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Quantitative determination of the macrolide antibiotics erythromycin, roxithromycin, azithromycin and clarithromycin in human serum by high-performance liquid chromatography using pre-column derivatization with 9-fluorenylmethyloxycarbonyl chloride and fluorescence detection

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Abstract

A validated, highly sensitive and precise high-performance liquid chromatographic (HPLC) method for the determination of the macrolides erythromycin, azithromycin, clarithromycin and roxithromycin in human serum is described. A diethyl ether extract, obtained from serum using a saturated sodium carbonate solution, was treated with 9-fluorenylmethyloxycarbonyl chloride (FMOC–Cl) for 40 min at 40°C and chromatographed on a base-deactivated octadecyl column, maintained at 50°C during elution, using an eluent composed of acetonitrile–hydrogenphosphate buffer, pH 7.5, with 0.125% triethylamine (3:2, v/v). Fluorescence detection was used at an excitation wavelength of 255 nm and an emission wavelength of 315 nm. Erythromycin, clarithromycin, roxithromycin and azithromycin were found to have retention times of 8.8, 15.7, 17.1 and 20.7 min, respectively. Recoveries ranging from 93 to 104% were found with reproducibility coefficients of variation of 1.1–5%. Mean correlation coefficients of 0.9997, 0.9998, 0.9996 and 0.9994 were found for the linear calibration curves ($n=2$) of erythromycin (0.320–16.1 mg/l), roxithromycin (3.24–19.4 mg/l), clarithromycin (0.190–19.4 mg/l) and azithromycin (0.0988–4.94 mg/l), respectively.

Keywords: Derivatization, LC; Erythromycin; Roxithromycin; Azithromycin; Clarithromycin

1. Introduction

Erythromycin [(2*R*,3*S*,4*S*,5*R*,6*R*,8*R*,10*R*,11*R*,12*S*, 13*R*)-5-(3-Amino-3, 4, 6-trideoxy-*N*,*N*-dimethyl-β-D-xylo-hexopyranosyloxy)-3-(2, 6-dideoxy-3-*C*, 3-*O*-dimethyl- α -L-ribo-hexopyranosyloxy)- 13 -ethyl- 6, 11, 12-trihydroxy-2, 4,6, 8,10, 12-hexamethyl-9-oxotridecan-13-olide], roxithromycin [erythromycin 9-{*O*(2-methoxyethoxy)methyl}oximycin], clarithromycin [6-*O*-methylerythromycin] and azithromycin [9-deoxo-9a-aza-9a-methyl-9a-homoerythromycin A dihydrate] (Fig. 1) are widely used macrolide antibiotics indicated for the use of infections with Gram positive bacteria such as *Streptococci*, *Staphylococcus aureus*, *Propionibacterium acnes*, *Listeria monocytogenes* and *Corynobacteria* species, as well as Gram negative bacteria, such as *Legionella pneumo-*

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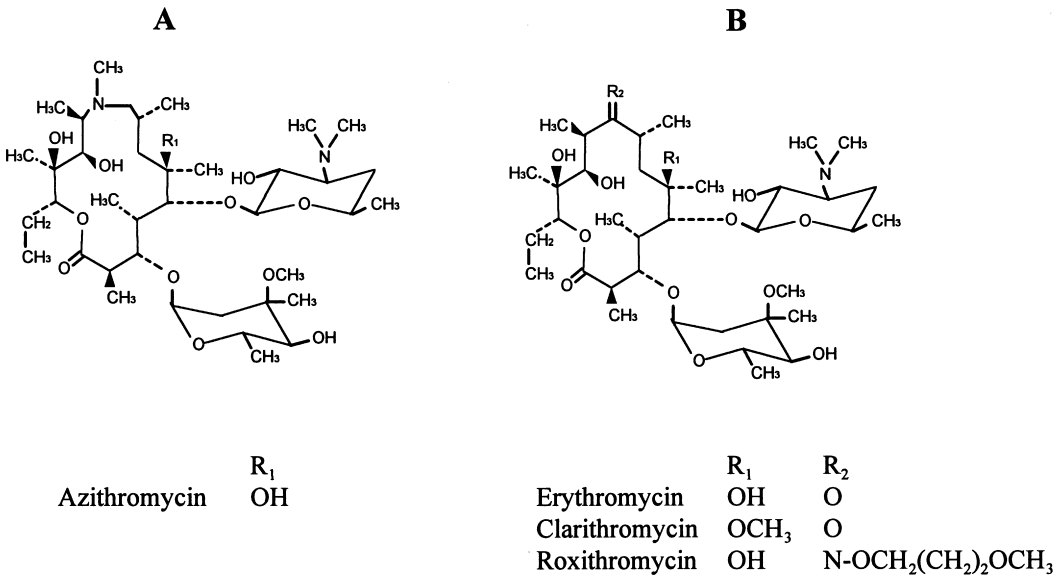

