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Note

Analysis of erythromycin and roxithromycin in plasma or serum by high-performance liquid chromatography using electrochemical detection

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Erythromycin and roxithromycin are macrolide antibiotics, the former being used since 1952 and the latter a relatively new antibiotic which is a derivative of erythromycin.

A number of methods used in the past for the quantitation of erythromycin were microbiological [1] or thin-layer chromatographic [2] in nature, both of which are lengthy and tedious compared to the faster and specific methods based on high-performance liquid chromatography (HPLC). Although HPLC methods using fluorescence and UV detection have been developed [3,4], these methods are not practical because of a complex post-column derivatization procedure used with fluorescence detection and a lack of sensitivity with UV detection because of the poor absorbance of erythromycin. The most sensitive method of detection for erythromycin and related compounds is electrochemical detection (ED) and several HPLC procedures using this detection system have been published [5–7]. Only one of these [7] indicates chromatography conditions for roxithromycin where it was used as an internal standard.

The method described here was developed to quantitate erythromycin or roxithromycin in plasma or serum in a study that investigated absorption of single and multiple doses of both drugs.

EXPERIMENTAL

Reagents

Erythromycin (analytical-reagent grade) was obtained from Sigma (St. Louis, MO, U.S.A.). The roxithromycin used had a purity of at least 955 μg/mg deter-

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mined by microbiological assay and was supplied by Roussel Uclaf (Paris, France). Acetonitrile and methanol were of HPLC grade and supplied by Mallinckrodt (Melbourne, Australia). All other reagents used were analytical-reagent grade.

Instrumentation

The HPLC system consisted of an ICI–Kortec dual-piston pump, an ESA Coulochem electrochemical detector (Model 5100A) fitted with a Model 5010 flow cell, an in-line carbon filter to the detector and a WISP autoinjector (Waters). The chromatography was recorded on a BBC metrawatt dual-pen recorder with the pens set at 100 and 500 mV for full scale deflection.

Chromatographic conditions

The columns used for the chromatography were an octyl (25 cm×4.6 mm, 5 μm) column for erythromycin and a methyl (25 cm×4.6 mm, 5 μm) column for roxithromycin both of which were supplied by ICI Australia (Instrument Division, Adelaide, Australia). The mobile phase used for erythromycin analysis (octyl column) consisted of 40% acetonitrile in distilled water containing 15 m*M* diammonium hydrogenphosphate as buffer with the pH adjusted to 6.75. The mobile phase used for roxithromycin analysis (methyl column) consisted of 42% acetonitrile in distilled water containing 15 m*M* diammonium hydrogenphosphate as buffer with the pH adjusted to 7.0. The adjustment of pH was achieved by the dropwise addition of concentrated phosphoric acid to the mobile phase and all pH changes were monitored with a pH meter.

Prior to use, the mobile phases were filtered through a 0.22-μm Nylon 66 membrane under reduced pressure and then left at least 12 h recycling through the electrochemical cell to reach a constant background current. The potentials applied to the detector for solvent recycling or analysis were +0.60 V for cell 1 and +0.75 V for cell 2, with the gain set at 5.

The solvent flow-rate was 2.0 ml/min and all chromatography was performed at ambient temperature. The mobile phase remained stable for five days when kept recycling and the detector showed no loss of sensitivity until after four weeks of constant use. Sensitivity was restored by washing the cells with 50% nitric acid, rinsing with distilled water, then washing with 1.0 *M* sodium hydroxide and then finally rinsing with distilled water.

Standards

Bulk standards having a concentration of 1000 mg/l for erythromycin and roxithromycin were prepared by dissolving 50 mg of each compound in 50 ml of methanol. These solutions were prepared fresh every four weeks. Working standards for each compound containing 1, 5, 10, 25, 50 and 100 mg/l were prepared by diluting the bulk standard with methanol–water (1:1). These solutions were prepared daily. Plasma standards were prepared by diluting 0.1 ml of each of the working standards with 0.9 ml of drug-free plasma, to give standards containing 0.1, 0.5, 1.0, 2.5, 5.0 and 10.0 mg/l. These also were prepared fresh daily.

