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# Determination of dirithromycin purity and related substances by high-performance liquid chromatography

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ABSTRACT

High-performance liquid chromatographic methods for the characterization of dirithromycin, a macrolide antibiotic derived from erythromycin, are described. Chromatography is performed on a Hypersil ODS column using a mobile phase consisting of acetonitrile–methanol–50 m*M* potassium phosphate buffer, pH 7.5 (44:19:37) with ultraviolet detection at 205 nm. The strength of the phosphate buffer can be used to control the selectivity of the separation of dirithromycin and related substances, especially the separation of erythromycylamine. The addition of methanol to the mobile phase improves peak shapes for the compounds of interest. Validation data for purity and related substances methods are described.

## INTRODUCTION

Dirithromycin, an oxazine derivative of erythromycin, is a new gram-positive macrolide antibiotic currently undergoing clinical evaluation. Accurate, well-characterized methods for the determination of dirithromycin purity and related substances are necessary for development and quality control of the bulk drug production process and assessment of bulk drug stability. The purity method must be free from interference by process-related impurities and degradation products. The related substances method must be able to detect and quantify these same impurities.

High-performance liquid chromatography (HPLC) has been widely used for the analysis of erythromycin and related macrolides because of its ability to separate closely related compounds arising from biosynthetic, synthetic, or metabolic processes [1–16]. Methods for biological fluid analysis have been designed primarily for quantification of the drug itself rather than low levels of related impurities [11–16]. The emphasis of methods for quality control has been separation of the main component from stucturally similar impurities and adequate quantification of these impurities [1–9]. Common problems encountered with methods for erythromycin and derivatives include peak tailing, poor resolution of impurities, and short column life due to the use of high-pH mobile phases and/or high column temperature [1–4,6,9,10]. A dependence of separation on column history or conditioning has also been observed [1,10].

The structures of dirithromycin and related substances are given in Fig. 1. Dirithromycin B is produced from the corresponding erythromycin B factor present in the starting material. Erythromycin hydrazone and erythromycylamine are precursors in the synthesis of dirithromycin and may be present at low levels in samples. Erythromycylamine may also arise from hydrolysis of dirithromycin in neutral or slightly acidic solution. Dirithromycin forms an equilibrium amount of epi-dirithromycin in solution with epimerization accelerated under acidic conditions [17].

The determination of dirithromycin and related substances represents a distinct challenge because of the lack of a UV chromophore in the molecule, the tendency to produce tailing peaks, and the instability of dirithromycin under neutral or slightly acidic

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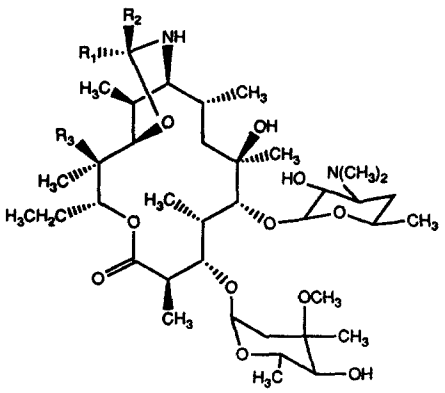
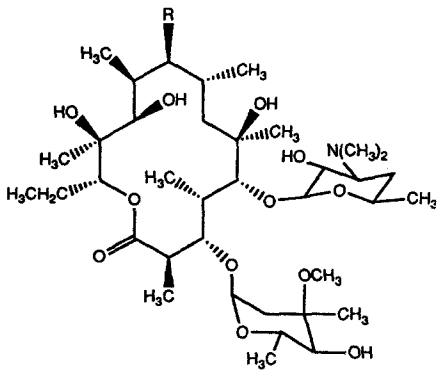


| Compound | R1 | R2 | R3 |
|---|---|---|---|
| Dirithromcyin | $CH_3OCH_2CH_2OCH_2$ | H | OH |
| Epi-dirithromycin | H | $CH_3OCH_2CH_2OCH_2$ | OH |
| Dirithromycin B | $CH_3OCH_2CH_2OCH_2$ | H | H |



| Compound | R |
|---|---|
| Erythromycylamine | $NH_2$ |
| Erythromycin hydrazone | $NNH_2$ |

Fig. 1. Structures of dirithromycin and related substances.

conditions. Although dirithromycin has been determined by HPLC with electrochemical detection [12], this detection mode is not as easily established in quality control laboratories as UV detection. Ultraviolet detection at low wavelengths limits the mobile phase modifiers that can be used to enhance selectivity and peak shape. Also, the need to use a relatively high-pH mobile phase results in concerns about column life and method performance over time. This report describes methods for dealing with the above problems to provide suitable assay procedures for the determination of dirithromycin and related substances in the bulk drug.