	R_1
Azithromycin	OH

	R_1	R_2
Erythromycin	OH	O
Clarithromycin	OCH_3	O
Roxithromycin	OH	N-$OCH_2(CH_2)_2OCH_3$

Fig. 1. Chemical structures of azithromycin (A) and erythromycin, clarithromycin and roxithromycin (B).

philia and *Haemophilus* species. Furthermore, the macrolide antibiotics are increasingly used for opportunistic infections in patients with AIDS.

Macrolides interfere with RNA-dependent bacterial protein synthesis, resulting in a bacteriostatic effect on pathogens.

Efficacy can generally be expected at drug concentrations exceeding the minimal inhibitory concentration for a pathogen with a factor two-three at the site of infection. Due to pharmacokinetic causes, such as variable drug absorption from the gastrointestinal tract and drug–drug interactions leading to altered drug elimination, effective drug concentrations might not always be reached in clinical situations [1].

Therefore, an analytical assay for the determination of macrolide concentrations in biological specimens is needed to control and assure drug efficacy.

Several high-performance liquid chromatography (HPLC) methods for the determination of macrolides in human serum have been reported [2–12], merely for erythromycin and occasionally for the newer macrolides. Most of these methods require electrochemical detection because of the insensitivity of the macrolides with UV detection.

Tsuji [12] described a method for the quantitation of erythromycin with fluorescence detection using derivatization. However, this method has several disadvantages, such as requiring a complex analytical system with post-column derivatization, a need for a large sample volume for clean up and a large injection volume in order to achieve the desired sensitivity. More recently, Zierfels and Petz [13] described a method for the determination of erythromycin in foods using fluorescence detection after derivatization with 9-fluorenylmethyloxycarbonyl chloride (FMOC–Cl). We have made this method applicable for the quantitation of erythromycin and the newer macrolides in serum.

In the current manuscript, a validated, sensitive and precise HPLC method is described for the quantitation of erythromycin, azithromycin, clarithromycin and roxithromycin in human serum.

2. Experimental

2.1. Chemicals and reagents

Erythromycin, as a base (purity 95.9%), roxithromycin (90%) and 9-fluorenylmethyloxycarbonyl–chloride (FMOC–Cl) were obtained from Sigma (Zwijndrecht, The Netherlands). Clarit-

hromycin (98.4%) and azithromycin (99.9%) were kind gifts from Abbott (Kent, UK) and Pfizer (Groton, USA) respectively. Sterile distilled water was from Braun-NPBI (Emmer-Compascuum, The Netherlands). Diethyl ether (p.a.), acetonitrile (gradient grade), triethylamine (for synthesis), sodium carbonate (p.a.), potassium hydroxide pellets (p.a.) and potassium dihydrogenphosphate (p.a.) were purchased from Merck (Amsterdam, The Netherlands).

2.2. Chromatography

The mobile phase was prepared by mixing 600 ml acetonitrile of with 400 ml of a solution comprising potassium dihydrogenphosphate (0.05 mol/l water) and 500 μl of triethylamine, adjusted to pH 7.5 with a 10% potassium hydroxide solution. The solvent was filtered and degassed through a 0.22-μm membrane filter from Millipore (Etten-Leur, The Netherlands).

