Determination of Erythromycin and Related Substances in Enteric-Coated Tablet Formulations by Reversed-Phase Liquid Chromatography

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ABSTRACT: An isocratic method for the identification and quantitation of erythromycin and related substances in enteric-coated tablet formulations using high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection at 205 nm is described. A novel method for sample preparation using a molecular weight centrifuge filter to reduce the interferences observed from polymeric tablet coating material is also presented. Erythromycin HPLC assays are best run at high pH; therefore, various polymer columns were evaluated. The resulting HPLC method that was developed has several advantages over current pharmacopeial assay methods for enteric-coated erythromycin tablets. Comparative data from both methods for the same batch of EryTab® tablets are presented. The method can also be applied to various other erythromycin formulations, including particle-coated tablets, erythromycin stearate tablets, and erythromycin ethylsuccinate suspensions and fermentation broths. A C18 Polymeric column is used with a mobile phase composition of 0.02 *M* potassium phosphate dibasic buffer (pH 9): acetonitrile (60:40) and flow rate of 1 mL/min. This method is more sensitive, specific, and rugged than the pharmacopeial method. © 2000 Wiley-Liss, Inc. and the American Pharmaceutical Association J Pharm Sci 89: 1097–1105, 2000

Keywords: erythromycin; HPLC; polymer column; enteric-coated

INTRODUCTION

Erythromycin base is marketed in various tablet and liquid formulations, all of which are designed to physically protect the drug from gastric degradation and thus increase bioavailability. The potency of erythromycin containing products is defined by the USP[1] as:

Erythromycin (mg) = Erythromycin A (mg) + Erythromycin B (mg) + Erythromycin C (mg)

where the major component is erythromycin A. Potency has historically been determined by a microbiological assay measuring the inhibition of

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growth of *Staphylococcus Aureus*. However, this assay measures the total biological activity of erythromycin, which may also be due to related substances such as erythromycin D, E and F.

Erythromycin has at least three known degradation products: anhydroerythromycin A (ANH), formed from the loss of one water molecule in acid;[2,3] erythromycin A enol ether (EEA), formed in equilibrium in acidic aqueous media;[4] and pseudoerythromycin A enol ether (PsEEA), formed in either neutral or alkaline conditions. Several manufacturing impurities may also be present, including erythromycin D, E, F, erythronolide B (EB), and *N*-demethyl erythromycin A (NDM). The structures of these related substances and the common chemical modifications made to erythromycin are shown in Figures 1 and 2.

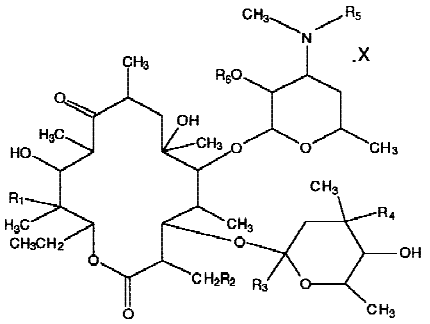
Erythromycin chromatography is best run at high pH. A mobile phase at acidic pH cannot be used because erythromycin is extremely acid labile. Raising the pH of the mobile phase to neutral produces high-performance liquid chromatography (HPLC) methods that can be run on a "pH stable" silica column for ~2 weeks before column life is sharply decreased and resolution is lost.[5] Several liquid chromatography (LC) methods for the assay of erythromycin have been developed and are being used for routine analysis using polymer columns.[1,6] The limitations of existing methods, however, include limited sensitivity, difficult mobile phase preparation, variable retention times, and lack of resolution. Many of these problems were addressed by the introduction of gradient elution methods,[7] column switching, [8,9] electrochemical detection[10–12] (particularly for biomedical analyses), or chemiluminescence detection.[13] Some attempts have also been made to improve upon available analytical methods for the detection of erythromycin by the introduction of newer techniques such as capillary electrophoresis,[14] which, although demonstrated to effectively distinguish between manufacturing processes, still lacks the sensitivity and resolution needed to accurately quantify low levels of impurities.

To date, a sufficiently rugged method does not exist. The aim of this research, therefore, was to develop a sensitive isocratic HPLC method (method A) for the detection and quantitation of erythromycin and related substances that is rugged enough to be used on a routine basis for drug products. The current British Pharmacopeial (BP) method for enteric-coated erythromycin tablets[15] was optimized (method B) and samples were run for comparison purposes (Table 1).