## EXPERIMENTAL

### *Reagents*

HPLC-grade acetonitrile and methanol were obtained from EM Science (Gibbstown, NJ, USA). Buffer solutions were prepared using appropriate concentrations of potassium phosphate, mono and dibasic, also from EM Science. Water for mobile phase and sample solutions was purified with a Milli-Q system from Millipore (Milford, MA, USA).

Dirithromycin and the related substances shown in Fig. 1 were prepared at Eli Lilly. Sample solutions were prepared as described in the text.

### *Apparatus*

The chromatographic system consisted of a Model 600 pump with column heater (Waters, Bedford, MA, USA), a Model 728 autoinjector (Alcott, Norcross, GA, USA) with a 10-$\mu$l fixed-loop injection valve (Valco, Houston, TX, USA), and a Model 787 UV detector set at 205 nm (Applied Biosystems, Ramsey, NJ, USA). Chromatograms were recorded using an in-house data aquisition system. The column temperature was maintained at 40°C, except during temperature effect studies. The flow-rate was 2.0 ml/min. The following columns were investigated: Hypersil ODS (Jones Chromatography, Littleton, CO, USA), YMC Basic and YMC ODS (YMC, Morris Plains, NJ, USA), Capcell Pak $C_{18}$ (Dychrom, Sunnyvale, CA, USA), Zorbax RX and Zorbax ODS (MacMod Analytical, Chadds Ford, PA, USA), Supelcosil LC-18-DB (Supelco, Bellefonte, PA, USA), Ultrasphere ODS (Beckman, San Ramon, CA, USA), Asahipak ODP-50 (Advanced Separation Technologies, Whippany, NJ, USA), ACT-1, 150 mm × 4.6 mm I.D. (Anspec, Ann Arbor, MI, USA), and Unisphere-PDB, 8 $\mu$m particle size (Biotage, Charlottesville, VA, USA). Unless indicated otherwise, all column dimensions were 250 mm × 4.6 mm I.D. with 5-$\mu$m particles.

## RESULTS AND DISCUSSION

### *Column choice*

Initial assay development indicated that a mobile

phase pH greater than 7.0 and elevated column temperature were necessary to achieve adequate separation and peak shape. These conditions, however, promoted short column life and associated reproducibility problems. Polymer columns with greater stability to high-pH eluents (Asahipak ODP-50, ACT-1) were investigated, but poor resolution and peak shape similar to that observed by Kibwage *et al.* [4], for erythromycins was obtained. A polymer-coated column (Capcell Pak $C_{18}$) was investigated using a pH 8.0 mobile phase. It provided acceptable resolution initially, but retention times drifted and selectivity was lost in 2–3 d. A polybutadiene-coated alumina column (Unisphere-PBD) was also used without success.

Efforts were then focussed on identification of silica-based reversed-phase columns that would provide the necessary selectivity with good stability. Columns investigated include the following: Zorbax ODS, Zorbax RX, YMC ODS, YMC Basic, Supelcosil LC-18-DB, Ultrasphere ODS, and Hypersil ODS. Of these, the Ultrasphere ODS and Hypersil ODS columns provided the best combination of selectivity and stability. These two columns displayed similar selectivity for the compounds of interest. The Hypersil ODS column provided longer column life, so it was used for further optimization.

*Organic modifier*

It was possible to control the retention of dirithromycin using acetonitrile to adjust eluent solvent strength, but badly tailing peaks were obtained. The retention and peak shape of dirithromycin and related substances were investigated as methanol was added to the mobile phase. Toluene was also included in the component mixture as a possible impurity from the production process. Fig. 2 shows chromatograms obtained as the methanol content was increased at the expense of the phosphate buffer. The peak shape of the macrolides improved dramatically with increases in methanol and, unexpectedly, their retention times remained almost constant. Plots of ln $k'$ (capacity factor) *vs.* % organic modifier are shown in Fig. 3. A linear plot with negative slope was obtained for toluene, indicating normal behavior for a simple hydrophobic retention mechanism. In contrast, the plots for the macrolides were almost flat. The phosphate buffer and methanol have roughly equal eluting strength