The chromatography system consisted of a high precision pump (Gynkotek model 480) at a flow-rate of 2.0 ml/min, a fluorescence detector (Jasco model FP-920) set at an excitation wavelength of 255 nm, an emission wavelength of 315 nm and a gain of one, a Marathon automatic injector with a built-in column oven set at 50°C from Separations (Hendrik Ido Ambacht, The Netherlands) and a Millennium chromatographic data system from Waters (Etten-Leur).

The separation was performed on a 125×4.6 mm base-deactivated Supelcosil silica C_{18} column packed with 5 μm particles, with a guard column (base-deactivated C_{18}; 20×4.6 mm with 5 μm particles) from Supelco (Zwijndrecht, The Netherlands).

2.3. Optimization of the derivatization conditions

The reaction of the macrolides with FMOC–Cl was optimized using 20 mg/l solutions with erythromycin and roxithromycin in acetonitrile as model compounds. The reaction time, the proportion of acetonitrile–water, the pH and temperature of the solution were varied around the expected optimal values [13]: Buffer solutions ranging from pH 5.4 to 10.5 were used to adjust the pH to the desired values. The reaction time was optimized by following the reaction for a total of 80 min.

Various acetonitrile–water proportions, ranging from 2:1 to 5:1, were used to optimize the polarity of the reaction solution. An excess of FMOC–Cl was added and the volumes of the test solutions were adjusted to 500 μl in 1.8 ml glass reaction vials. The mixtures were allowed to react in a warmed water-bath at temperatures ranging from 20–80°C. After derivatization, all solutions were chromatographed on the HPLC system described. Relative peak heights were measured to find the optimal conditions.

Since the reaction between the hydroxyl groups of the model compounds and FMOC–Cl is similar for all macrolides, the optimal conditions found were also applied for derivatization of the two other macrolides used.

2.4. Sample preparation

Since macrolides are not prescribed together to patients, one macrolide could be used as the internal standard for the assay of the other macrolides. In the prescribed method, roxithromycin was used as an internal standard for the erythromycin, clarithromycin and azithromycin assay, and clarithromycin was used for the roxithromycin assay.

A 20.0-μl volume of internal standard was added to a 1.0-ml sample containing the macrolide, in a 10-ml disposable glass tube, and 200 μl of a warmed saturated sodiumcarbonate solution (0.5 g/ml, with a pH of approximately 12) was added and the solution was vortex-mixed for 10 s. A volume of 6 ml of diethyl ether was added to the sample solution. After shaking for 15 min at a frequency of 270 min^{-1}, the solution was centrifuged for 5 min at 2700 *g*. The solution was allowed to freeze at −20°C in an ethanol bath. The organic layer was transferred to a dry disposable glass tube and evaporated at 40°C under a flow of nitrogen. The residue was dissolved in 200.0 μl of acetonitrile and vortex-mixed for 30 s. The solution was centrifuged at 2700 *g* and quantitatively transferred to a 1.8-ml reaction vial. A 100.0-μl volume of a 2.5-mg FMOC–Cl/ml acetonitrile solution and 75.0 μl of a 0.1-*M* phosphate buffer in water, adjusted to a pH of 7.5 with a 10% potassium hydroxide solution, were added. The reaction vial

was incubated in a waterbath of 40°C for 40 min. After derivatization, 50 μl of the solution was injected onto the chromatographic system.

2.5. Validation of the macrolide assay

2.5.1. Specificity and selectivity

For the examination on the presence of disturbing endogenous components, human serum from six different, drug-free persons was tested. These samples were pre-treated according to the sample preparation procedure, apart from the addition of internal standard solution. A chromatogram of a reference solution containing the four macrolides in serum was prepared and compared with the blank solutions.

2.5.2. Recovery

Three standards prepared in human blank serum, with concentrations ranging between the limits of quantitation of the substance to be assayed, were determined in quintuplicate and compared with an unpretreated reference solution in acetonitrile, prepared with the same concentration as the standards.

