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Liquid chromatographic determination of the macrolide antibiotics roxithromycin and clarithromycin in plasma by automated solid-phase extraction and electrochemical detection

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Abstract

A liquid chromatographic method for the determination of the macrolide antibiotics, roxithromycin and clarithromycin, in plasma is described. The method is fully automated, employing on-line solid-phase extraction for sample clean-up, using the Prospekt unit. Plasma samples, mixed with internal standard, were injected onto exchangeable CN cartridges. After washing, the compounds were eluted and transferred to a C_{18} analytical column for separation and electrochemical detection. Clarithromycin was used as internal standard when assaying roxithromycin and vice versa. The recovery of the solid-phase extraction method was 90% and higher, and the relative standard deviation was about 3%. The limit of quantitation was 0.5 μmol/l when 25 μl of plasma was injected. Comparison with a liquid–liquid extraction method for sample clean-up showed good agreement.

1. Introduction

Roxithromycin and clarithromycin are macrolide antibiotics (see Fig. 1 for structures) with an antibacterial spectrum similar to that of erythromycin. A limited number of papers can be found in the literature concerning the analysis of roxithromycin [1,2] and clarithromycin [3,4] in biological fluids, whereas the documentation of erythromycin is more extensive. Previously, erythromycin and its derivatives in biological fluids have been determined by microbiological assays [5,6]. These methods are, however, laborious and, in the presence of metabolites with antibacterial activity, also less selective than methods employing liquid chromatography.

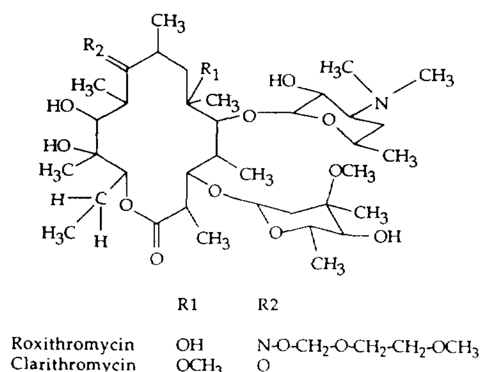

Fig. 1. Structures of roxithromycin and clarithromycin.

Because of the poor UV absorbance of macrolides and the need for derivatization prior to liquid chromatography when using fluorescence detection, electrochemical detection seems to be the most appropriate mode for monitoring these substances [1–4,7–11]. Due to the high potential

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required for oxidation of macrolides and the subsequent high background current, a Coulochem detector appears to be the most advantageous, but also other amperometric detectors have been used [7,8,11].

Sample clean-up by liquid–liquid extraction (LLE) at alkaline pH, followed by evaporation and reconstitution, has been employed in most of the previous assays. This has given recoveries for roxithromycin and clarithromycin ranging from 68 to 90% [1–3,7]. So far only one paper has been published regarding the use of solid-phase extraction (SPE) for sample preparation of erythromycin in serum and urine [9].

We have developed a fast and simple method for the determination of roxithromycin and clarithromycin in plasma for pharmacokinetic studies by using the Prospekt on-line SPE unit. This fully automated system, which has previously been described for analysis of other drugs [12–14], consists of a microprocessor, one to three six-port valves, a solvent-delivery unit and an autosampler.

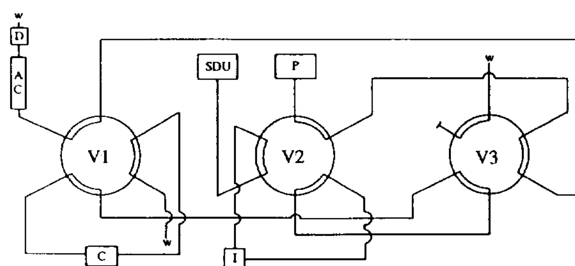

Fig. 2. Schematic disposition of the chromatographic system. V1, V2, V3 = Valves belonging to the Prospekt unit; SDU = solvent-delivery unit; P = pump; I = injector; C = cartridge; AC = analytical column; D = detector; w = waste.

2. Experimental

2.1. Chemicals

All solvents were of HPLC grade (Rathburn, Walkerburn, UK) and reagents of analytical grade (Merck, Darmstadt, Germany). Clarithromycin was provided by Abbot Labs. (Abbot Park, IL, USA) and roxithromycin by Roussel Uclaf (Romainville, France). Water was from an ELGA (Wycombe, UK) purification system.

2.2. Chromatographic system

The chromatographic system comprised a Pharmacia LKB HPLC pump 2248 (Bromma, Sweden), a CMA/200 refrigerated autosampler (CMA Microdialysis, Stockholm, Sweden), a Spark Holland (Emmen, Netherlands) Prospekt module with a microprocessor, a cartridge transport system, three six-port valves and a solvent-delivery unit (SDU), with the capability of delivering up to six solvents. Glass vials (0.3 ml) were from Chromacol (London, UK). A schematic disposition is shown in Fig. 2.

