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MICRO-METHOD FOR THE DETERMINATION OF ROXITHROMYCIN IN HUMAN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING ELECTROCHEMICAL DETECTION

F.M. DEMOTES-MAINAIRD*, G.A. VINÇON, C.H. JARRY and H.C. ALBIN

Department of Clinical Pharmacology, Hôpital Pellegrin, 33076 Bordeaux (France)

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SUMMARY

A simple and sensitive high-performance liquid chromatographic micro-method for the determination of roxithromycin in human plasma and urine is described. A dichloromethane extract of the sample was chromatographed on a C_{18} reversed-phase column with acetonitrile–83 m*M* ammonium acetate–methanol (55.23:22, v/v) adjusted to pH 7.5 with acetic acid as the mobile phase. Roxithromycin and the internal standard, erythromycin, were detected by dual coulometric electrodes operated in the oxidative screen mode. The applied cell potential of the screen electrode was set at +0.7 V and the sample electrode at +0.9 V. The intra- and inter-assay coefficients of variation were $\leqslant 7.0\%$. The detection limit (signal-to-noise ratio=3) was 0.1 μg/ml for both plasma and urine. A study of drug stability during sample storage at 4, 20 and 37°C showed no degradation of roxithromycin. The method is convenient for clinical monitoring and pharmacokinetic studies.

INTRODUCTION

Roxithromycin (Fig. 1) is a new macrolide antibiotic with an antibacterial spectrum of activity similar to that of erythromycin [1,2], but it has different pharmacokinetic properties. Roxithromycin is well absorbed and gives plasma levels that are more sustained and higher than those of erythromycin [3]. Therefore, it should be effective at lower doses with less frequent administration, which is of considerable clinical interest. This efficacy can be enhanced by the ability to determine roxithromycin concentrations in biological fluids

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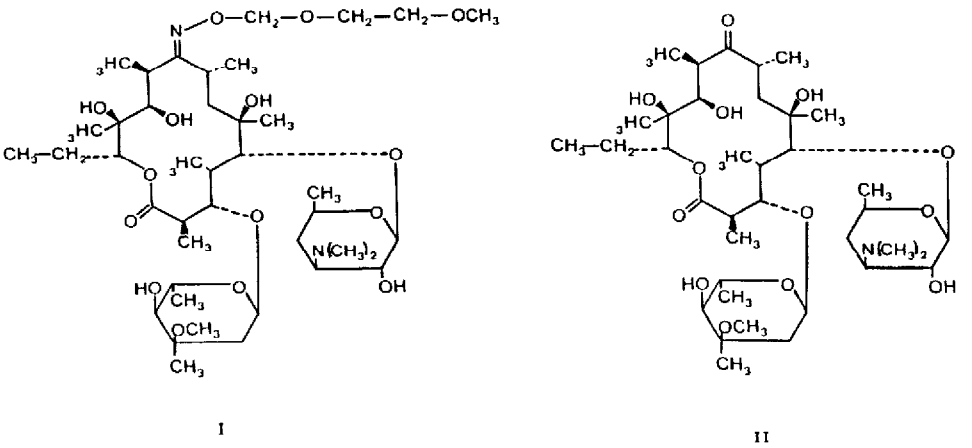

Fig. 1. Structures of roxithromycin (I) and erythromycin (II).

accurately and rapidly, thereby ensuring that bactericidal levels are achieved and maintained.

At present, only a microbiological assay for the determination of roxithromycin in plasma, urine and milk has been described [4]. Most pharmacokinetic data have been obtained using this method with *Sarcina lutea* as the assay strain. In comparison with bioassay procedures, high-performance liquid chromatography (HPLC) has several advantages, such as rapidity and selectivity, especially when the drug is analysed in the presence of active metabolites or other antibiotics.

Some methods based on reversed-phase HPLC have been developed for the quantitation of the macrolides, particularly erythromycin. UV absorption [5,6], fluorescence [7] and electrochemical [8–11] detection methods have been used.

