Determination of macrolides in biological matrices by high-performance liquid chromatography with electrochemical detection

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Abstract

A liquid chromatographic method for the determination of macrolide antibiotics is described using a cyanopropyl column which proved to be as efficient or superior to the normally used apolar reversed-phase columns. The recovery of the macrolides from water and plasma was 80–90%. Using 0.5 ml of plasma, 30 ng/ml of clarithromycin, 50 ng/ml of roxithromycin and 10 ng/ml of azithromycin could be determined with acceptable precision and accuracy. The method has been employed in pharmacokinetic studies in humans for the determination of roxithromycin, clarithromycin and azithromycin in plasma, serum and other biological matrices. The particular selectivity of the cyanopropyl phase may also allow the simultaneous determination of erythromycin and its prodrug esters. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

Macrolide antibiotics are macrocyclic lactones isolated first from *Streptomyces* spp. The common skeleton is a 12–16-membered lactone ring with attached neutral and/or amino sugars [1]. Until the late 1980s the 14-membered macrolide erythromycin, available since 1952, remained the only widely used macrolide antibiotic. However, its absorption after oral administration is highly variable due to instability in the acid environment of the stomach, and resulting plasma concentrations are unpredictable. In the last decade semisynthetic derivatives of erythromycin such as roxithromycin, clarithromycin, and azithromycin (Fig. 1) have been developed which are more stable to acid (c.f. [2]). Therefore, they exhibit better oral bioavailability, and a more favourable pharmacokinetic behaviour.

Besides their activity against common Gram-positive and Gram-negative cocci, these new compounds have gained interest, because of their activity against nonclassical pathogens such as *Helicobacter pylori*
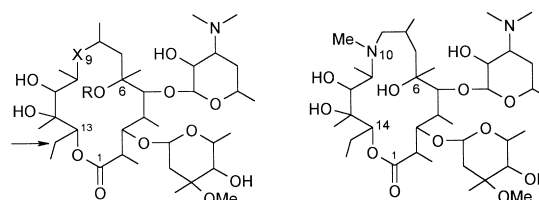
	R	X	
Erythromycin	H	C=O	
Clarithromycin	CH₃	C=O	
Roxithromycin	H	C=N-OCH₂OCH₂CH₂OCH₃	

Fig. 1. Chemical structure of erythromycin and its semisynthetic derivatives clarithromycin, roxithromycin, and azithromycin. (Clarithromycin forms in vivo an active metabolite, 14-hydroxy clarithromycin. The position in the molecule is indicated by the arrow.)

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(HP) and their therapeutic efficacy in HP-induced peptic ulcer disease.

Macrolides have only a weak UV absorbance in the low wavelength range (<220 nm), and with a few exceptions [3–5] HPLC with photometric detection does not allow the sensitive determination of macrolides in biological fluids. Sensitive methods have been reported based on fluorimetry [6,7] or chemiluminescence [8], but these methods need derivatization and/or special complex equipment. In the future, LC–MS methods [9,10] may be used. The first paper on the determination of a macrolide by HPLC and electrochemical detection (EC), namely erythromycin, appeared in 1983 [11]. Today, reversed-phase HPLC–ECD has become the standard method for the determination of macrolide antibiotics in biological matrices. Before chromatography, the macrolides are extracted from plasma into organic solvents [12–23], or separated by solid-phase extraction [24–26]. A wide spectrum of reversed-phase materials have been employed for separation, such as C_{18}, C_8, C_1 [9,13,14,16,17,19,20,22,23,25], diphenyl [12,18,21], PRP-1 [14,21,25] and alumina [21]. We describe a method using cyanopropyl silica as stationary phase which proved to be at least as efficient, and of better selectivity than the apolar reversed-phases.