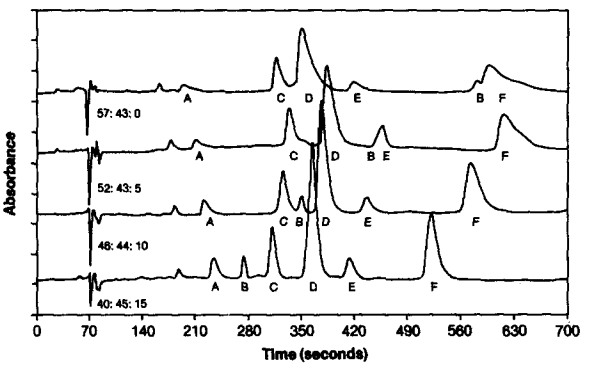



Fig. 2. Effect of methanol on separation and peak shape of dirithromycin and related substances. Mobile phase: 50 m*M* potassium phosphate, pH 7.3–acetonitrile–methanol in the ratios indicated; A = erythromycylamine (4 μg), B = toluene (0.02 μg), C = erythromycin hydrazone (2 μg), D = dirithromycin (20 μg), E = epidirithromycin (4 μg), F = unknown.

for dirithromycin and related substances, suggesting that retention for these compounds is controlled by a combination of hydrophobic and ion-exchange mechanisms.

*Buffer concentration*

The buffer concentration was also found to be a key parameter in controlling the separation of

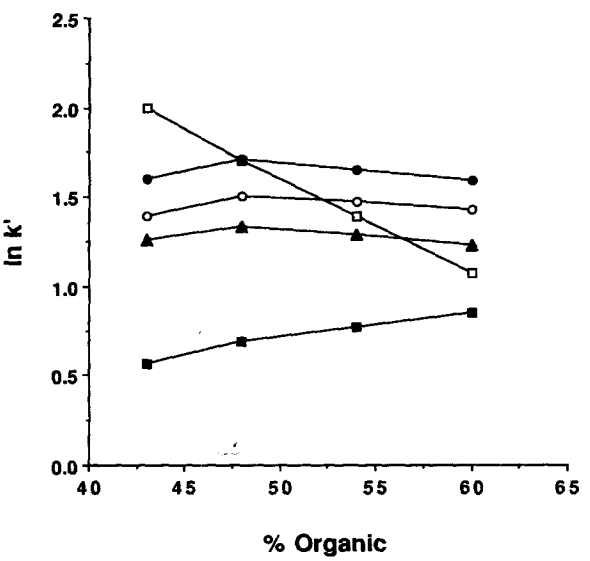



Fig. 3. Ln $k'$ *vs.* % organic solvent in mobile phase: □ = toluene, ■ = erythromycylamine, ▲ = erythromycin hydrazone, ○ = dirithromycin, ● = epidirithromycin.

dirithromycin and related substances. This is consistent with the influence of the aqueous portion of the eluent described above. Capacity factors are plotted as a function of buffer concentration in Fig. 4. While increases in buffer concentration produced slight decreases in retention for most sample components, the retention of erythromycylamine was dramatically affected. As evident in Fig. 4, the buffer concentration could be adjusted to elute erythromycylamine in the desired position relative to the other components.

*pH*

The pH dependence of the separation is illustrated by the plot of *k' vs.* pH at constant buffer concentration in Fig. 5. Mobile phase pH values greater than 7.0 were necessary for optimum resolution and peak shape. As has been observed with other macrolides, increases in pH produced longer retention and better resolution [14,15]. Better peak shape was also observed at higher pH for dirithromycin and related substances. As expected, pH had no effect on toluene retention. Another concern about the mobile phase pH was on-column epimerization of dirithromycin. This would be manifested as a distortion in the trailing side of the dirithromycin peak,