Like erythromycin, roxithromycin has a very weak UV absorbance in the low UV wavelength range ($\leqslant$235 nm). When using this mode of detection, a large volume of biological fluid (2–3 ml) is needed in order to have sufficient sensitivity. Moreover, these low wavelengths are associated with considerable background noise and large interferences from co-extracted compounds. Tsuji [7] reported a complex fluorimetric determination that required an analytical column maintained at 70°C and special instrumentation for post-column derivatization.

For these reasons, we have developed a rapid and sensitive HPLC method with electrochemical detection. The electrochemical response is due to oxidation of the tertiary amino group present in the macrolide chemical structure (Fig. 1). This paper describes a micro-method that allows the determination of roxithromycin in plasma and urine using the same chromatographic conditions. The stability of these macrolides during sample storage was studied.

EXPERIMENTAL

Chemicals

Roxithromycin (RU 28965) and erythromycin base were supplied by Roussel Uclaf (Paris, France). Acetonitrile was supplied by Rathburn (Walkerburn, U.K.) and methanol by Prolabo (Paris, France). Water was deionized and doubly glass-distilled. All other chemicals (ammonium acetate, acetic acid, sodium monophosphate, sodium diphosphate and dichloromethane) were of analytical-reagent grade (E. Merck, Darmstadt, F.R.G.).

HPLC conditions

The chromatographic system was composed of a solvent delivery pump (Type 364.000; Knauer Berlin, F.R.G.), a manual injector (Model U6K; Waters Assoc., Milford, MA, U.S.A.) and a μBondapak C_{18} reversed-phase column (30 cm × 3.9 mm I.D., particle size 10 μm) (Waters Assoc.). A dual-electrode electrochemical detector (ESA Model 5100 A, Coulochem Environmental Sciences, Bedford, MA, U.S.A.) was connected to an Omniscribe recorder (Houston Instruments, Houston, TX, U.S.A.). This electrochemical detector was equipped with an ESA Model 5020 guard cell (1.0 V) placed in-line before the injector in order to electrolyse components of the mobile phase. The Model 5010 dual-electrode cell was operated in the oxidative screen mode. The applied cell potential of the screen electrode E_1 was set at +0.7 V and the sample electrode E_2 at +0.9 V. To protect the graphite electrodes, in-line filters (0.5-μm carbon filter, ESA No. 5100-A-50) were placed before the guard cell and the analytical cell.

The mobile phase was acetonitrile–83 m*M* ammonium acetate–methanol (55:23:22, v/v), the pH being adjusted to 7.5 with acetic acid. Before use, the mobile phase was filtered through a Durapore 0.22-μm filter (Millipore, Milford, MA, U.S.A.). The flow-rate was 1.0 ml/min at ambient temperature (20–25°C).

Standard solutions

Stock solutions of roxithromycin and erythromycin (1 mg/ml) were prepared in methanol and could be stored at −20°C for twelve months without degradation. Appropriate dilutions of the roxithromycin stock solution were made in drug-free human plasma or urine to provide concentrations of 1–20 μg/ml. Urine samples were diluted 1:2 (v/v) with isotonic sodium chloride solution. The internal standard concentration was 10 μg/ml in doubly distilled water.

The chromatograms were recorded at a chart speed of 0.25 cm/min and peak-height ratios of roxithromycin to erythromycin were measured. When the response of electrode decreased, the guard and analytical cells were flushed with 6 *M* nitric acid for 20 min according to the manufacturer.

Sample preparation

Aliquots of plasma or diluted urine (200 μl) were pipetted into 10-ml glass extraction tubes. After the addition of 100 μl of internal standard (10 μg/ml), 600 μl of phosphate buffer (pH 9) and 3 ml of dichloromethane, each tube was stoppered and shaken for 10 min. Following centrifugation at 2000 *g* for 5 min, the upper layer was discarded. The organic phase (2.5 ml) was transferred into another tube and evaporated to dryness at ambient temperature under a stream of nitrogen. The residue was reconstituted with 50 μl of methanol and vortexed for 10 s to facilitate dissolution of the compounds. A 15-μl aliquot of this sample was injected into the chromatograph.