2. Experimental

2.1. Apparatus

The HPLC system comprised a Pharmacia model 2248 pump with additional pulse dampener (bourdon pipe of 80-cm length), a Shimadzu autoinjector SIL-9A with system controller SCL-6B, Waters column oven TCM (set at 30°C), a cyanopropyl silica column Zorbax SB CN 5 μm (150×4.6 mm I.D.), ESA Coulochem II detector equipped with guard cell and analytical cell 5010, and a Shimadzu integrator C-R4A.

2.2. Chemicals and reagents

Clarithromycin and 14-hydroxy clarithromycin were gifts from Abbott (Wiesbaden, Germany), roxithromycin from Hoechst Marion Roussel (Bad Soden, Germany), azithromycin from Pfizer (Karlsruhe, Germany). Erythromycin, erythromycin ethylsuccinate, and erythromycin estolate were obtained from Sigma (Deisenhofen, Germany), acetonitrile and methanol (both HPLC reagent) from Baker (Gross-Gerau, Germany), and *tert*.-butyl methyl ether (for HPLC) from Fluka (Neu-Ulm, Germany). Water was deionized and further purified for HPLC with a Milli-Q system (Millipore, Eschborn, Germany). All other chemicals (analytical-reagent grade) were obtained from Merck (Darmstadt, Germany).

2.3. Standard solutions

Stock solutions (100 μg/ml) of erythromycin, clarithromycin, 14-hydroxyclarithromycin, roxithromycin and azithromycin were prepared in methanol–water (50:50, v/v), stored in a refrigerator at 4°C, and warmed up to room temperature before use. They remained stable for at least 4 weeks. Working standards were prepared by dilution of the stock solutions with methanol–water (50:50, v/v). Solutions containing erythromycin ethylsuccinate or erythromycin estolate were prepared in acetonitrile, because the esters are hydrolysed in protic solvents to erythromycin with half-lives at 25°C in methanol–water, 50:50 of 1.7 h (ethylsuccinate) and 8.2 h (estolate), respectively.

2.4. Chromatographic conditions

The mobile phases were mixtures of 50 mM Na_2HPO_4–NaH_2PO_4–acetonitrile–methanol. The apparent pH of the mobile phase varied between 6.5 and 7.5, depending on the respective macrolide (exact pH see legends to Figs. 3–6). Before use, the mobile phase was degassed by vacuum filtration through a 0.45-μm filter. The flow-rate was 1.0 ml/min. The settings of the coulometric detector were as follows: guard cell $+1$ V; screening electrode (E1) $+0.50$–0.60 V, sample electrode (E2) $+0.80$–0.90 V.

2.5. Sample preparation

The procedure as described here for plasma, is representative also for other body fluids such as saliva, gastric juice or lysate of leucocytes in buffer.

Gastric mucosa (5–20 mg tissue) was homogenized by ultrasonication in 300 µl 10 m*M* sodium phosphate buffer (pH 7.4), and then treated like plasma. A 500-µl aliquot of plasma or serum was transferred to a 12-ml conical glass centrifugal tube. Typically, 500 ng internal standard (in methanol–water, 50:50, v/v): clarithromycin for roxithromycin assay and vice versa; clarithromycin or roxithromycin for azithromycin assay) was added. After vortexing, 200 µl 0.1 *M* Na_2CO_3, and 3 ml *tert.*-butyl methyl ether were added. The mixture was thoroughly shaken (5×2 s SMI Multi-tube vortexer, American Dade), centrifuged (5 min at 1000 *g*), and the aqueous layer was frozen out by placing the tubes for a few seconds in liquid nitrogen or for 15 min in a freezer at −70°C. Then, the upper organic layer was transferred to another glass tube, and the solvent was evaporated to dryness at room temperature (Centrifugal vacuum evaporator RC 10.22, Jouan). The residue was reconstituted in 250 µl methanol–water (50:50, v/v). An aliquot of 20–50 µl was injected into the HPLC system.

