Fig. 1), the salt form of CLD (Cleocin Phosphate®, brand of CLD phosphate sterile solution, Upjohn), is used parenterally and undergoes hydrolysis in the body to form active CLD [3].

Both gas chromatography (GC) and microbiological analytical procedures exist for detecting CLD in biological fluids [4, 5]. Both, however, are time-consuming and labor-intensive. The GC assay requires lengthy extraction and derivatization, while the microbiological assay is non-specific and less accurate. Previous high-performance liquid chromatographic (HPLC) assays for CLD were rapid, but were developed only for analysis of sterile fluids or water [6–10]. Our HPLC assay, to our knowledge, is the only one that successfully separates CLD from biological fluids. No extraction is required, making this method extremely rapid.

EXPERIMENTAL

Apparatus

The HPLC system (Pro-840 with Model 510 pump; Waters Assoc., Milford, MA, U.S.A.) was equipped with a Kratos Model 783 variable-wavelength UV detector (Spectros, Ramsey, NJ, U.S.A.), a Nova-Pak C_{18} octadecylsilane column (5 μm, 15 cm $\times$ 3.8 mm; Waters) a WISP 710 B autoinjector (Waters) and

a Pro-840 computer/printer for data collection, system operation and data storage/retrieval (Waters). The monochromator of the UV detector was continuously purged with purified nitrogen. The detector wavelength of 198 nm was attenuated to 0.001 a.u.f.s. with a filter response time of 0.1 s. Calibrated syringes (Hamilton, Reno, NV, U.S.A.) (1.00, 5.00, 10.0, 25.0, 50.0 and 100 μl) were used during sample preparation.

Reagents

Sterile water was deionized and filtered through Milli-Q and Nanopure (0.45 μm) systems (Millipore, Milford, MA, U.S.A.). Acetonitrile, UV grade (Cat. No. 015, Burdick and Jackson Labs., Muskegon, MI, U.S.A.), was distilled in glass. The orthophosphoric acid (85%, Lot KTGY, Mallinckrodt, Paris, KY, U.S.A.), tetramethylammonium chloride (TMA) (Lot 241574 685; Fluka, Waupaugh, NY, U.S.A.), CLD hydrochloride [Lot 267PY, 86.4% relative purity (expressed as free rather than salt); Upjohn, Kalamazoo, MI, U.S.A.] (I, Fig. 1), CLD B (Lot 9658-RDB-60; Upjohn) (IV, Fig. 1), CLD phosphate (Upjohn) (III, Fig. 1), N-demethyl-(4-pentyl)-CLD [Lot 10891-RBJ-54(1), Upjohn] (V, Fig. 1) and TRI (Lot 111, Upjohn) were used as received.

Mobile phase

The mobile phase was composed of acetonitrile–water–phosphoric acid–7.6 m*M* TMA (30:70:0.2:0.075) with a final apparent pH of 6.7. Mobile phase was prepared in 2-l quantities in the following manner. A 600-ml volume of acetonitrile was transferred to a 2-l graduated cylinder. Nanopure water was then poured into the cylinder until the fluid level reached the 1900-ml mark. Phosphoric acid (4 ml) was added along with 15 ml of a previously prepared 10% TMA (1 g TMA salt in 9 ml water) solution. Water was then added to the 2-l mark, and the contents filtered under vacuum with a 0.45-μm membrane filter. The pH was adjusted to 6.7 with 1 *M* sodium hydroxide, then the solution was pumped through the column at a flow-rate of 1.0 ml/min at a pressure of 130 bar. A constant slow bubbling of helium through the mobile phase during operation was required to keep oxygen in the system at a minimum. Prior to starting analysis, 2 h at the above conditions were required to reach system equilibrium.

Sample preparation and analysis

Proteins in heparinized plasma and serum samples (0.2 ml) were precipitated with 0.5 ml of acetonitrile containing the internal standard TRI (44 ng per 100 ml). After the samples were vortexed for 20 s and centrifuged at 3000 *g* for 10 min, the resulting supernatant was poured off the protein pellet and evaporated under nitrogen to a volume of 250 μl. A WISP autoinjector was used to deliver 15–30 μl of the concentrated supernatant onto the HPLC system.