RESULTS AND DISCUSSION

LC separation

Typical chromatograms of plasma and urine samples are shown in Fig. 2. Under the described chromatographic conditions, the retention times of roxithromycin and erythromycin base were 9.8 and 7.0 min, respectively, and these drugs were well resolved from endogenous plasma or urine compounds. The retention times of various macrolides and some drugs with a tertiary amino group present in their molecule are listed in Table I.

As noted by previous investigators [7–11], the pH of the mobile phase was found to have a great effect on the retention time of erythromycin. The same effect was noted with roxithromycin, i.e., the retention time increased when the mobile phase was made alkaline (Fig. 3). At higher pH the electrochemical oxidation of macrolides is facilitated [10]. Further, erythromycin degrades mostly to an enol ether at pH <6.3 [7]. For these reasons, the pH of the mobile phase was maintained at 7.5, the optimum value for separating erythromycin from endogenous compounds. An increase in the percentage of acetonitrile and methanol in the mobile phase decreased the retention times and concentrations of 55 and 22%, respectively, were chosen to give the optimum separation of the peaks with reasonable chromatographic retention times.

The low ionic strength of the buffer used in the mobile phase (83 m*M*) provided adequate conductivity for electrochemical detection and minimized background current from the detector.

Detection

Macrolide antibiotics which contained a tertiary amino group were detectable by electrochemical oxidation, and roxithromycin and erythromycin could be detected by this procedure. However, these compounds exhibited a relatively high oxidation potential (Fig. 4), and it was necessary to use an electrochemical detection system with dual coulometric electrodes in the oxidative screen mode. In this mode, many compounds in the extracted plasma and urine samples were irreversibly oxidized at the first electrode E_1 (0.7 V) without a

