Journal of Chromatography, 571 (1991) 199–208
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6047

Simultaneous determination of clarithromycin and 14(*R*)-hydroxyclarithromycin in plasma and urine using high-performance liquid chromatography with electrochemical detection

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(First received March 26th, 1991; revised manuscript received June 20th, 1991)

ABSTRACT

A sensitive method for the simultaneous high-performance liquid chromatographic determination of clarithromycin and its active metabolite in plasma and urine is described. Alkalinized samples were coextracted with an internal standard and analyzed on a C_8 column using electrochemical detection. Recoveries were $\geq 85\%$ and consistent. Standard curves for plasma were linear in the range 0–2 μg/ml for both compounds ($r > 0.99$), with limits of quantification of ~10.03 μg/ml (0.5-ml sample). Within-day and day-to-day precision were good, with coefficients of variation mostly within ±5%; accuracy for both compounds were routinely within 90–110% of theoretical values. Standard curves for urine were linear in the range 0–100 μg/ml with limits of quantification of 0.5 μg/ml (0.2-ml sample). Urine assays also had similar within-day and day-to-day precisions and accuracy.

INTRODUCTION

Clarithromycin (6-O-methylerythromycin A) (Fig. 1), a novel macrolide with a broad spectrum of antibacterial activity [1–5], is currently under development by

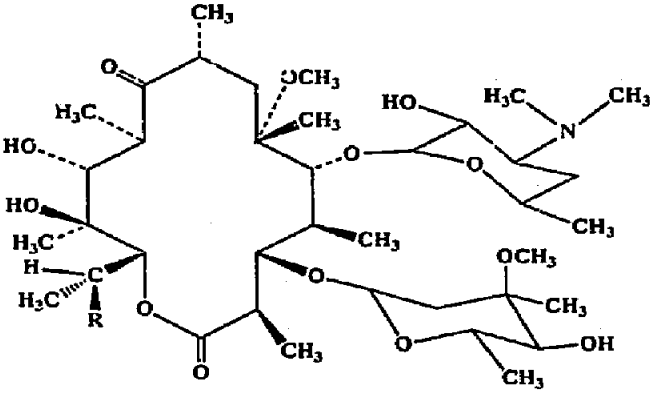

Fig. 1. Chemical structures of clarithromycin (R = H) and 14(*R*)-hydroxyclarithromycin (R = OH).

0378-4347/91/$03.50

Abbott Labs. 14(*R*)-Hydroxy-6-O-methylerythromycin A (14(*R*)-hydroxyclarithromycin, Abbott 62671) has been recovered in human urine and identified as one of the major metabolites of clarithromycin. Like the parent drug, it has been shown to have substantial antimicrobial acitivity [6]. To measure the circulating levels of the parent drug and its metabolite, as well as their extents of recovery from urine, a suitable method for measuring the two compounds in plasma and urine was needed. Previously, erythromycin derivatives were shown to elicit good electrochemical responses using a porous graphite electrode [7,8]. Since this mode of detection was also appropriate for clarithromycin and its 14-hydroxylated metabolite, we developed a sensitive method to measure both compounds simultaneously in biological fluids using high-performance liquid chromatography with electrochemical detection (HPLC–ED).

EXPERIMENTAL

Chemicals

All solvents were HPLC grade and reagents were analytical grade from Fisher (Fairlawn, NJ, USA) or Mallinckrodt (St. Louis, MO, USA). Clarithromycin, erythromycin A 9-O-methyloxime (internal standard, Abbott 41036), and erythromycin B (Abbott 24091) were provided by Abbott Labs. (Abbott Park, IL, USA). 14(*R*)-Hydroxyclarithromycin, descladinosylclarithromycin, 14(*S*)-hydroxyclarithromycin, 14(*R*)-hydroxydescladinosylclarithromycin, and another metabolite designated M-7 (empirical formula $C_{38}H_{69}NO_{14}$) were obtained from Taisho (Tokyo, Japan).