For the determination of erythromycin recovery, 1.00 ml of 0.991, 2.97 and 8.06 mg/l erythromycin in serum solutions with 20.0 μl of a 0.0510-mg roxithromycin/l acetonitrile solution as internal standard were used. The recoveries of azithromycin standards were determined using 1.00 ml of 0.269 mg/l, 2.96 mg/l and 4.93 mg/l serum solutions, with 20.0 μl of a 0.160-mg roxithromycin/l acetonitrile solution as internal standard. For clarithromycin recoveries, 1.00-ml of 0.971 mg/l, 9.71 mg/l and 19.4 mg/l clarithromycin serum solutions with 20.0 μl of 0.160 mg roxithromycin/l acetonitrile, as internal standard, were used. The roxithromycin recoveries were determined using 1.00 ml of 3.24, 9.72 and 19.5 mg/l roxithromycin in serum solutions with 20.0 μl of a 0.840 mg clarithromycin/l acetonitrile solution as the internal standard.

The recoveries of the internal standard were determined absolutely, while the other recoveries were quantified as ratios (macrolide/internal standard). For reliable quantification, all macrolide recoveries, including the internal standard, should be between 85–115%.

2.5.3. Repeatability

The concentrations of the macrolides found in the samples assayed for the determination of the recoveries were also used to calculate the repeatability.

The repeatability is defined as:

$$\text{repeatability} - \text{C.V.} = \frac{\sqrt{\text{MSwg}}}{\text{mean}} \cdot 100\%$$

where MSwg represents the mean square within groups and C.V. is the coefficient of variation.

The mean square within groups was determined by the ANOVA test, performed using the statistical software program SPSS (version 6.1.3, SPSS, USA).

To perform the assay under repeatable quantification conditions, the repeatability coefficient of variation should not exceed the 15% limit.

2.5.4. Reproducibility

One of the samples used for the determination of the repeatability was assayed twice again under varying conditions, such as the use of another chromatographic system with the same characteristics, and performing of the assay on different days. The variation between the three sets of results obtained were submitted to the ANOVA test; the mean square within groups and the mean square between groups were calculated. The reproducibility is defined as:

$$\text{reproducibility} - \text{C.V.} = \frac{\sqrt{\frac{\text{MSbg} - \text{MSwg}}{n}}}{\text{mean}} \cdot 100\%$$

where MSwg represents the mean square within groups, MSbg the mean square between groups and n is the number of analysis of the sample quantified in one run.

The concentrations of the test solutions used were: 2.96 mg/l erythromycin, 9.71 mg/l clarithromycin, 9.72 mg/l roxithromycin and 2.96 mg/l azithromycin.

In order to perform the assay with reliable reproducibility, the reproducibility coefficient of variation should not exceed the 15% limit.

2.5.5. Limit of quantitation

The lower limit of quantitation (LLQ) is defined as the concentration that can be determined with a given precision. The LLQ is appointed at the con-

centration equal to $S/N=5$. The macrolide peak of a pretreated serum sample resembling this concentration should be clearly distinguishable from noise peaks.

The higher limit of quantitation (HLQ) is defined as twice the highest concentration to be expected in routine samples. Clinical values for the serum concentrations of macrolides are only occasionally given in literature. Maximal serum concentrations of 4.66, 12, 0.63 and 6.5 mg/l were found after a single dose of 1200 mg of clarithromycin, 1000 mg of roxithromycin, 500 mg of azithromycin and 1000 mg of erythromycin, respectively, and are indicative of the range of expected concentrations [1,15–24].

2.5.6. Linearity

The linearity of the assay was tested for each macrolide for concentrations ranging from the low therapeutic to twice the highest therapeutic concentration to be expected.

For each macrolide, five standards were assayed in duplicate. The results were submitted to the two-sided Students *t*-test using the statistical program STATCAL 6.50 (University of Amsterdam, The Netherlands).

This program calculates the probability of the calibration curve's order performing the *t*-test to polynoma with different degrees. For a linear relationship, no significance ($p<0.05$) should be found for orders surpassing the first degree. Furthermore, the correlation coefficients of the curves should be >0.995.