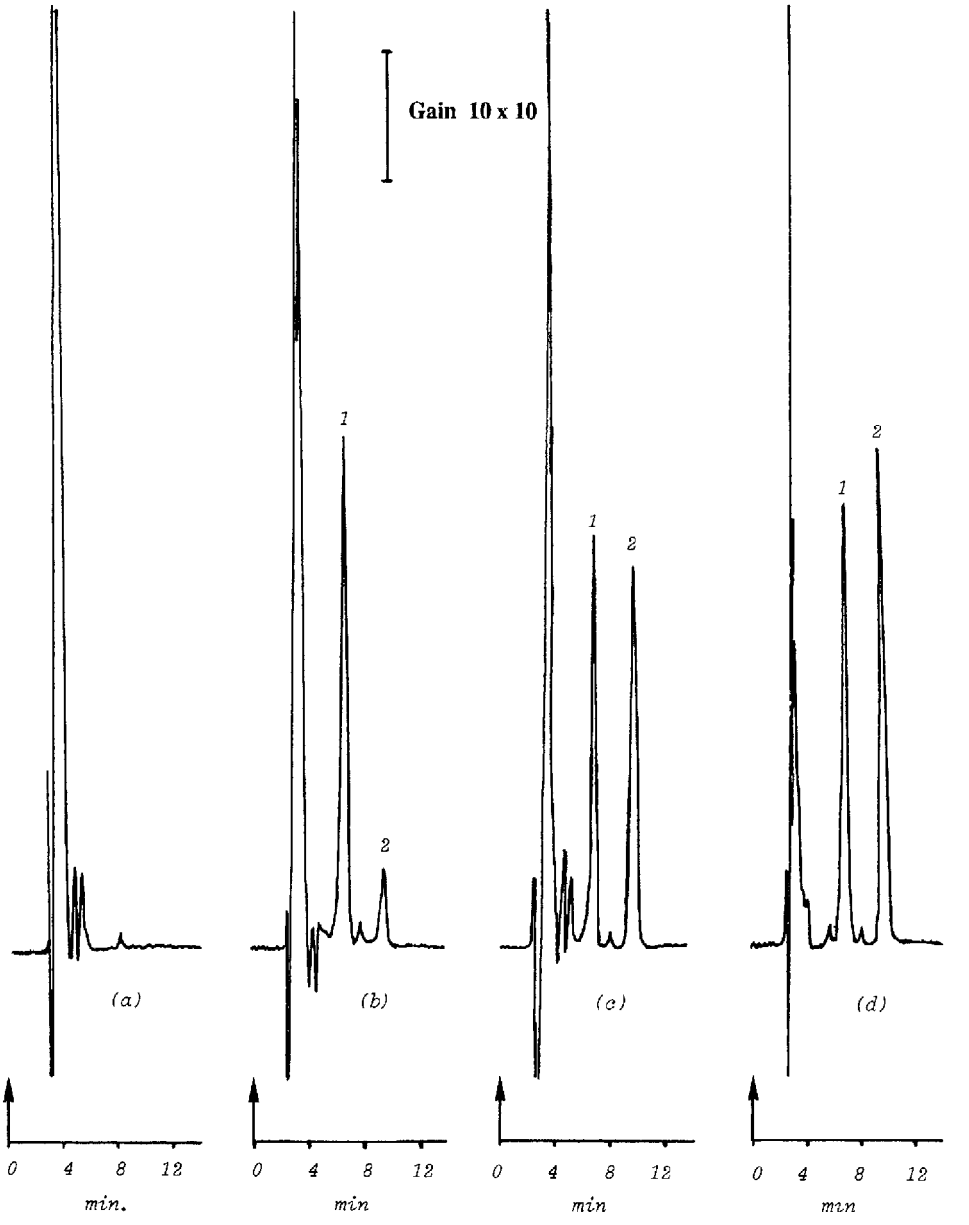

Fig. 2. Chromatograms of (a) human drug-free plasma; (b) human plasma spiked with 0.8 μg/ml; (c) human plasma spiked with 4.9 μg/ml; (d) human urine spiked with 5.7 μg/ml. Peaks 1 = erythromycin; 2 = roxithromycin.

TABLE I

RETENTION TIME OF SOME DRUGS

Amount injected, 500 ng; retention times relative to roxithromycin (9.8 min).

Drug	Relative retention time	Drug	Relative retention time
Lidocaine	0.45	Erythromycin stearate	1.10
Disopyramide	0 60	Imipramine	1.25
Erythromycin	0.71	Amitriptyline	1.40
Erythromycin ethylsuccinate	0 85	Clomipramine	1.45
Spiramycin	0.90	Erythromycin estolate	1.65
Josamycin	0.93		

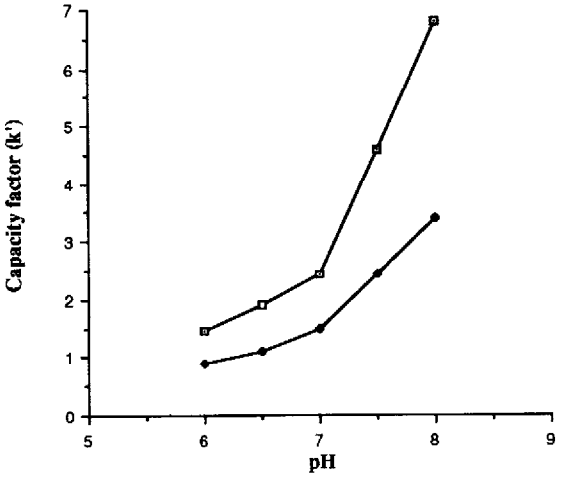

Fig. 3. Effect of pH on the retention of (□) roxithromycin and (●) erythromycin.

decrease in the response to roxithromycin and the internal standard. A potential of +0.9 V was chosen for the second electrode E_2, as significant background noise occurred at the maximum value (+0.95 V).

Linearity

There was a linear relationship between concentration and response up to 25 μg/ml in plasma samples ($r=0.9989$, $n=10$) and diluted urine samples ($r=0.9994$, $n=10$).