2.6. Quantitation

The concentrations of the macrolides were calculated using peak height ratios of internal standard and sample peaks. The evaluation by the peak height method was more precise at low concentrations, as compared to the peak area method.

To assess linearity blank plasma was spiked with standard solutions of the macrolides to give concentrations in the range 0.1–5 µg/ml (clarithromycin, roxithromycin) or 0.025–1 µg/ml (azithromycin), and processed in duplicate as described above. Calibration lines (equation: $y=mx$) were constructed by plotting peak height of each drug to that of internal standard against the respective concentration.

In routine use, spiked plasma samples of middle concentration (1 µg/ml for clarithromycin, 2–4 µg/ml for roxithromycin and 200 ng/ml for azithromycin) was processed with each assay for calibration. Intra-day and inter-day precision was assessed by analysis of control samples (high, low concentration) in plasma with each run in duplicate.

The limit of quantification was estimated by analysing mixtures of the macrolides at progressively lower concentrations starting at the lower end of the calibration curves. The limit of quantification for each antibiotic was defined as that concentration level where accuracy and precision were still better than 20%.

To determine the limit of detection on column, a dilute (50 ng/ml) solution of the drugs in methanol–water (50:50, v/v) was injected into the HPLC system. The limit of detection was then defined as the amount of antibiotic which caused a signal three times the noise (S/N=3/1).

The recovery from plasma or buffer was determined by processing spiked samples. The results were compared with those obtained after direct injection of an aqueous–methanolic (50:50) reference solution of the analytes.

3. Results and discussion

3.1. Recovery, linearity and sensitivity

The recovery of the macrolides from water and plasma was 80–90%. The assay showed good linearity ($r>0.9996$ for clarithromycin, $r>0.9991$ for roxithromycin, $r>0.998$ for azithromycin, $n=6$ calibration lines) over the whole range (clarithromycin and roxithromycin 0.1–5 µg/ml; azithromycin 25–1000 ng/ml) which covered the concentrations typically found in plasma of man after administration of therapeutical doses. The $R.S.D._{inter}$, $R.S.D._{intra}$ and accuracy were between 0.3–9.1%, 1.3–6.1% and 0.6–7.3% for clarithromycin, 0.7–10.3%, 2.4–5.7% and 0.6–8.6% for roxithromycin, and 1.5–15.7%, 6.2–14.2% and 1.1–12.4% for azithromycin.

Using 0.5 ml of plasma 30 ng/ml of clarithromycin (found, $R.S.D._{inter}$, $R.S.D._{intra}$: 34.7 ng/ml, 4.1%, 16%), 50 ng/ml of roxithromycin (53.3, 3.1, 4.7), and 10 ng/ml for azithromycin (11.7, 9.4, 17) could be determined with acceptable precision and accuracy.

The limit of detection on column (S/N=3) varied between 0.4 and 1.5 ng, being lowest for azithromycin, and depending on the performance of the analytical cell of the detector.

3.2. Performance of the analytical column

Macrolides are lipophilic compounds with basic character. For separation base deactivated reversed-phase materials are to be preferred, as it has been reported earlier [23,25,27,28]. The mobile phases consist typically of mixtures of aqueous phosphate or acetate buffer with 40–80% organic modifier such as acetonitrile and/or methanol. The pH varies between 6.5 and 7.5 or between 10 and 11, when PRP-1 or alumina phases are used [14,21,25].

Initially, we used LiChrospher RP select B and Novapak C_{18} (primed with di-*n*-butylamine according to [29]) materials. However, we observed that the local anesthetic, lidocaine, which was present in high concentrations in plasma and bronchial mucosa of patients undergoing bronchoscopy, interfered with the determination of 14-hydroxy clarithromycin, the active metabolite of clarithromycin. We could circumvent this interference by using a cyanopropyl phase, namely Zorbax SB CN [30]. This column also exhibited excellent peak shape and plate number. We found that also other 5-μm cyano materials (Spherisorb CN, Ultrasep CN, Nucleosil CN, Novapak CN) would be suitable for the analysis of macrolides. The plates/m for clarithromycin and roxithromycin were good to excellent (15 000–20 000 for Ultrasep and Nucleosil, 25 000–30 000 for Spherisorb, Zorbax SB), and moderate for erythromycin and its esters (up to 50% lower).