A calibration curve containing standards of 0.320, 0.640, 0.800, 1.20, 2.01, 4.02, 6.02, 8.03, 12.0 and 16.1 mg/l erythromycin in serum and 1.03 mg/l roxithromycin as internal standard were used for the determination of the linearity of the erythromycin calibration curve. Standards with 3.24, 4.86, 8.10, 9.72, 16.2 and 19.4 mg/l roxithromycin in serum and 16.3 mg/l clarithromycin as internal standard were used for the determination of the roxithromycin calibration curve's linearity. A calibration curve using 0.190, 0.970, 2.91, 9.71, 14.6 and 19.4 mg/l clarithromycin solutions as standards and 3.12 mg/l roxithromycin serum as internal standard were used for the determination of the clarithromycin calibration curve's linearity. The linearity of the azithromycin calibration curve was performed using 0.0988, 0.296, 0.790, 2.96 and 4.94 mg/l azithromycin solutions as standards with 3.12 mg roxithromycin/l serum as the internal standard.

2.5.7. Stability

Stock solutions of the macrolides in acetonitrile were prepared at concentrations of 1 mg erythromycin/l, 10 mg roxithromycin/l, 10 mg clarithromycin/l and 3 mg azithromycin/l, stored at 4°C and assayed at appropriate time intervals. The macrolide peak-heights were quantitatively compared with peak-heights from macrolides in freshly prepared stock solutions.

To estimate the stability of derivatized solutions, the derivatized macrolide solutions were assayed every hour over a period of 24 h. The peak-heights of the macrolides at different time intervals were compared with those of the initial values.

Spiked serum samples containing 2.79 mg/l erythromycin, 9.69 mg/l roxithromycin, 9.98 mg/l clarithromycin and 2.80 mg/l azithromycin, stored at 4°C and −20°C, were assayed daily over a one-week period to test the stability in human serum.

3. Results

3.1. Optimization of the derivatization conditions

The derivatization reaction appeared to be highly dependent on pH (Fig. 2), reaction temperature (Fig. 3) and reaction time (Fig. 4). Furthermore, there was some dependency on the composition of the reaction mixture (Fig. 5). The optimal conditions were found to be: a pH of 6.5–7.5, a reaction temperature of 40°C for 40 min and a reaction solution consisting of water–acetonitrile (1:3 to 1:4, v/v).

3.2. Validation

3.2.1. Specificity and selectivity

A representative chromatogram of blank serum, spiked with the four macrolides is shown in Fig. 6. Endogenous components chromatographed within 2 min and no endogenous components were found to interfere with the elution of the macrolides.

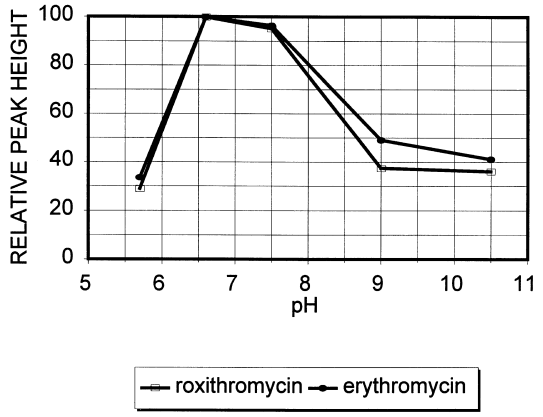

Fig. 2. Optimization of the reaction pH for the reaction between FMOC–Cl and the macrolides erythromycin and roxithromycin ($n=2$). Solutions containing 20 mg/l of the macrolides and 250 μg of FMOC–Cl were used with a water–acetonitrile ratio of 1:4, adjusted to 500 μl, and a reaction temperature of 40°C for 40 min.