Precision and accuracy

The instrumental precision (2.3%) was determined by repeated injection ($n=8$) of 500 ng of roxithromycin. The accuracy and precision of the method were determined on drug-free human plasma samples spiked with roxithro-

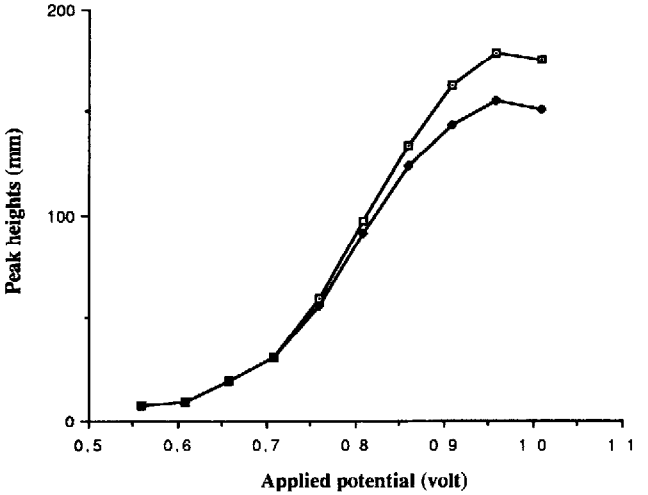

Fig. 4. Hydrodynamic voltammograms of (●) roxithromycin and (□) erythromycin. The on-column amount injected was 0.5 μg. The applied potential of E_1 was increased by 0.05 to 1.0 V and that of E_2 was set at 0.0 V.

TABLE II

ACCURACY AND PRECISION FOR PLASMA SPIKED WITH ROXITHROMYCIN

Spiked concentration (μg/ml)	Intra-assay determination ($n=10$)			Inter-assay determination ($n=10$)		
	Mean (μg/ml)	C.V. (%)	Accuracy[a] (%)	Mean (μg/ml)	C.V. (%)	Accuracy[a] (%)
2.0	2.05	5.5	102.5	2.0	7 0	100.0
9.5	9.31	1.9	99.7	9.35	2.1	98.4
15.0	15.31	1.6	102.0	14.86	2.2	99.0

[a]Accuracy = (amount found/amount added) · 100.

mycin at different concentrations (2, 9.5 and 15 μg/ml). Accuracy was defined as (amount found/amount added) · 100 (Table II).

Recovery

Plasma samples spiked to a final concentration of 8 μg/ml were extracted with different organic solvents and recoveries of roxithromycin and erythromycin were determined by comparing the response of known amounts of drug (Table III). Dichloromethane was chosen as roxithromycin and erythromycin were well extracted with mean values of 68.2 ± 1.0 and 78.8 ± 2.4%, respectively, and no interfering peak was noted. The detection limit (signal-to-noise ratio = 3) was 0.1 μg/ml for both plasma and urine.

TABLE III

RECOVERIES OF ROXITHROMYCIN AND ERYTHROMYCIN ADDED TO DRUG-FREE HUMAN PLASMA ($n=5$)

Solvent	Recovery (%)	
	Roxithromycin	Erythromycin
Dichloromethane	68.2 ± 1.0	78.8 ± 2.4
Diethyl ether	56.4 ± 1.5	47.5 ± 0.9
Toluene	54.7 ± 3.6	45.8 ± 0.6
Ethyl acetate	41.1 ± 0.6	47.6 ± 3.9
Benzene	34.6 ± 1.0	Interfering peak
Chloroform	27.1 ± 1.6	39.1 ± 2.3
n-Hexane	22.3 ± 2.7	21.5 ± 1.9

Stability

The stability of roxithromycin during sample storage at different temperatures was studied. One plasma sample containing 9 μg/ml was kept for two days at 4, 20 or 37°C. In each instance, fourteen determinations were made during 48 h; the coefficient of variation (C.V.) ($n=14$) was 2.5% (mean ± S.D. = 8.98 ± 0.23 μg/ml) for the sample kept at 4°C, 3.0% (mean ± S.D. = 9.09 ± 0.28 μg/ml) for the sample kept at 20°C and 2.2% (mean ± S.D.