The Zorbax SB CN was finally selected for all assays which differed from the other cyano materials by its higher selectivity between erythromycin, clarithromycin and roxithromycin, and by its 3–4-fold higher retention. The retention of the macrolides at Zorbax SB CN was similar to that at Novapak C_{18}. But the cyanopropyl material seems more suitable than C_{18} silica for the simultaneous determination of erythromycin and its esters. The retention time of the erythromycin esters is similar at Zorbax SB CN compared to erythromycin (Fig. 2), but much longer at C_{18} silica [13]. Moreover, the selectivity varies with pH providing an additional parameter for tuning of the mobile phase.

The standard chromatographic system consisted of the Zorbax SB CN column with eluent consisting of 450 ml 50 mM sodium phosphate, 50 ml methanol and 300 ml (clarithromycin), 350 ml (roxithromycin)
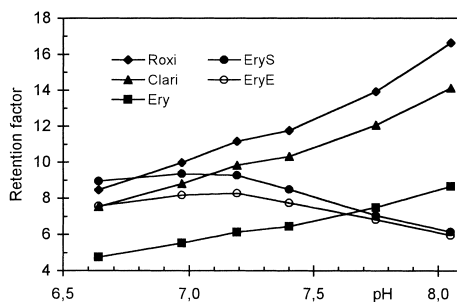

Fig. 2. Changes of retention factors of macrolides with pH of the mobile phase. Chromatographic conditions: Column Zorbax SB CN (150×4.6 mm I.D.) with eluent of 50 mM sodium phosphate–acetonitrile–methanol (450:300:50, v/v/v), apparent pH 6.6–8.0. Roxi=roxithromycin, Clari=clarithromycin, Ery=erythromycin, EryS=erythromycin ethylsuccinate, EryE=erythromycin estolate.
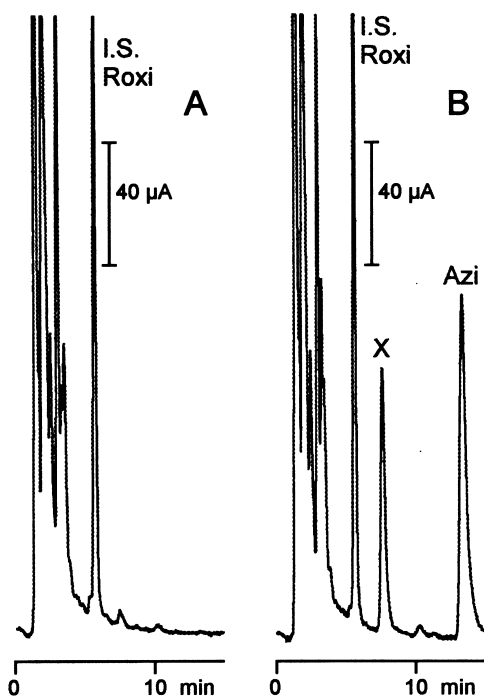

Fig. 3. Chromatograms obtained from plasma of a volunteer before (A) and following oral administration of azithromycin (B: Azi=0.48 μg/ml). The peak at X may be an unidentified metabolite of azithromycin. Chromatographic conditions: column Zorbax SB CN (150×4.6 mm I.D.) with eluent of 50 mM sodium phosphate–acetonitrile–methanol (500:600:50, v/v/v), apparent pH 6.8. I.S. Roxi=internal standard roxithromycin. Azi= azithromycin.

respectively 500 ml (azithromycin) acetonitrile. The apparent pH was adjusted to 6.5–6.8 (azithromycin) and 7.0–7.5 (clarithromycin, roxithromycin).