RESULTS

Separation

Fig. 2a represents a typical chromatogram for a blank human plasma sample with the addition of the internal standard TRI. Fig. 2b and c represent plasma

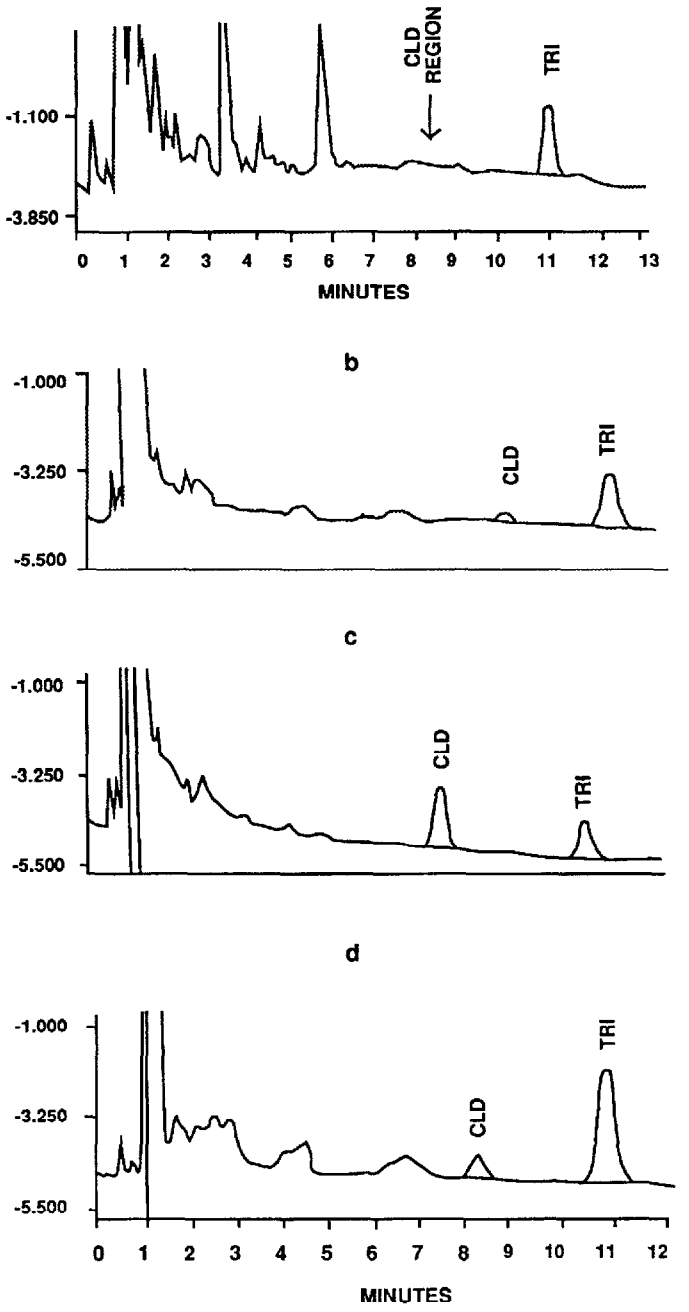

Fig. 2. Chromatograms of clindamycin in human plasma. (a) Blank plasma spiked with internal standard, TRI; (b) plasma concentration of 1.5 μg/ml; (c) plasma concentration of 12 μg/ml; (d) 12-h clinical sample following a 1200-mg intravenous dose.

	Peak	Retention time	Area	Height	Type	Amount	R_F
a	CLD	8.60	0		0	0.000	0.0000e+00
	TRI	11.60	33374	1856	BB	0.000	0.0000e+00
d	CLD	8.32	10225	454	BB	6.483	1.5582e+08
	TRI	11.13	67615	2459	BB	Internal standard	1.2584e+09

samples spiked with CLD at 1.5 and 12 μg/ml. Fig. 2d represents CLD in a 62-kg healthy volunteer, 12 h after 1200 mg intravenous dosing. The CLD eluted at 8.4 min and the internal standard TRI eluted at 11.3 min.

Calculations

Standard curves were constructed using unweighted least-squares ($y=mx+b$) method with the origin as a data point, but not forced through zero (floating intercept). Superior performance is achieved if a split curve approach using the concentrations 0.17–1.73 and 1.73–17.3 μg/ml is employed. All chromatograms must be re-calculated with a forced baseline as provided in a special chromatographic software package (Waters Assoc.). The use of peak height provided better linearity than peak area, when applied over the entire standard curve.

Linearity

Table I depicts the linear relationship between the drug plasma concentration and the peak-height ratio of CLD over TRI. Linear regression of peak-height ratio versus concentration gives a coefficient of determination (r^2) of 0.9998, a slope of 0.1105 and a y-intercept of 0.0055. The right-most columns give the calculated concentration values from the linear regression curves.

Eight standard curves were produced over a seven-week period. There was no significant trend in the slopes of the high or low standard curves, but three out of eight y-intercepts for the low curve were significantly different from zero ($p \leqslant 0.02$). At 0.17 μg/ml, six out of seven standard concentrations were within 10% of theory; at 0.35 μg/ml five out of six were within 10% of theory.