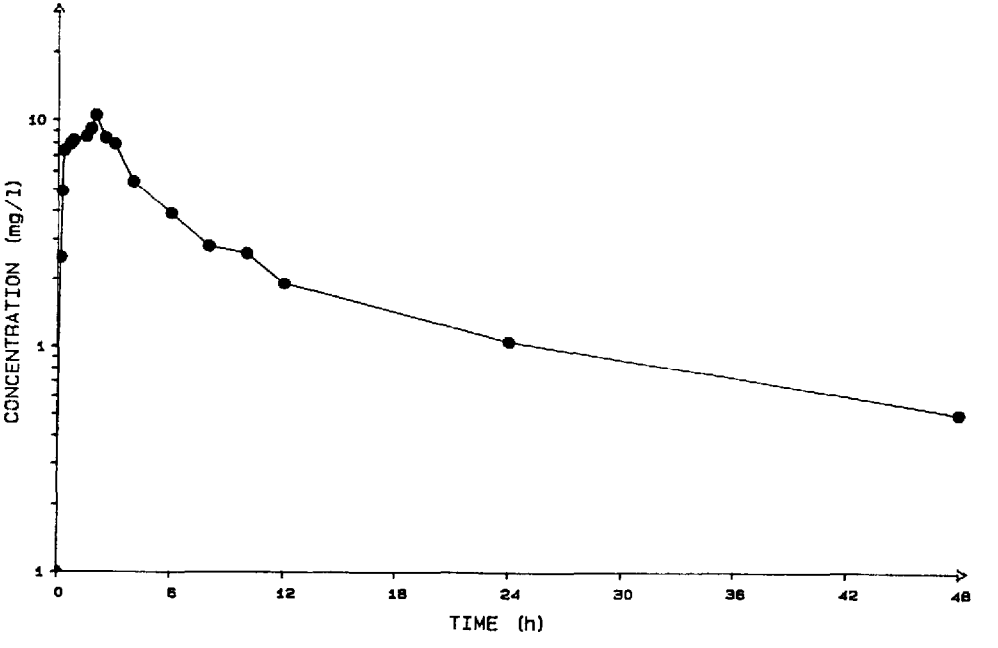

Fig. 5. Plasma levels of roxithromycin after oral administration of 150 mg to a volunteer

$= 9.06 \pm 0.20$ μg/ml) for the sample kept at 37°C. These results show that there was no degradation of roxithromycin.

Application

The method was used for the determination of roxithromycin in plasma from volunteer after administration of 150 mg orally (Fig. 5). The drug was rapidly absorbed and the maximum plasma concentration (10.5 μg/ml) was reached 2 h after dosing. The curve showed a two-phase decrease with a terminal half-life of 14 h. These values agree with previous published studies [3].

CONCLUSION

The method presented here has been used in our laboratory over twelve months and during this period, no change was observed in the chromatographic retention times of roxithromycin and erythromycin. The lifetime of the column was excellent as the performance did not decrease in this period. In order to enhance the detector stability it was necessary to operate the HPLC system continuously and to use the electrochemical detector daily. On repeated injections (> 500) of sample extracts, a decrease in electrode response may occur, but it can be restored by flushing the electrodes with acid for 10 min.

This rapid and sensitive micro-method may be useful for pharmacokinetic studies and for monitoring the drug in patients undergoing treatment, particularly in paediatrics where sample sizes are limited.

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REFERENCES

1 T. Barlam and H.C Neu, Antimicrob. Agents Chemother., 25 (1984) 529.
2 R.N. Jones, A.L. Barry and C. Thornsberry, Antimicrob. Agents Chemother., 24 (1983) 209.
3 H.B. Lassman, S.K. Puri, I. Ho, R. Sabo and M.J. Mezzino, J. Clin. Pharmacol., 28 (1988) 141.
4 A.L. Barry and R.P. Packer, Eur. J. Clin. Microbiol., 5 (1986) 536.
5 Th. Cachet, I.O. Kibwage, E. Roets, J. Hoogmartens and H. Vanderhaeghe, J. Chromatogr., 409 (1987) 91.
6 C. Stubbs, J.M. Haigh and I Kanfer, J. Pharm. Sci., 74 (1985) 1126.
7 K. Tsuji, J. Chromatogr., 158 (1978) 337.
8 M.-L. Chen and W.L. Chiou, J. Chromatogr., 278 (1983) 91.
9 D. Croteau, F. Vallée, M.G. Bergeron and M. Lebel, J. Chromatogr., 419 (1987) 205.
10 G.S. Duthu, J. Liq. Chromatogr., 7 (1984) 1023.
11 L.-G. Nilsson, B. Walldorf and O. Paulsen, J. Chromatogr., 423 (1987) 189.