The assay was employed for the analysis of clarithromycin, roxithromycin and azithromycin in various biological matrices such as plasma, saliva, white blood cells, bronchial secretion, humour of the eye, gastric juice, bronchial mucosa, and gastric mucosa. The samples were obtained from pharmacokinetic studies in volunteers and patients.

Chromatograms of the investigated macrolides are depicted in Figs. 3–6. All macrolides were well separated from endogeneous compounds. The retention times of all macrolides increased with aging of the column, and unfortunately the selectivity between clarithromycin and roxithromycin (mutual internal standard) decreased. Slight modifications of the acetonitrile content (<10%) and pH (<0.3) in the eluent were needed to achieve and maintain satisfactory resolution. The column had to be exchanged after 500–800 injections of biological specimens, because of insufficient selectivity and peak broadening.
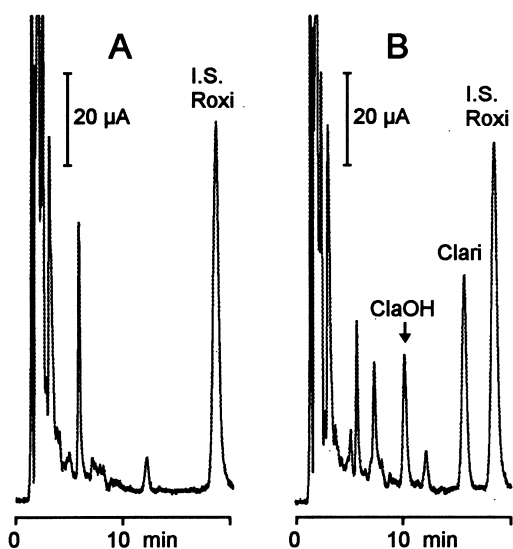

Fig. 5. Chromatograms obtained from plasma of a volunteer before (A) and following oral administration of roxithromycin (B: Roxi=2.1 µg/ml). Chromatographic conditions: column Zorbax SB CN (150×4.6 mm I.D.) with eluent of 50 m*M* sodium phosphate–acetonitrile–methanol (450:350:50, v/v/v), apparent pH 7.0. I.S. Clari=internal standard clarithromycin, Roxi= roxithromycin.

3.3. Performance of the detector

Most of the HPLC–EC methods employ coulometric detection by the ESA Coulochem detector. Obviously, this type of detector with a guard and a screen cell before the analytical cell provides better baseline stability at high oxidation potentials and high organic contents of the mobile phase, as compared to amperometric detectors (c.f. [21,25]). We used eluents based on phosphate buffer, because the background noise with eluents containing ammonium acetate or tetrabutyl ammonium salts [12,16,18,21,22], e.g. to improve peak shape on C_{18} columns, was considerably higher. The eluent could be recycled for at least 1 week corresponding to 150–250 injections.

The standard settings of the Coulochem II detector were: guard cell +1.0 V; screening cell E1+0.50 V; analytical cell E2+0.80 V. When the response of the detector decreased, the potential was increased stepwise up to 0.60 at E1 and 0.90 V at E2, provided the signal-to-noise ratio remained acceptable. The detec-