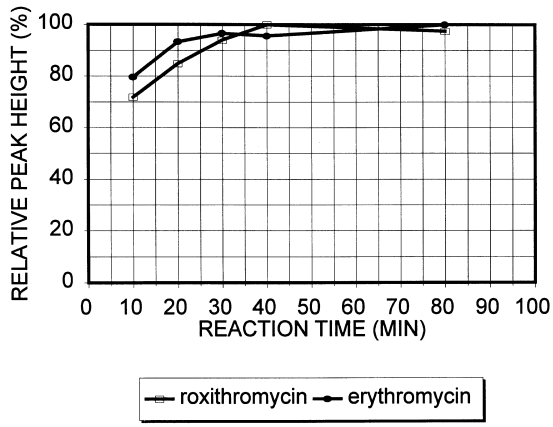

Fig. 4. Optimization of the reaction time ($n=2$). Test solutions containing 20 mg/l erythromycin and roxithromycin were used. These macrolides were derivatized with 250 μg of FMOC–Cl using a pH of 7.5, a water–acetonitrile ratio of 1:4, adjusted to 500 μl, and a reaction temperature of 40°C.

3.2.2. Recovery

The results of the recovery study are summarized in Table 1. Recoveries ranging from 93–104% were found for the macrolides. The recoveries of the internal standards were 101% with 1.03 mg roxithromycin/l as internal standard for erythromycin, 98% with 3.12 mg roxithromycin/l as internal standard for clarithromycin and azithromycin, and 97% with 16.3 mg clarithromycin/l as internal standard for roxithromycin.

3.2.3. Repeatability

The results of the repeatability study are summarized in Table 1. The repeatability coefficients of

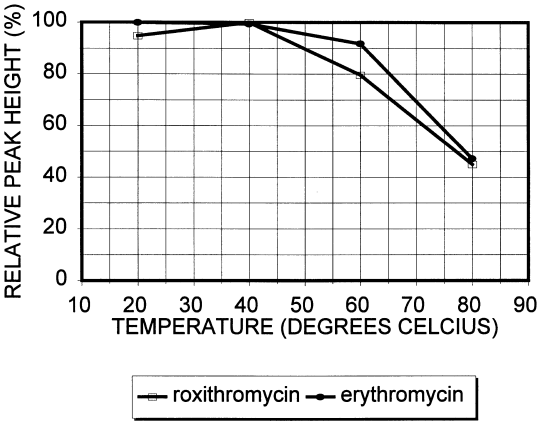

Fig. 3. Optimization of the reaction temperature ($n=2$). The reaction was performed using 20 mg/l erythromycin and roxithromycin solutions with 250 μg of FMOC–Cl at pH 7.5 and a water–acetonitrile ratio of 1:4, adjusted to 500 μl, and reacted for 40 min.

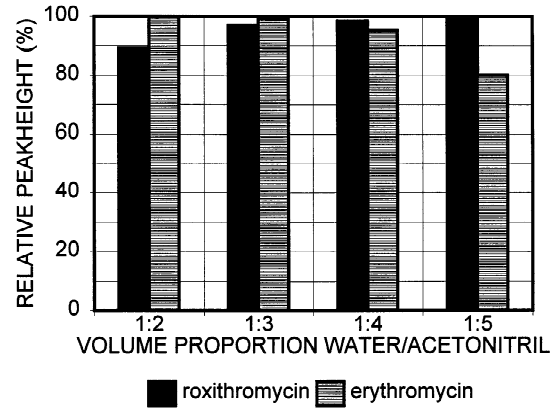

Fig. 5. Optimization of the water– acetonitrile volume ratio ($n=2$) using 20 mg/l erythromycin and roxithromycin solutions as test compounds. The derivatization was performed using 250 μg of FMOC–Cl, a pH of 7.5 of solutions adjusted to 500 μl and a reaction temperature of 40°C for 40 min.