Chromatographic system

Solvent delivery was accomplished using a standard low-pulse HPLC pump (Waters M-6000A, Millipore, Milford, MA, USA). A pulse dampener (Lo-Pulse, SSI, State College, PA, USA) was used to minimize pump noise. Coulometric detector (Model 5100A, Environmental Sciences Assoc., Bedford, MA, USA) was used with the electric potential of the screening electrode at about +0.5 V and the working electrode at about +0.78 ± 0.04 V. The signal output was monitored using a computing integrator (SP-4200, Spectra-Physics, San Jose, CA, USA) or computer data system (Chromatography Data System, Nelson Analytical, Cupertino, CA, USA).

Several octyl-bonded (C_8) reversed-phase HPLC columns, including 5 μm Spherisorb, Nucleosil (both from Alltech Assoc., Deerfield, IL, USA) and Sepralyte (Analytichem International, Harbor City, CA, USA) were suitable. The mobile phase for Nucleosil C_8 (5 μm, 250 mm × 4.6 mm I.D.) consisted of acetonitrile–methanol–water (39:9:52, v/v) containing 0.04 *M* NaH_2PO_4 and NaOH to bring the pH to 6.8. The flow-rate was set at 1.2–1.4 ml/min. Slight modifications of the organic contents and the pH in the eluent were sometimes needed to achieve satisfactory resolution because of column-to-column variabilities in retentivity for macrolide derivatives.

Extraction procedure for plasma

Procedures using 0.5 ml of plasma, as described here, are representative. A 0.5-ml aliquot of plasma was transferred to a clean tube. About 750 ng of internal standard (*e.g.* 75 μl of 10 μg/ml internal standard in a 1:1, v/v, acetonitrile–water mixture), 0.2 ml of sodium carbonate solution (0.1 *M*), and 3 ml of ethyl acetate–hexane (1:1, v/v) were added to each tube. The contents of the tubes were stirred vigorously for 1 min and centrifuged at 800 *g* for 5 min. The organic layer was transferred to a clean tube, and the solvent was evaporated to dryness at 45°C under a stream of air. The residue was dissolved in 200–400 μl of 50% (v/v) solution of acetonitrile in water, and portions of the solution (20–80 μl) were injected into the HPLC system and analyzed.

Standard curve for plasma samples

Standard solution of clarithromycin and 14-hydroxyclarithromycin were prepared individually in acetonitrile at concentrations of approximately 1 mg/ml; they were refrigerated until used. Usually, 100 μl of each of the standard solutions were mixed with 9.8 ml of normal human plasma to provide a standard solution of both compounds in plasma at 10 μg/ml each. This was serially diluted with normal plasma to provide a set of standard samples containing both compounds. The set used for method validation had concentrations of 0.1, 0.2, 0.5, 1.0, and 2.0 μg/ml each. Standard curves were constructed by weighted (1/concentration) linear regression of the observed peak-height ratios and the concentrations. Calibrators were prepared similarly by dilution of a separate set of stock solutions of clarithromycin and 14-hydroxyclarithromycin; the stock solutions were different from those used for the standard curves. The calibrators (sometimes referred to as quality control samples) were assayed as the unknown and the results were used to monitor the accuracy of daily analysis.

Extraction procedure for urine samples

The procedure for urine was very similar to that described for plasma. The following procedure using 0.2-ml aliquots of urine is representative. Aliquots (0.2 ml) of urine were transferred individually to clean test tubes. To each tube, the following were added: 3 μg of internal standard (usually 300 μl of internal standard at 10 μg/ml in 50% aqueous acetonitrile), 0.1 ml of sodium carbonate solution (0.1 *M*), and 3–4 ml of ethyl acetate–hexane solution (1:1, v/v). The tubes were stirred vigorously for 1 min on a vortex mixer (American Scientific Supply, McCaw Park, IL, USA) and centrifuged at 800 *g* for 5 min. The organic layer (2–3.5 ml) was transferred to a clean tube and the solvent was removed by evaporation at about 45°C under a stream of air. The residues were dissolved in 0.8–1.2 ml of 50% aqueous acetonitrile, and portions of this solution were injected into the HPLC system.

Standard curves for urine were typically constructed with standards at concentrations of 0, 1.0, 2.5, 5.0, 10, 25, 50, and 100 μg/ml for each compound. Cali-

brators were prepared as usual; the stock solutions were different from those for the standard curve samples.