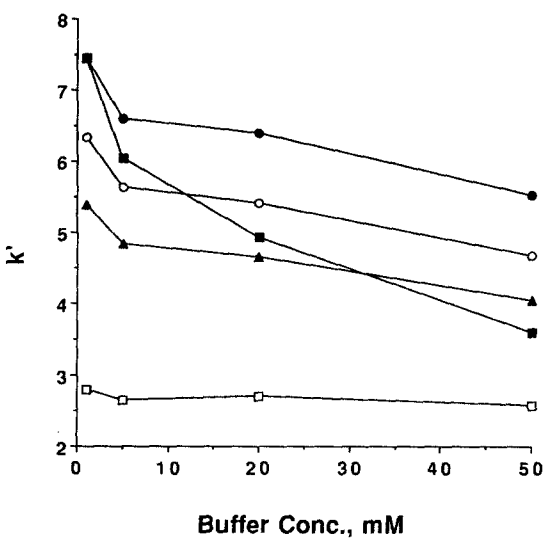



Fig. 4. Effect of buffer concentration on retention of dirithromycin and related substances. Buffer = potassium phosphate, pH 8.0; mobile phase = buffer–acetonitrile–methanol (36:50:14); column temperature = 45°C; symbols as in Fig. 3.

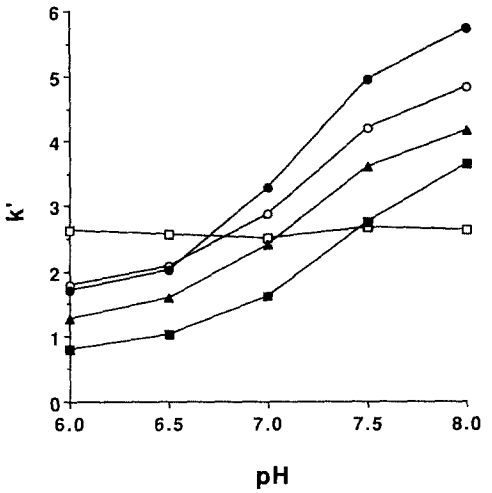



Fig. 5. Effect of pH on retention of dirithromycin and related substances. Mobile phase = 50 m*M* potassium phosphate–acetonitrile–methanol (36:50:14); column temperature = 45°C; symbols as in Fig. 3.

since the epimer was eluted closely after. No significant distortion was observed, indicating that little epimerization occurred during the time between injection and exit from the column at a flow-rate of 2.0 ml/min.

*Temperature*

Column temperature also affected dirithromycin retention, although not as significantly as other parameters. Retention times increased slightly with increases in temperature rather than decreased as might normally be expected. This inverse temperature dependence of retention has been observed for other erythromycin derivatives [1] and tylosin, another macrolide [18].

*Final conditions*

A number of factors were considered when choosing the final conditions for dirithromycin analysis. Although high mobile phase pH and high column temperature gave the best separations, the conditions led to short column life. Values of 7.5 for pH and 40°C for column temperature were chosen as a compromise between column lifetime and resolution. Acceptable resolution was obtained and columns could normally be used for over 300 h.

A mobile phase composition of 50 m*M* phosphate

buffer, pH 7.5–acetonitrile–methanol (37:44:19) was chosen to provide the best separation and peak shape for dirithromycin and known impurities. As shown in Fig. 6, these conditions gave good selectivity and peak shape. Erythromycylamine was of particular concern since it is a degradation product as well as a process-related impurity. Under the conditions chosen, it was separated from other known and unknown impurities that appeared in most samples.

*Other considerations*

It is often impractical or impossible to use reference standards to quantify each related substance in a sample such as dirithromycin, so a common practice is to determine related substances relative to a diluted standard of the main component [2,19]. Consideration of the response of the related substance relative to the main component is important when using this approach. A relative response factor, if significantly different from 1.0, can be applied to correct for differences in absorptivity at the detection wavelength. This was necessary if toluene or erythromycin hydrazone were observed in dirithromycin samples. They absorb much more strongly than dirithromycin as indicated by relative response factors of 64 and 4.1, respectively. Normally, these impurities were not observed in samples. All other impurities were determined relative to a dilute dirithromycin standard with no response correction.

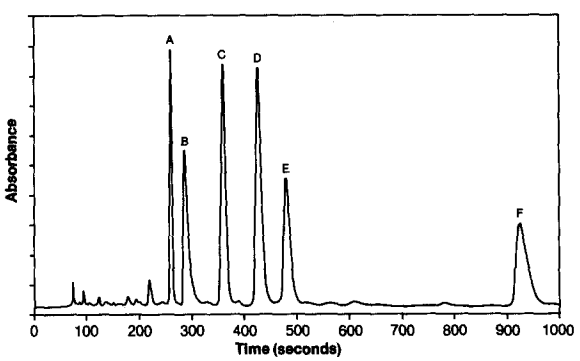



Fig. 6. Separation of dirithromycin and related substances using final assay conditions. Mobile phase = 50 m*M* potassium phosphate buffer, pH 7.5–acetonitrile–methanol (37:44:19); A = toluene (0.1 μg), B = erythromycylamine (10 μg), C = erythromycin hydrazone (4 μg), D = dirithromycin (20 μg), E = epidirithromycin (10 μg), F = dirithromycin B (10 μg).