The SPE cartridges (10 × 2 mm I.D.) contained 20 mg of Baker CN packings (30–40 μm) and were distributed by Spark Holland. The analytical column Hypersil BDS C_{18} (3 μm, 100 × 4.6 mm I.D.) came from Shandon (Astmoor, UK) and was thermostatted at 55°C.

The mobile phase pH 7 (ionic strength I = 0.025) contained 4.5 mM NaH_2PO_4, 6.8 mM Na_2HPO_4 and 54% acetonitrile. The three solutions used for conditioning and washing the cartridges were (1) 100% methanol, (2) 10% methanol in water and (3) phosphate buffer pH 10.5 (I = 0.10) containing 29 mM Na_2HPO_4, 1.9 mM Na_3PO_4 and 10% acetonitrile. Prior to use all buffer solutions were filtered through a Millipore (Milford, MA, USA) filter (HA, 0.22 μm) and ultrasonicated for 30 min. The flow-rate over the analytical column was 1.0 ml/min and the eluent was monitored by an ESA (Bedford, MA, USA) Coulochem II electrochemical detector. A dual analytical cell Model 5011 was used with the upstream potential set at + 0.65 V (cell 1) and the downstream potential at + 0.85 V (cell 2). The signal was monitored at the second cell. An ESA carbon in-line filter was placed before the analytical cell to protect it. The potential applied on cell 1 was used to reduce the background current at the second cell. Data were monitored and processed by a Multichrom chromatographic

data system (VG Data Systems, Altrincham, UK).

2.3. Sample preparation

The frozen plasma samples were thawed at room temperature, mixed and centrifuged for 5 min at 1300 g. An aliquot of 100 μl of the samples was pipetted into 1.5-ml glass vials and 100 μl of the internal standard in phosphate buffer pH 10.5 ($I=0.10$), with 10% of acetonitrile, were added. After mixing, the samples were transferred to glass vials (0.3 ml) and placed in the autosampler. A volume of 20–100 μl of the samples was injected.

Reference plasma samples were prepared by mixing 100 μl of drug-free plasma and 100 μl of a solution containing both reference compound and internal standard in phosphate buffer pH 10.5 ($I=0.10$), with 10% of acetonitrile.

2.4. Solid-phase extraction

A new extraction cartridge (connected to valve 1 in Fig. 2) was automatically inserted for each sample and conditioned with 2 ml of solvent 1 (100% methanol) followed by 2 ml of solvent 2 (10% methanol in water) and ca. 4 ml of solvent 3 (phosphate buffer pH 10.5, $I=0.10$, with 10% of acetonitrile) using the SDU. As described earlier [14], the CMA/200 autosampler had in the meantime prepared the sample for injection, and by a signal from the microprocessor the plasma sample was injected onto the SPE cartridge. Prior to injection the flow-rate over the cartridge was changed from 2 to 0.5 ml/min.

After 5 min of loading, when most of the plasma matrix had been washed off the cartridge, valve 1 was switched and the compounds were transferred backwards by the mobile phase onto the analytical column for separation, followed by electrochemical detection. At the same time data collection was started. Valve 1 was switched back after 3 min of elution and the SDU started to wash the capillaries with 2 ml of solvent 2 and 2 ml of solvent 1 at a flow-rate of 2 ml/min. After a total time of 15 min the procedure was repeated. The SPE procedure is summarized in Table 1.

By switching valve 2, the autosampler was directly connected to the analytical column (see Fig. 2). Injections could be made directly onto the analytical column for calculation of extraction recoveries and control of chromatographic conditions. It is also possible to use the third valve of the Prospekt unit for washing the analytical column after a completed series of plasma samples.

Table 1
Solid-phase extraction procedure

Time (min:s)	Switch valve No.	Solvent	Flow-rate (ml/min)	Comment
00:00		1	2.0	Change of cartridge; activation with 100% methanol
01:00		2		Activation with 10% methanol in water
02:00		3		Conditioning with phosphate buffer pH 10.5, $I=0.10$, and 10% acetonitrile
03:50			0.5	Adjusting flow-rate over cartridge
04:00				Injection of sample
09:00	1			Start of elution; start of data collection
12:00	1	2	2.0	End of elution; washing of capillaries with 10% methanol in water
13:00		1		Washing with 100% methanol
14:00				End of washing

3. Results and discussion

3.1. Chromatography

The electrochemical response for the macrolides, measured as the peak areas, increased twofold by increasing pH of the phosphate buffer, used in the mobile phase, from 6 to 7. A further raise of the pH to 8 did not affect the response much and a buffer solution of pH 7 was chosen. The retention of clarithromycin and roxithromycin increased with increasing pH and the theoretical plate count was improved, but at a pH above 7 this effect was less pronounced. In order to further improve the peak shape the column was thermostatted at 55°C. When increasing the temperature from 35 to 55°C an increase of ca. 50% in the theoretical plate count was found for both clarithromycin and roxithromycin. An increase in retention was also observed.