TABLE I

LINEARITY OF CLINDAMYCIN STANDARD CURVE IN HUMAN PLASMA

CLD stock: 2 mg per 10 ml water × 0.864 (purity factor) = 0.173 mg/ml.

CLD spiked (μl)	Spiked concentration (μg/ml)	Peak-height ratio (CLD/TRI)	Calculated concentration (μg/ml)		
			Full curve*	Split curve**	
0	0	0	−0.05	−0.08	−0.04
0.20	0.17	0.0264	0.19	0.16	
0.40	0.35	0.0529	0.43	0.40	
0.60	0.52	0.0662	0.55	0.53	
1.0	0.86	0.0938	0.80	0.78	
2.0	1.73	0.2019	1.78	1.76	1.79
4.0	3.46	0.3861	3.44		3.45
8.0	6.91	0.7645	6.87		6.87
12.0	10.4	1.1440	10.3		10.3
16.0	13.8	1.5411	13.9		13.9

*$y=0.1105x+0.0055$, $r^2=0.9998$.

**Low curve: $y=0.1100x+0.0084$, $r^2=0.9998$; high curve: $y=0.1107x+0.0041$, $r^2=0.9998$.

Selectivity

Under the above chromatographic conditions, no endogenous peaks interfere with CLD or TRI in the following biological fluids: human plasma, serum and urine; rabbit serum; dog plasma and pig plasma. A number of drugs and CLD analogues were also injected into the system (Table II), both to seek potential internal standards and to note any interfering compounds. No interferences were observed. No supply of N-demethyl-CLD, an active metabolite, was available for evaluation. N-Desmethyl-CLD did not interfere in plasma samples collected from volunteers given CLD. Based on the behavior of the other, structurally related moieties it can be inferred that the metabolite would not have interfered with the peaks of interest, if present.

TABLE II

SELECTIVITY: RETENTION TIMES OF OTHER COMPOUNDS

Class of drug	Compound	Retention time (min)
Cephalosporins	Cefoperazone	< 1.5
	Cefotaxime	< 1.5
	Cephalothin	< 1.5
Xanthines	8-Chlorotheophylline	1.1
Penicillins	Ticarcillin	1.3
	Mezlocillin	16.4
Sedative-hypnotics	Phenobarbital	6.3
	Desmethyldiazepam	15.9
	Diazepam	3.2
	Oxazepam	19.3
	Triazolam (internal standard)	11.3
Clindamycin analogues	Clindamycin B	3.8
	Clindamycin phosphate	< 1.5
	N-Demethyl-(4-pentyl)-clindamycin	< 1.5
	Clindamycin	8.4

TABLE III

INTRA-DAY PRECISION FOR CLINDAMYCIN IN PLASMA

	Spiked concentration (μg/ml)	Found concentration (mean $\pm$ S.D.) (μg/ml)	Coefficient of variation (%)
High	6.91	6.94 ± 0.286	4.13
Medium	2.10	2.13 ± 0.051	2.41
Low	0.69	0.66 ± 0.031	4.66

TABLE IV

INTER-DAY PRECISION FOR CLINDAMYCIN IN PLASMA

	Spiked concentration (μg/ml)	Found concentration (mean $\pm$S.D.) (μg/ml)	Coefficient of variation (%)
High	6.91	6.94 ± 0.230	3.32
Medium	2.10	2.20 ± 0.122	5.52
Low	0.69	0.69 ± 0.041	5.71

TABLE V

RECOVERY OF CLINDAMYCIN FROM HUMAN PLASMA

	Concentration (μg/ml)	Peak-height ratio		Recovery* (%)
		Water sample	Plasma sample	
Low	0.86	0.1361	0.1555	118
Medium	4.32	0.7430	0.7494	101
High	8.64	1.448	1.392	96

*Recovery $= \frac{\text{peak-height ratio plasma sample}}{\text{peak-height ratio water sample}} \times 100\%$.

Variation

Intra-day and inter-day variation of the method for low (0.69 μg/ml), medium (2.1 μg/ml) and high (6.9 μg/ml) plasma concentrations were determined using replicate spiked samples. The ranges for the coefficient of variation (C.V.) were between 2.4 and 5.7%, as shown in Tables III and IV.

Recovery

Recovery data were obtained by analysis of spiked samples prepared as described, except that the internal standard was not added until the nitrogen evaporation step. Peak-height ratios in water and plasma were compared as follows:

$$\text{Recovery} = \frac{(\text{peak-height ratio for CLD in plasma})}{(\text{peak-height ratio for CLD in water})} \times 100\%$$

The recovery range was from 96 to 118%, as shown in Table V. This procedure was repeated twice, with the same recovery results. Both plastic and glass labware were used in the method.