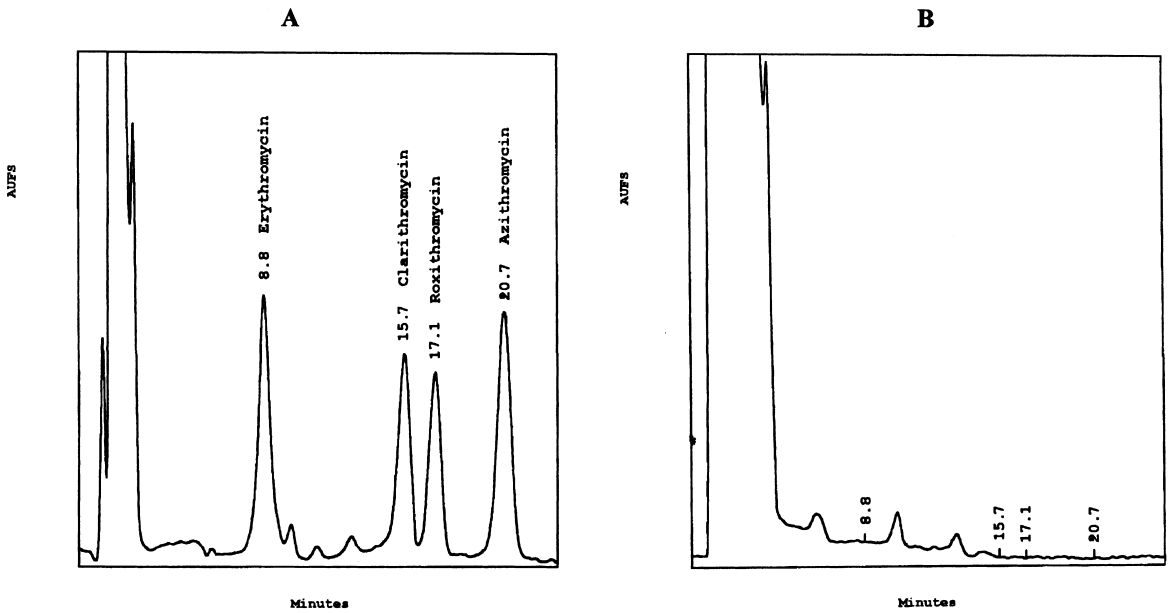

Fig. 6. Chromatograms of an extract of serum (A) containing 4 mg/l of the macrolides erythromycin, clarithromycin, roxithromycin, azithromycin and a blank serum extract (B).

variation do not exceed the 15% limit. For the internal standards, the coefficients of variation were 4, 2 and 5% at 1.03 mg roxithromycin/l, 16.3 mg clarithromycin/l and 3.12 mg roxithromycin/l, respectively.

3.2.4. Reproducibility

The results of the reproducibility study are summarized in Table 1. The samples assayed were found to have reproducibility coefficients of variation that did not exceed the 15% limit; No significant addi-

Table 1
Recoveries ($n=5$) of the macrolides from serum samples and repeatability ($n=5$) and reproducibility ($n=15$) results

Compound	Concentration (mg/l)	Recovery (%)	Repeatability coefficient of variation (%)	Reproducibility coefficient of variation (%)
Azithromycin	0.269	93	6	
	2.96	98	5	5
	4.93	98	3	
Roxithromycin	3.24	102	5	
	9.72	97	3	1.1
	19.5	102	6	
Clarithromycin	0.971	104	8	
	9.71	104	4	4
	19.4	104	1.2	
Erythromycin	0.991	96	2	
	2.97	102	5	4
	8.06	96	2	

tional variation was found when performing the assay under reproducibility conditions.

3.2.5. Limits of quantitation

The lower limits of quantitation were calculated to be 0.20 mg/l for erythromycin, 0.065 mg/l for roxithromycin, 0.20 mg/l for clarithromycin and 0.092 mg/l for azithromycin. The chromatogram peaks resembling these concentrations were all clearly distinguishable from noise peaks. The higher limits of quantitation were estimated to be 16.0 mg/l for erythromycin, 19.5 mg/l for roxithromycin, 20.0 mg/l for clarithromycin and 5.0 mg/l for azithromycin.

3.2.6. Linearity

The calibration curves of erythromycin, roxithromycin, clarithromycin and azithromycin resulted in correlation coefficients of 0.9997, 0.9998, 0.9996 and 0.9994, respectively ($n=2$). First degree regression was found to be the most suitable for all four macrolide calibration curves ($p<0.05$).

3.2.7. Stability

No decrease of the macrolide concentrations of stock solutions containing erythromycin and roxithromycin was observed over a period of 26 days when stored at 4°C. The azithromycin and clarithromycin stock solutions, kept at 4°C, were stable over a period of at least 27 and 21 days, respectively.