3.2. Solid-phase extraction

We chose a pH (10.5) well above the pK_a values (ca. 9) of roxithromycin and clarithromycin in the loading buffer (solvent 3), in order to get strong retardation on the cartridge. To use a high pH at the SPE did not cause any problems, as a new cartridge was inserted for every new sample.

Two different packing materials of the cartridges were investigated, C_8 and CN. The chromatographic peaks were broader after SPE on C_8 cartridges, compared with CN cartridges. When using C_8 cartridges the theoretical plate count, N, was about 1300 for clarithromycin and roxithromycin, at a loading time of 5 min. For CN cartridges the corresponding value for N was about 5600. CN cartridges were chosen for this method.

The effects of the amount of acetonitrile in the loading buffer and of the loading time on the recovery, were investigated by experimental design [15]. A statistical full factorial design was set up with two factors: (i) the loading time, varied between 2 and 5 min and (ii) the amount of acetonitrile in solvent 3, varied between 10 and 20%. The model comprised 4 (2^2) experiments and 3 centerpoints, that is a loading time of 3.5 min and an amount of acetonitrile of 15%. The recovery was determined by comparing the peak areas after SPE with those obtained by injecting the same amount of an aqueous solution directly onto the analytical column. It was found that a combination of long loading time (5 min) and a high amount of acetonitrile (20%) gave low recoveries of clarithromycin and roxithromycin, below 50%. At the low level of acetonitrile (10%), an increase in the loading time from 2 to 5 min did not affect the recoveries. As the front peaks of the chromatograms were smaller at 5 min than at 2 min, a loading time of 5 min was chosen. The recovery and variability when using 10% of acetonitrile were further examined and are reported under *Quantitation and accuracy*.

3.3. Detection

The response of the electrochemical cell decreased during a series of analyses. It was thus important to have an internal standard that behaved similar to the analyte and compensated for the loss in sensitivity. This was the case with the couple of roxithromycin and clarithromycin. A few hours after a completed series of plasma samples the electrochemical response had regained its initial level. The cell was cleaned once a week by flushing with 6 *M* nitric acid followed by 1 *M* sodium hydroxide.

After injection of ca. 200 plasma samples a decrease in column efficiency could be seen. By adding new packing material onto the top of the column, it was possible to restore the column efficiency, at least temporarily. Washing the column with a mixture of water and methanol and changing the top frit also increased the life time. This was done once a week while the cell was cleaned.

3.4. Stability

Plasma samples containing roxithromycin and clarithromycin were stable at −20 and −70°C for at least 2 months. Plasma samples, containing phosphate buffer pH 10.5 ($I = 0.10$), with 10%

acetonitrile, were stable in a refrigerated autosampler for at least 24 h. Working reference solutions of roxithromycin and clarithromycin in phosphate buffer pH 10.5 ($I = 0.10$), with 10% acetonitrile, were stable for 24 h at room temperature and at least for two days in a refrigerator when kept from light.

3.5. Quantitation and accuracy

The yield of extraction was calculated by comparing the peak areas of a reference plasma sample with the peak areas of the same amount of analyte in a reference aqueous solution injected directly onto the analytical column. When comparing SPE with direct injection, area measurements were used to eliminate any difference in theoretical plate count. The yields of extraction were 89.4 ± 5.5% ($n = 11$) for roxithromycin and 99.0 ± 5.3% ($n = 11$) for clarithromycin at concentrations of 7.0 and 5.0 μM, respectively.

The ratios of the peak height of the analyte to that of the internal standard in the reference plasma samples were measured and used for calculation of plasma concentrations. The within-day variability was ± 3.0% ($n = 10$), when assaying plasma samples containing 5.7 μM roxithromycin, and ± 1.9% ($n = 10$) for clarithromycin at a concentration of 5.0 μM. The between-day relative standard deviation (R.S.D.) was ± 4.0% ($n = 15$) for a plasma sample containing 3.2 μM roxithromycin and ± 5.8% ($n = 8$) for clarithromycin at a concentration of 3.0 μM.