RESULTS AND DISCUSSION

Erythromycin A 9-O-methyloxime, the internal standard, gave two chromatographic peaks, including a minor peak due to impurity, but both were well resolved from clarithromycin and 14-hydroxyclarithromycin. This compound was stable under the work-up conditions. The extraction recoveries for all three compounds were consistent and estimated to be better than 85%. It was noted that 14-hydroxyclarithromycin could be readily converted to M-7 (an anhydro derivative) when its solution in ethyl acetate–hexane was heated to 45°C in the presence of sodium carbonate. This emphasized that care should be taken to avoid transferring any aqueous phase along with the organic solution during the sample extraction. With such care, no significant formation of M-7 was observed.

Fig. 2 depicts typical chromatograms from normal human plasma, plasma spiked with both clarithromycin and 14-hydroxyclarithromycin at 0.5 μg/ml each, and plasma from a subject 2 h after he received a 600-mg oral dose of clarithromycin. A Nucleosil C_8 (5 μm, 250 mm × 4.6 mm I.D.) column was used.

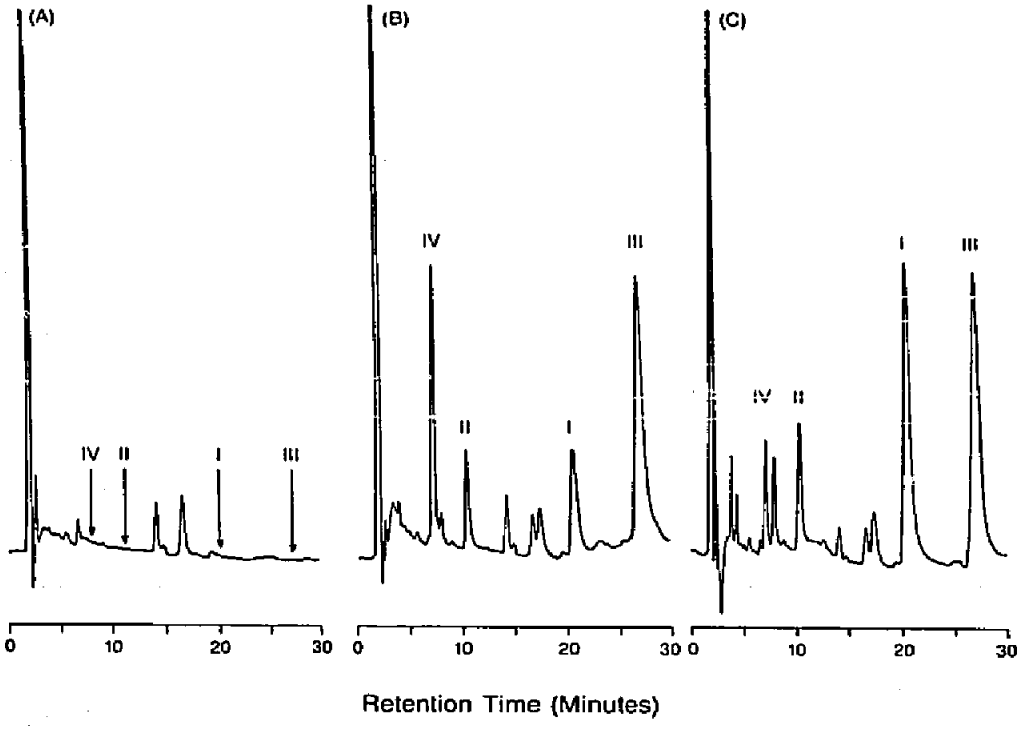

Fig. 2. Typical chromatograms from plasma samples using a Nucleosil C_8 column. (A) Normal human plasma (NHP); (B) NHP spiked with clarithromycin (I), 14-hydroxyclarithromycin (II), internal standard (III), and descladinosylated clarithromycin (IV); (C) plasma collected from a subject 2 h after administration of a 600-mg oral dose of clarithromycin.

There were no apparent interferences at the retention times of parent drug, the internal standard and 14-hydroxyclarithromycin from normal pooled human plasma. In chromatogram B, descladinosylclarithromycin was included to show its retention time (7.2 min). A peak at this retention time was also observed in the chromatogram of post-clarithromycin plasma, suggesting the presence of this metabolite in human plasma. Usually, one unknown late-eluting peak appeared at about 60 min under the above conditions. The retention of this peak was monitored periodically to allow adjustment of the injection intervals (usually 30–35 min).