Stability

No apparent degradation of CLD in samples spiked at low to high concentrations in human plasma stored at $-20°$C was noted for up to 56 days, as is shown in Table VI. Ongoing studies indicate that samples stored at $-20°$C suffer approximately 10% degradation in one year and 25–50% degradation in two years.

TABLE VI

STABILITY OF CLINDAMYCIN IN HUMAN PLASMA AT −20°C

Day	Clindamycin concentration (μg/ml)		
	0.69 μg/ml (low)	2.16 μg/ml (medium)	6.91 μg/ml (high)
0	0.74	2.20	7.18
2	0.74	2.15	6.79
14	0.73	2.43	7.20
15	0.72	2.25	6.99
21	0.71	2.19	6.52
22	0.73	2.12	6.28
31	0.66	2.20	7.02
48	0.75	1.98	6.72
56	0.71	2.16	7.05

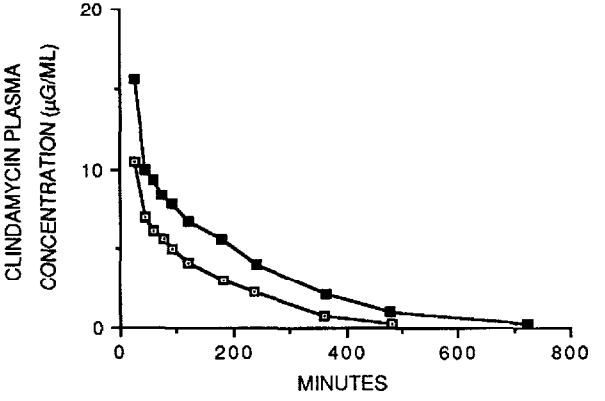

Fig. 3. Concentration versus time plot for clindamycin in a healthy volunteer, following 600-mg (□) and 1200-mg (■) intravenous doses.

Clinical study

A cross-over study in which six healthy subjects were given, by intravenous infusion over a 30-min period, 600, 900 and 1200 mg CLD has been completed by our group. Fig. 3 is a representative concentration versus time plot for the 600 and 1200-mg dose in one of these subjects.

DISCUSSION

Initial investigation revealed that CLD, a highly polar compound, would come off a reversed-phase column too quickly to clear the high large endogenous plasma peaks typical of low-wavelength UV detection. Addition of ion-pairing reagents both lengthened retention times and contributed to peak symmetry. A promising initial combination of sodium dodecyl sulfate (SDS) with a C_8 column allowed the drug to clear the plasma peaks, but peaks were not yet symmetrical. SDS

needed to be purified, and the large concentrations required (2 g/l of solvent) shortened column life considerably. A better system proved to be TMA on a C_{18} column. The combination allowed for a two-fold reduction of the acetonitrile required for the mobile phase. The pH was tested for optimum separation; using phosphoric acid the optimum was found to be pH 6.7.

Wavelengths from 220 to 195 nm were tested with a standard spectrophotometer and the Kratos detector. With the Kratos, we found that the 198-nm setting gave a two-fold increase in the peak signal over the 204-nm setting and a ten-fold increase over the 214-nm setting. Below 198 nm, increased baseline noise overcame any advantages of increased peak height.

At 198 nm, problems with oxygen quenching and temperature-drift instability become much greater, but these were partially overcome by the use of a constant stream of dry, purified nitrogen through the detector's monochromator and a constant gentle bubbling of pure helium through the mobile phase. Because of temperature dependency, the column and surrounding stainless-steel tubing were wrapped with insulative materials such as styrofoam. Best results were obtained on automated overnight runs, when activity in the laboratory and demand on circuitry were minimal.

The internal standard, TRI, was selected primarily because no endogenous plasma peaks of the listed biological fluids interfered with TRI. TRI also has a better peak shape than the other compounds tested as possible internal standards. In this method, TRI is merely correcting for system variability. Since the method using TRI requires no extraction or derivatization, this chemically unrelated compound may be as appropriate as internal standard as other, more closely related species.

The Waters Pro 840 chromatographic system, with its sophisticated data collection, storage and manipulation software programs, allowed for a more accurate rendering of chromatograms than was possible with other integrators and recorders currently in use at our facilities. All baselines were forced "by hand" using a special scanning program, which allowed us to even eliminate noise associated with the M-510 pump.

CONCLUSION

In conclusion, the use of a low-wavelength setting combined with simple removal of oxygen from critical areas of the system allows for accurate, precise and reliable measurement of CLD concentrations in human plasma for up to 12 h after administration of an intravenous infusion of 1200 mg to healthy volunteers. The HPLC method described here has significant advantages over other techniques currently used for measuring CLD in biological fluids. It is fast, simple and requires no extraction.

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