In this study erythromycin and roxithromycin were not given concomitantly. Therefore roxithromycin was used as an internal standard for the analysis of

erythromycin and erythromycin as the internal standard for the analysis of roxithromycin. The concentration of each compound in aqueous solution when used as an internal standard was 25 mg/l.

Method

To a labelled 15-ml culture tube fitted with a PTFE-lined screw cap was added 1 ml of sample (plasma or serum) or standard. To this was added 0.1 ml of internal standard solution, 0.05 ml of 1 *M* sodium carbonate solution and 6 ml of extraction solvent (diethyl ether–isopentane, 3:2). The tube contents were mixed by inversion for 20 min and then separation of aqueous and organic layers was achieved by centrifugation for 5 min at 1500 *g*. The lower aqueous layer was frozen by immersion in a dry ice–acetone bath and the upper organic layer transferred to an appropriately labelled conical glass tube. The organic solvent was removed by vortex evaporation and the residue reconstituted with 0.20 ml of a buffered methanol solution (methanol–15 m*M* phosphate buffer, pH 6.5, 1:1). This was followed by the addition of 1 ml of hexane–octanol (95:5). The tube contents were vortex-mixed and then the aqueous and organic layers separated by centrifugation for 5 min at 1500 *g*. The lower aqueous layer was transferred to an insert tube for the WISP and 0.075–0.1 ml was injected.

RESULTS AND DISCUSSION

Chromatography

Chromatograms for erythromycin and roxithromycin are illustrated in Figs. 1 and 2, respectively, where (A) represents blank plasma, (B) a prepared standard containing 1.0 mg/l analyte and (C) a plasma sample from a subject. With the analysis of erythromycin (Fig. 1) the retention times for erythromycin and roxithromycin were 8.8 and 23.8 min, respectively. Both peaks were well resolved of endogenous material that separated under these conditions. Similarly for the analysis of roxithromycin (Fig. 2) the retention times for erythromycin and roxithromycin were 8.05 and 12 min, respectively. Roxithromycin elutes well clear of any potentially interfering endogenous material, but erythromycin elutes very near to the peak of an endogenous compound. With a new column erythromycin is adequately resolved from this potential interference, but with use separation decreases. However, for concentrations above 0.5 mg/l the interference is minimal, with a small shoulder appearing near the baseline and when erythromycin is used as internal standard (0.1 ml of 25 mg/l) the interference is not observable. For roxithromycin, a methyl column is preferable because of the much shorter retention times and the resulting sharp peaks enhance the sensitivity of the assay.

Detection

A number of methods for the quantitation of erythromycin using HPLC and the ESA dual-cell electrochemical detector have been published [5–7]. Cell potentials between +0.8 and +0.9 V have been used for the sample cell. These potentials were found to be suitable for the quantitation of roxithromycin but endogenous plasma constituents were also detected at these high oxidative po-

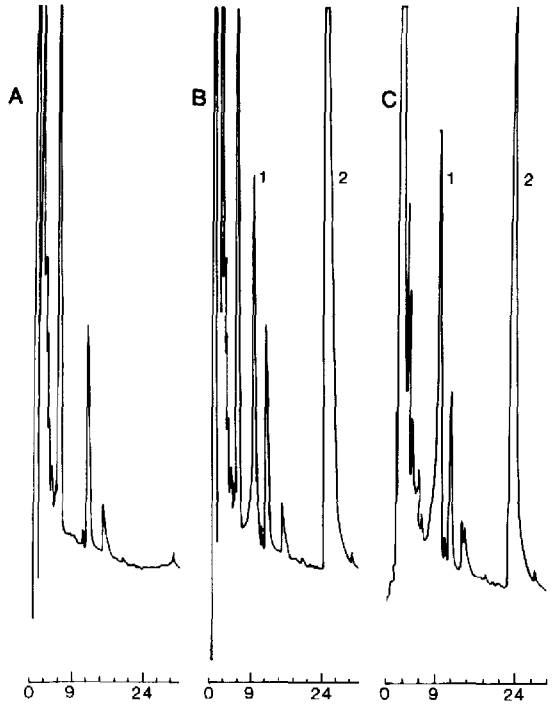

Fig. 1. Chromatograms obtained from extracted human plasma analysed using the system for quantitating erythromycin. (A) Blank human plasma; (B) plasma standard containing 1.0 mg/l erythromycin (peak 1) and the internal standard roxithromycin (peak 2); (C) plasma sample from a subject.