Under the proposed HPLC conditions, the available metabolites such as descladinosylclarithromycin (described above), 14(*S*)-hydroxyclarithromycin, 14(*R*)-hydroxydescladinosylclarithromycin (M-6), and an anhydro derivative

TABLE I

STANDARD CURVE DATA FOR HPLC–ED ASSAY OF CLARITHROMYCIN AND ITS 14-HYDROXY METABOLITE IN PLASMA AND URINE

Theoretical concentration (μg/ml)	Calculated concentration			
	Clarithromycin		Metabolite	
	μg/ml	% of theory	μg/ml	% of theory
Plasma				
0.0	(0.01)	–	(0.00)	–
0.1	0.101	101	0.106	106
0.2	0.195	97.5	0.202	101
0.5	0.506	101	0.455	91.0
1.0	0.999	99.9	0.994	99.4
2.0	2.00	100	2.04	102
(0.80)[a]	0.833[c]	104	0.829[c]	104
(0.40)[b]	0.394[c]	98.5	0.396[c]	99.0
Urine				
1.0	1.13	113	1.02	102
2.5	2.45	98.0	2.39	95.6
5.0	4.90	98.0	4.85	97.0
10.0	9.50	95.0	10.6	106
25.0	23.8	95.2	25.1	100
50.0	49.3	98.6	49.2	98.4
100.0	102.4	102	100.3	100
(40)[a]	39.2[c]	98	40.0[c]	100
(20)[b]	21.7[c]	109	21.6[c]	108

[a] High calibrator.
[b] Low calibrator.
[c] Concentrations were calculated based on the standard curves.

(M-7) were all well resolved from clarithromycin, 14-hydroxyclarithromycin, and internal standard. Typical relative retentions of these compounds using a Nucleosil C_8 column were: clarithromycin, 1.00 (retention volume of about 20.2 ml); 14-hydroxyclarithromycin, 0.62; 6-O-methylerythromycin B, 1.27; internal standard, 1.18; N-desmethylclarithromycin, 0.75; descladinosylclarithromycin, 0.48; M-6, 0.41; and M-7, 0.45. These values varied somewhat when different columns were used or when the eluent was modified (especially the pH), but in general the compounds were eluted in the same order.

Table I illustrates representative standard curve data for the determination of both clarithromycin and 14-hydroxyclarithromycin in human plasma and urine. The best-fit equation for clarithromycin in plasma was $y = 0.7680x - 0.0112$, with the y-intercept of -0.0112 not significantly different from zero. The correlation coefficient was 0.9999, showing excellent linearity. The results for the two calibrators were 98.5 and 104% of the theoretical values, supporting the accuracy of the method.

Similarly, the least-squares best-fit equation for 14-hydroxyclarithromycin was $y = 0.7943x - 0.0036$, with the y-intercept of -0.0036 not significantly different from zero. The correlation coefficient was 0.9989. Results for the calibrators indicated good accuracy of the assay for 14-hydroxyclarithromycin (99–104% of theoretical values) in plasma. The estimated limit of quantification (LOQ) of the proposed procedure was 0.03 μg/ml for either clarithromycin or 14-hydroxyclarithromycin using 0.5 ml of plasma. This LOQ was confirmed in a separate experiment using a calibrator with nominal concentration of either compound at 0.04 μg/ml. The mean assay result was 0.038 μg/ml ($n = 5$) for either clarithromycin or 14-hydroxyclarithromycin, with a coefficient of variation (C.V.) of ± 12%, verifying acceptable accuracy and precision at near the LOQ of the method. This LOQ estimate can be routinely achieved if good detector sensitivity is maintained and sample background is not abnormally high.

To demonstrate the within-day precision, two calibrators were assayed in triplicate using the proposed method. The mean (± S.D.) calculated concentrations for the low calibrator (nominal concentration: 0.2 μg/ml for each compound) were 0.203 ± 0.002 μg/ml for the parent drug and 0.200 ± 0.006 μg/ml for the metabolite. For the high calibrator (1.0 μg/ml for each compound), the mean (± S.D.) assayed concentrations were 1.02 ± 0.034 μg/ml for the parent drug and 1.02 ± 0.037 μg/ml for the metabolite. The C.V.s were all within ± 3.6%.