Fig. 4. Chromatograms obtained from plasma of a volunteer before (A) and following oral administration of clarithromycin (B: Clari=0.39 µg/ml; ClaOH=0.23 µg/ml). Chromatographic conditions: Column Zorbax SB CN (150×4.6 mm I.D.) with eluent of 50 m*M* sodium phosphate–acetonitrile–methanol (450:300:50, v/v/v), apparent pH 7.5. I.S.=internal standard roxithromycin; ClaOH=14-hydroxy clarithromycin.
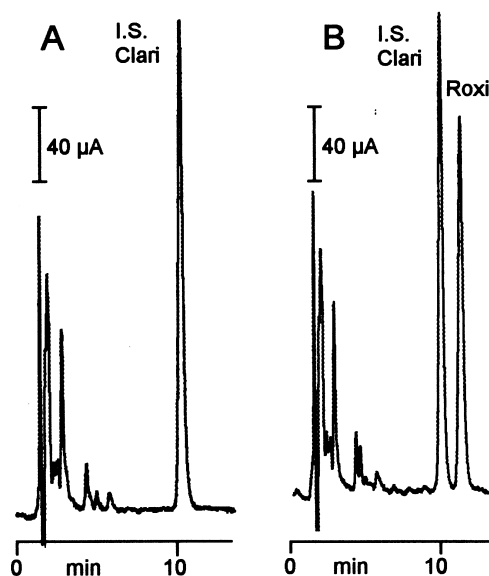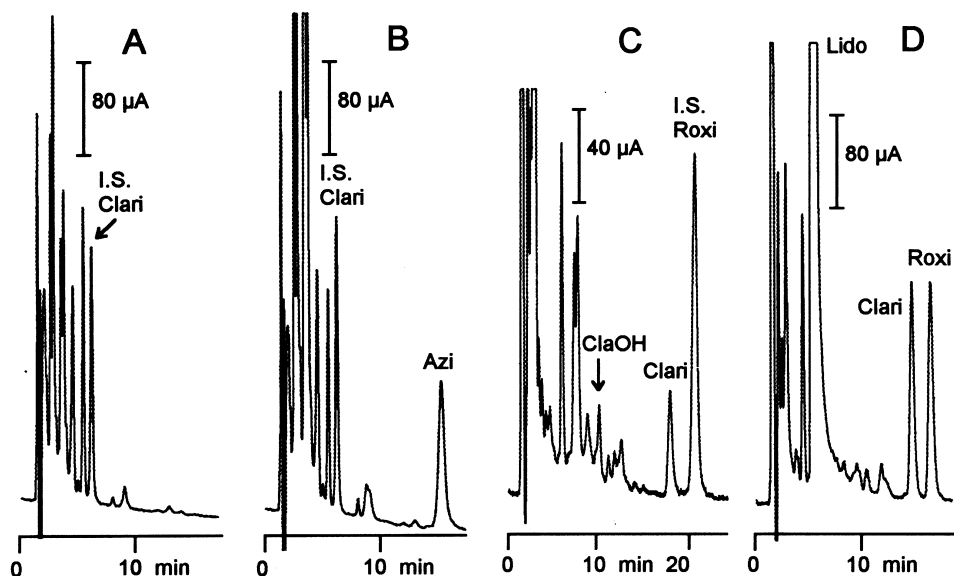
Fig. 6. Chromatograms obtained from analysis of about $4 \cdot 10^6$ leucocytes (polymorphonuclear neutrophils, A, B), 500 µl vitreous humour of the eye (C), and 18 mg gastric mucosa (D) of volunteers or patients following oral administration of azithromycin (A: blank before administration, B: Azi=44 µg/ml cell volume), clarithromycin (C: Clari=45 ng/ml, ClaOH=20 ng/ml), or roxithromycin (D: Roxi=7.3 µg/g). Chromatographic conditions: column Zorbax SB CN (150×4.6 mm I.D.) with eluent of 50 mM sodium phosphate–acetonitrile–methanol (500:600:50, v/v/v) (A, B), pH 6.5; (450:300:50) (C), pH 7.4; (450:320:50) (D) pH 7.4. Clari=clarithromycin; ClaOH=14-hydroxy clarithromycin; Roxi=roxithromycin; Azi=azithromycin; I.S.=internal standard.

tor recovered at least partially after flushing with methanol (30–60 min at a flow-rate of 1 ml/min), and also after an overnight pause. Deterioration could occur within 1 working day. Therefore, the use of an internal standard is mandatory.

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