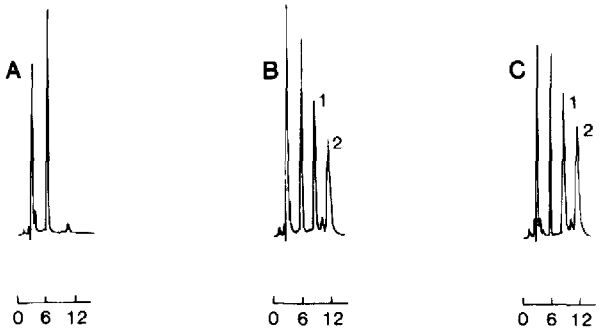

Fig. 2. Chromatograms obtained from extracted human plasma analysed using the system for quantitating roxithromycin (A) Blank human plasma; (B) plasma standard containing 1.0 mg/l roxithromycin (peak 2) and the internal standard erythromycin (peak 1); (C) plasma sample from a subject.

tentials. The peak that partially interferes with erythromycin can be diminished in size by reducing the potential to +0.75 V without significantly reducing the peak height of erythromycin or roxithromycin. Cell 1, the screen cell, was set at +0.6 V.

Extraction procedure

Most published non-microbiological procedures for the analysis of erythromycin have an extraction step [2–7], and the solvent of choice for the extraction of erythromycin from plasma or blood is diethyl ether. During method development, it was observed that dichloromethane and chlorobutane quantitatively extract erythromycin and roxithromycin. These solvents did produce cleaner extracts than diethyl ether with ED, but had the minor disadvantage of being denser than water (dichloromethane) and having a relatively high boiling point (chlorobutane). These characteristics would increase the analysis time by requiring an aspiration step if dichloromethane were used or a longer time for solvent evaporation if chlorobutane were used. *tert.*-Butyl ether has been used to extract erythromycin [6] and may give cleaner extracts than diethyl ether, because of the increased lipophilic nature of the molecule, but its cost and availability were a disadvantage. Cleaner extracts were eventually obtained by introducing isopentane into the extraction solvent and a mixture of diethyl ether–isopentane (3:2) gave cleaner extracts without reducing the quantitative extraction of erythromycin and roxithromycin.

After the extraction solvent was removed by vortex evaporation, the residue was reconstituted with methanol–15 m*M* phosphate buffer, pH 6.5 (1:1) and, finally, the reconstitution solvent was extracted with 1 ml of 5% octanol in hexane to remove any potentially interfering lipophilic material. A similar reconstitution procedure was used by Duthu [6] and is essential in reducing the amount of interferences seen with ED to acceptable levels.

Recovery of analytes

Recoveries were performed in duplicate at each of the five calibrator concentrations (0.1, 0.5, 1.0, 2.5 and 5.0 mg/l) for each analyte. The peak heights for erythromycin and roxithromycin at each standard concentration were compared with those prepared in the mobile phase used for the analysis of that compound at five times the concentration of the calibration standards. The recoveries (mean $\pm$S.D., $n=10$) of erythromycin and roxithromycin were 85.6 ± 3.8 and $88.9 \pm 3.5\%$, respectively.

Accuracy and precision

To determine the intra-assay precision and accuracy of the method, plasma samples were prepared containing 0.10 and 5.0 mg/l of each analyte (erythromycin and roxithromycin). Samples of each analyte were analysed ten times on a within-day basis and the concentration (mean $\pm$S.D.) and coefficient of variation (C.V.) of each analyte in each control were calculated. For erythromycin, 0.1 mg/l, the mean $\pm$S.D. measured concentration was 0.101 ± 0.006 mg/l with a C.V. of 5.94%, while for erythromycin, 5.0 mg/l, the mean $\pm$S.D. measured concentration was 4.960 ± 0.091 mg/l with a C.V. of 1.83%.

For roxithromycin, 0.1 mg/l, the mean $\pm$S.D. measured concentration was 0.095 ± 0.004 mg/l with a C.V. of 4.21%, while for roxithromycin, 5.0 mg/l, the mean $\pm$S.D. concentration was 5.17 ± 0.081 mg/l with a C.V. of 1.57%.