Table II shows the day-to-day precision and accuracy for the assay of both clarithromycin and 14-hydroxyclarithromycin in plasma. A set of two calibrators (low and high) were analyzed over a nine-day period. The mean assay results for parent drug were 99.3 and 103% of theoretical values, and the C.V.s were within ± 3.4%. Similarly, the mean calculated concentrations for 14-hydroxyclarithromycin from the two calibrators were 101 and 104% of theoretical values, respectively, with C.V.s being within ± 5.1%.

Fig. 3 illustrates the chromatograms from urine samples. Normal pooled hu-

TABLE II

DAY-TO-DAY PRECISION AND ACCURACY FOR THE ASSAY OF CLARITHROMYCIN AND ITS 14-HYDROXY METABOLITE IN PLASMA AND URINE

Compound	Calculated concentration (μg/ml)		C.V. (%)	Percentage of theory
	Mean	Range		
Plasma (n = 6)[a]				
Low calibrator				
Parent	0.397	0.38–0.42	3.4	99.3
Metabolite	0.403	0.38–0.42	5.1	101
High calibrator				
Parent	0.823	0.81–0.86	2.4	103
Metabolite	0.833	0.79–0.88	3.8	104
Urine (n = 8)[b]				
Low calibrator				
Parent	21.1	19.5–22.3	4.6	105
Metabolite	21.0	19.6–21.9	3.9	105
High calibrator				
Parent	40.1	38.7–41.2	2.1	100
Metabolite	40.2	38.8–41.6	2.3	100

[a] Theoretical concentrations for both compounds were 0.40 μg/ml each in low calibrator and 0.80 μg/ml each in high calibrator.

[b] Theoretical concentrations of both compounds were 20.0 μg/ml each for low calibrator and 40 μg/ml each for high calibrator.

man urine showed no apparent interferences at the retention times of clarithromycin, 14-hydroxyclarithromycin, and internal standard. Standard curve data in urine are shown in Table I. The best-fit equation for clarithromycin was $y = 0.08017x - 0.0200$, with the y-intercept of -0.0200 not significantly different from zero. The data showed excellent linearity with a correlation coefficient of 0.9995. The results of the calibrators assayed using the above curve were in good agreement with theoretical values (98 and 109%). Similarly, an equation of $y = 0.07508x + 0.0230$ was obtained for 14-hydroxyclarithromycin, in which the y-intercept of 0.0230 was not significantly different from zero and the correlation coefficient was 0.9998. The calculated concentrations for the calibrators were 108% of theoretical values at the 20 μg/ml level and 100% at the 40 μg/ml for either clarithromycin or 14-hydroxyclarithromycin.

Within-run precision of the method was assessed by analyzing two urine calibrators with clarithromycin and 14-hydroxyclarithromycin at 10 and 40 μg/ml in quadruplicate. The mean analytical recoveries at 10 μg/ml were 97.3% for clarithromycin (C.V. = ±2.1%) and 104.8% for 14-hydroxyclarithromycin (C.V. =