Both the assay of roxithromycin and that of clarithromycin was linear up to 25 μM. The limit of quantitation was 0.5 μM when injecting 25 μl of plasma with an R.S.D. of ± 5.7% ($n = 8$) and ± 1.2% ($n = 8$), respectively. Chromatograms of authentic human plasma samples containing roxithromycin 3.6 μM and clarithromycin 1.8 μM are shown in Figs. 3 and 4, respectively.

3.6. Comparison with an LLE method

The method was validated against a method employing LLE for sample clean-up. When measuring roxithromycin, using that work-up procedure, erythromycin was chosen as internal standard because of more favourable chromatographic conditions. An aliquot of 500 μl of plasma containing roxithromycin or clarithromycin and the internal standard was extracted at alkaline pH with 5 ml hexane containing 20% of isobutanol. The organic phase was separated, evaporated to dryness and reconstituted in the

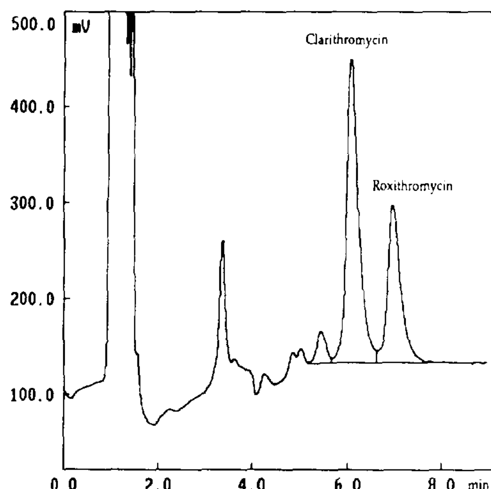

Fig. 3. Chromatogram of an authentic plasma sample containing roxithromycin (3.6 μM) and clarithromycin (internal standard, 4.8 μM). A 50-μl volume of sample was injected.

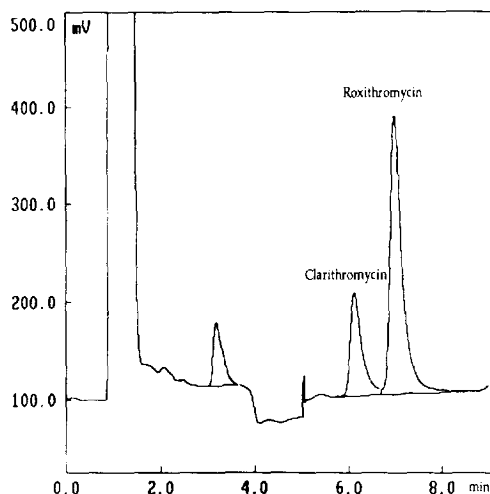

Fig. 4. Chromatogram of an authentic plasma sample containing clarithromycin (1.8 μM) and roxithromycin (internal standard, 7.0 μM). A 50-μl volume of sample was injected.

stituted in the mobile phase, which consisted of a phosphate buffer pH 7 and 45% of acetonitrile. The compounds were separated on a 3 μm Hypersil BDS C_{18} column (100 × 4.6 mm I.D.) at a flow-rate of 1.2 ml/min. The column was thermostatted at 55°C and the eluent was monitored by an ESA Coulochem I electrochemical detector with a dual analytical cell Model 5011.

In order to compare the LLE method with the SPE method, results from the analysis of 32 authentic plasma samples of clarithromycin and 28 samples of roxithromycin, ranging from 360 to 15 600 nmol/l, were used. It was assumed that the logarithmic transformation of the values follow a bivariate normal distribution. The estimated ratio of the methods and the 95% confidence interval (CI) were 1.011 CI (0.977, 1.046) for clarithromycin and 1.007 CI (0.981, 1.034) for roxithromycin. Accordingly, the results from the LLE and SPE methods showed excellent agreement.

3.7. Ruggedness

The ruggedness of the method was investigated using a statistical full factorial design. The factors tested for their effect on the extraction recovery were (i) the amount of acetonitrile in solvent 3, (ii) the loading time of the SPE and (iii) the elution time. These factors were varied in small intervals, that is (i) 8 to 12%, (ii) 4 to 6 min and (iii) 2 to 4 min, respectively. For 3 factors there are 8 (2^3) possible combinations of factor levels. Moreover three center points were investigated, that is, an amount of 10% acetonitrile, a loading time of 5 min and an elution time of 3 min. The levels of the center points were chosen according to the described method. A statistical analysis of variance (ANOVA) was performed to evaluate any effect of the experimental variables. No statistically significant effect on the extraction recoveries for either roxithromycin or clarithromycin was found when varying any of the factors chosen, and the method was proved rugged within the intervals studied.

4. Conclusions

The described method for determination of the macrolide antibiotics, roxithromycin and clarithromycin, in plasma samples yielded high recoveries and good precision. Validation of this automated SPE method against an LLE method gave results showing excellent agreement.

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