Inter-assay accuracy and precision were determined by analysing samples con-

taining 0.1 and 5.0 mg/l erythromycin and 0.25 and 5.0 mg/l roxithromycin on a between-day basis. For each control a 1.0-ml aliquot was taken at the time of preparation and dispensed into a 15-ml cultured tube fitted with a PTFE-lined screw cap. These tubes and contents were frozen at −20°C and a high and low control used for each analytical run for each analyte throughout the course of the pharmacokinetic study to check calibration data. Storage of the quality controls as predispensed aliquots reduces the likelyhood of decomposition of the analytes through repeated freezing and thawing of bulk controls.

For erythromycin, 0.10 mg/l, the mean ±S.D. measured concentration was 0.093±0.009 mg/l ($n=20$) with a C.V. of 9.67%, while at 5.0 mg/l, the mean ±S.D. measured concentration was 4.713±0.203 mg/l ($n=24$) with a C.V. of 4.31%.

For roxithromycin, 0.25 mg/l, the mean ±S.D. measured concentration was 0.232±0.015 mg/l ($n=24$) with a C.V. of 6.47%, while at 5.0 mg/l, the mean ±S.D. measured concentration was 5.015±0.150 mg/l ($n=24$) with a C.V. of 2.99%.

Linearity and limit of detection

Calibration data, when plotted as peak-height ratio (peak height of analyte divided by peak height of internal standard) versus concentration, gave a straight-line calibration curve for each analyte. Linear regression analysis was performed on each set of calibration data and sample concentrations were calculated from the equations obtained. The following linear equations are typical for a data set: erythromycin, $y=0.421x+0.001$ ($r=0.999$); roxithromycin, $y=0.324x-0.001$ ($r=0.999$), where x is the peak-height ratio and y is the concentration in mg/l.

The limit of detection, defined here as the concentration where the ratio of peak height of analyte to background is 10:1, is 0.01 mg/l for both erythromycin and roxithromycin.

Interferences

Although the subjects in this study were taking no other medication, various drugs were chromatographed on each system to evaluate potential interferences. Table I gives the retention-time ratios for the drugs listed relative to erythromycin (column A) and relative to roxithromycin (column B). The retention-time ratios shown for column A were calculated from retention data obtained from the HPLC system used to quantitate erythromycin. Similarly the retention-time ratios shown for column B were calculated from retention data obtained using the HPLC system to quantitate roxithromycin.

As can be seen from the data, the system used to quantitate roxithromycin is somewhat more susceptible to interference. In addition, the only peaks observed were those due to endogenous substances in the plasma and from the two drugs. No extra peaks were seen which could be attributed to drug metabolites or degradation products. The metabolites of these drugs were not available and therefore it was not possible to check their potential for interference on the two systems.

TABLE I

DRUGS TESTED FOR INTERFERENCE

Drug tested	Column A (retention time relative to erythromycin)	Column B (retention time relative to roxithromycin)
Erythromycin	1	0.76
Roxithromycin	2.23	1
Caffeine*	–	–
Alprenolol	0.99	0.53
Propranolol	1.11	0.54
Labetalol	0.79	0.47
Oxprenolol	0.83	0.53
Flecainide	1.51	0.10
Amiodarone*	–	–
Mexiletine*	–	–
Protriptylline	1.88	0.67
Amitriptylline	2.63	0.93
Disopyramide	0.81	0.79
Procainamide	0.39	0.67
Doxepin	1 67	0.82
Prazosin	0.57	0.40
Pindalol	0.49	0.45
Paracetamol*	0.24	–
Quinidine	0.98	0.80
Phenytoin*	–	–
Phenobarbitone*	–	–
Erythromycin estolate	2.42	1.04
Erythromycin ethylsuccinate	2.86	0.76
Oleandomycin	0.74	0.68

*Either not detected or eluted with the solvent front.

CONCLUSION

The method reported here for the analysis of erythromycin is as sensitive as other reported methods for this drug and the method for the analysis of roxithromycin has similar sensitivity. The performance of these assays proved to be excellent during the course of the pharmacokinetic study.

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