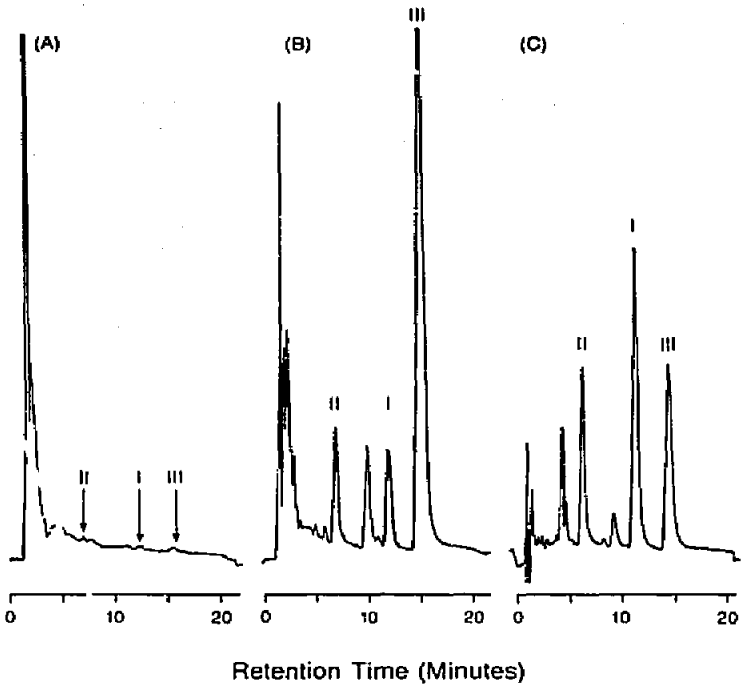

Fig. 3. Typical chromatograms for urine samples. (A) Normal human urine (NHU); (B) NHU spiked with clarithromycin (I) and 14-hydroxyclarithromycin (II) at 2.5 μg/ml each, worked up with internal standard (III); (C) human urine collected in the first hour after a single 400-mg oral dose of clarithromycin.

±0.8%). Those at 40 μg/ml were 95.3% for clarithromycin (C.V. = ±1.2%) and 103.1% for 14-hydroxyclarithromycin (C.V. = ±1.5%).

Day-to-day precision and accuracy data were generated by analyzing two urine calibrators over a seventeen-day period (eight assays). The results, as listed

TABLE III

RESULTS FROM PLASMA QC SAMPLES GENERATED DURING ROUTINE ANALYSES

Concentration (μg/ml)	Percentage of theory	C.V. (%)	*n*
Clarithromycin			
0.18	93.6	±7.6	24
0.72	99.4	±5.9	29
3.70	98.4	±6.0	28
14(R)-Hydroxyclarithromycin			
0.15	100	±7.3	27
0.60	103	±6.5	29
2.94	105	±6.6	28

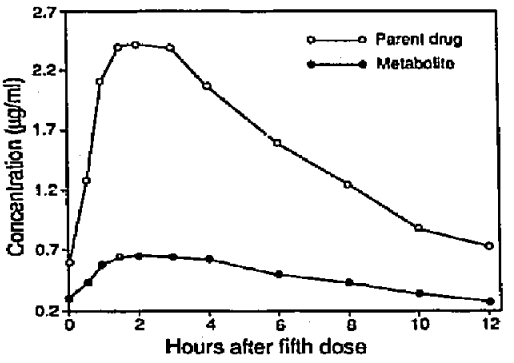

Fig. 4. Mean plasma concentrations of clarithromycin and 14(*R*)-hydroxyclarithromycin in healthy adult men after a fifth 500-mg oral dose of clarithromycin (taken twice daily).

in Table II, indicated that good accuracy could be maintained from day to day. The projected LOQ of the assay procedure was about 0.5 µg/ml, using 0.2 ml of urine. This could easily be improved by using a larger volume of urine or by injecting greater volumes of the extracts.

No changes in clarithromycin concentrations were detected in human plasma after two years of frozen storage and in human urine after one year, supporting the stability of the compound in both matrices. At least 85% of 14-hydroxyclarithromycin was recovered after six months of frozen storage in either matrix, showing adequate stability for this metabolite, as well. The standards in plasma and urine were stable for at least five freeze–thaw cycles and for at least 5 h at room temperature. The extracted samples were stable for at least 30 h at ambient temperature in the autosampler.

The proposed method has been used in several pharmacokinetic studies. During these studies, we have assayed numerous clinical samples for both clarithromycin and 14-hydroxyclarithromycin; the predose plasma and urine samples have verified the specificity of the method. Table III shows the typical results for plasma quality control (QC) samples obtained during one of those studies, using standard curves in the range 0.04–4 µg/ml for either parent drug or the metabolite. During these routine analyses, the results for individual QC samples in plasma and urine were mostly within 90—110% of theory, and the between-day precision were generally within ±7%. An example for the use of this assay in human pharmacokinetic studies is shown in Fig. 4, which illustrates the mean plasma concentrations of clarithromycin and the 14(*R*)-hydroxylated metabolite from twelve healthy adult men after the fifth 500-mg oral dose of clarithromycin, given twice daily. Under such conditions, the harmonic mean terminal plasma half-life was about 4 h for clarithromycin.

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