Sample concentration and injection volume were chosen to provide adequate response without overloading the column and degrading resolution. Concentrations of 2 mg/ml and 10 mg/ml were used for the purity and related substances methods, respectively. A 10-μl injection was used for both methods. Higher concentration or larger injection volume for the related substances method caused loss of resolution between the main peak and components that were eluted just before it.

The stability of sample solutions before injection was investigated. Sample solution stability was greatly dependent on the diluent employed. Epimerization was sufficiently rapid in aqueous buffer/organic diluents to preclude predissolution of samples for automated analysis. Stability of these solutions was increased using a refrigerated autoinjector at 4°C, but epimerization was still observed in less than 4 h. Solutions were stable for 4 h at room temperature, however, in a completely organic diluent composed of acetonitrile–methanol (70:30). Methanol was necessary for adequate solubility of the sample. Adverse effects on the chromatography, such as distorted peak shape, were not observed even though this diluent has a greater solvent strength than the mobile phase. Peaks were broadened only slightly compared to samples dissolved in mobile phase. For automated analysis, stability considerations outweighed the marginal loss of column efficiency, so the completely organic diluent was used.

*Method validation*

Linearity of the purity method was evaluated over a concentration range of 1.66–2.70 mg/ml which encompasses the nominal assay concentration of 2 mg/ml. A coefficient of determination of 0.9996, a relative standard deviation (R.S.D.) of 0.4%, and a log–log slope of 1.00 were obtained.

The reproducibility of the purity method was determined by repeated analysis of a control sample over a period of 6 months. During this time, the analysis was performed by three analysts, on four instruments, using seven columns. An R.S.D. ($n$ = 40) of 0.47% was obtained for the average of duplicate analyses done each day. This demonstrates excellent intralaboratory precision over a number of variables known to affect assay performance.

Linearity of the related substances method was evaluated at the low and high concentration ranges. Dilute standards of dirithromycin over a range of 0.01–0.2 mg/ml provided a coefficient of determination of 0.9996, an R.S.D. of 2.7%, and a log–log slope of 1.03. This range corresponds to 0.1–2.0% related substances in the sample solution. The linearity of total peak area for related substances in sample solutions over a range of 5–15 mg/ml was also determined. A coefficient of determination of 0.9926, an R.S.D. of 4.0%, and a log–log slope of 1.02 were obtained. The above data demonstrate the linearity of response for the standard and sample solutions used in the related substances method.

Reproducibility for related substances was also determined with a control sample. As with the purity method, this measure of precision incorporated variability due to different days, analysts, instruments, and columns. For a sample containing 1.9% related substances, the R.S.D. ($n$ = 29) over a 5-month period was 8%. As expected, the within-day R.S.D. of 2.4% was much lower because it was not affected by as many variables.

*System suitability*

Dirithromycin reacts in mobile phase solution to form epidirithromycin and erythromycylamine. These compounds provide an excellent means of checking the suitability of the chromatographic system each time the method is performed. The system suitability solution was prepared by allowing a 2.5 mg/ml solution of dirithromycin in mobile phase to stand at room temperature for 24 h. After this time, the relative concentrations of the three compounds remained constant for at least 1 month. Dirithromycin peak tailing and resolution from the degradation products were established as meaningful system suitability parameters. Based on data collected over a period of six months ($n$ = 149), criteria for an acceptable system were dirithromycin peak tailing of less than 2.0, resolution between dirithromycin and erythromycylamine of at least 5.0, and resolution between dirithromycin and epidirithromycin of at least 2.0. When these criteria were not met, the mobile phase was adjusted slightly or the column was replaced.

## CONCLUSIONS

A mobile phase consisting of methanol, acetonitrile and phosphate buffer with a Hypersil ODS column was shown to provide separation of dirithromycin and related substances. Methanol reduced peak tailing and the phosphate buffer concentration could be adjusted to control the retention of erythromycylamine relative to the other macrolides studied. The methods described for purity and related substances provided good precision and are suitable for quality control of the bulk drug substance.

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