The derivatized solutions were found to be stable (>95%) for 4 h. After seven days, the concentrations of erythromycin, roxithromycin, clarithromycin and azithromycin in serum stored at −20°C were found to be 96, 102, 104 and 97%, respectively, from the initial values.

4. Discussion and conclusion

The assay of erythromycin in foods, as described by Zierfels and Petz [13], was used as the basis for the development of an assay for the quantification of erythromycin, clarithromycin, roxithromycin and azithromycin in human serum. The fluorescent compound used, FMOC–Cl, is known to form stable derivatives with amino groups, which makes it a suitable reagent for detection of amino acids. In a similar way, it is also capable of forming stable ester-bonded derivatives with hydroxyl groups.

In this manuscript, the development and validation of a sensitive and precise HPLC-method for the determination of macrolides, using this derivatization technique, is described.

The conditions of the reaction between FMOC–Cl and the hydroxyl groups of the macrolides used was optimized. It was necessary to apply a reaction mixture composition with a high content of acetonitrile, due to the insolubility of FMOC–Cl and the derivatization products in hydrophilic solutions. An excess (e.g. 80%) of acetonitrile in the reaction mixture resulted in precipitation of the phosphate buffer, which was needed for the removal of hydrogen ions from the hydroxyl groups of the macrolides. At higher pH values, solvolysis dominated the reaction, which resulted in the formation of incomplete macrolide derivatives. The reaction could be accelerated by heating the reaction mixture in a waterbath. Overheating of the reaction mixture resulted in reduced formation of macrolide derivatives, probably caused by increasing the degradation reaction of the ester bond between the macrolide and FMOC.

Liquid–liquid extraction of the macrolides could not be performed using organic solvents in combination with regular buffer solutions such as phosphate, acetate or borate without losses of more than 15% in recovery of the macrolides. Using a heated saturated carbonate solution resulted in higher recoveries of the macrolides with most of the organic solvents. Diethyl ether in combination with the saturated carbonate solution was found to yield the highest concentrated and cleanest extract from serum for all of the macrolides used.

All four of the macrolide derivatives used chromatographed separately from each other as well as from other FMOC products, such as FMOC–OH, which is formed from FMOC–Cl and water. Column heating resulted not only in an increased performance of the analytical column, but also in a better separation of the erythromycin derivative from alternative FMOC products.

The chromatographic speed could be increased by removing the interfering FMOC compounds from the derivatized solution, performing an extraction of the

derivatized macrolides with organic solvents as performed in amino acid analysis [14]. By making the eluent less hydrophilic, the macrolide peaks could be chromatographed faster without loss of resolution due to the absence of interfering reagent products. This effect would also increase peak-heights in the chromatogram, which, in turn, could result in lowering the limit of detection. Furthermore, FMOC–Cl possibly reacts with free silanol groups of the column material in the front since we observed peak broadening, front peak tailing and even peak doubling after numerous injections with FMOC–Cl solutions. The remedy to this problem was inverting the guard column and analytical column during the wash step. Washing was performed with eluent for half an hour. After the washing procedure, the column could be used for a few days without changing its chromatographic performance.

Derivatized solutions were stable for 4 h at room temperature. After this period of time, rapid degradation of the derivatized compounds was observed, probably caused by decomposition of the ester bond between the macrolide and FMOC. Theoretically, this process should be reversible by repeating the derivatization step, although the sensitivity would decrease due to dilution of the macrolide solution. Therefore, we recommend performing the assay within the 4-h period of time during which the derivatives show maximal stability.

We limited the assay to the determination of parent compounds of the macrolides. The quantitation of the active 14-hydroxide metabolite of clarithromycin was not performed because of the relatively small amount ($<10\%$) of this substance in the human body and the scarcity of this compound as a reference sample. However, this compound could probably be assayed in a similar way as the metabolite shares similar molecular characteristics to those of the basic macrolides.

The bioanalytical assay is now applied in a pharmacokinetic study in patients receiving macrolides and appeared to be feasible for this purpose.

In conclusion, the current method appeared to be precise and sensitive for the determination of macrolide concentrations in human serum.

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