EXPERIMENTAL

Chemicals and Reagents

Abbott Reference Standards (ARS) of erythromycin base (containing 97.6% erythromycin A),



	R1	R2	R3	R4	R5	R6	X
Erythromycin A	OH	H	H	OCH_3	CH_3	H	-
Erythromycin B	H	H	H	OCH_3	CH_3	H	-
Erythromycin C	OH	H	H	OH	CH_3	H	-
Erythromycin D	H	H	H	OH	CH_3	H	-
Erythromycin E	OH	- O -		OCH_3	CH_3	H	-
Erythromycin F	OH	OH	H	OCH_3	CH_3	H	-
N-demethylerythromycin A	OH	H	H	OCH_3	H	H	-
Erythromycin Ethylsuccinate	OH	H	H	OCH_3	CH_3	$C_2H_5COOC(CH_2)_2CO$	_
Erythromycin Stearate	OH	H	H	OCH_3	CH_3	H	$CH_3(CH_2)_{16}CO_2H$

Figure 1. Chemical structure of erythromycin A and related compounds.

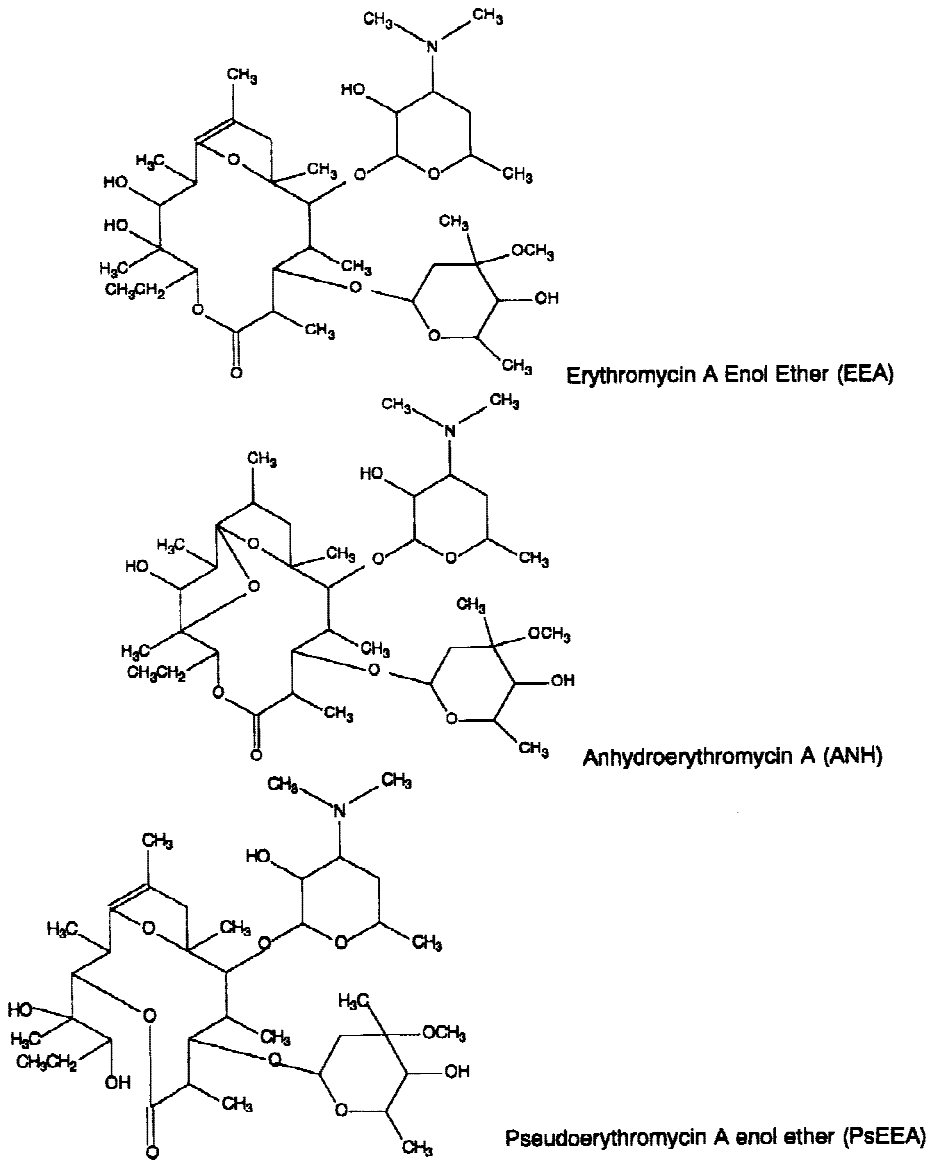


Figure 2. Known degradation products of erythromycin.

erythromycin B, erythromycin C, *N*-demethyl erythromycin A, anhydroerythromycin A, erythromycin enol ether, and erythromycin base check sample were used throughout the study. Water was distilled and potassium phosphate dibasic (AR grade; Baker), acetonitrile (HPLC grade; EM Science), *t*-butanol (HPLC grade, 99.5%+; Fisher), methanol (HPLC grade;EM Science), and phosphoric acid (85%; JT Baker) were used without further purification.

Solutions

Dilute phosphoric acid was prepared by dissolving a calculated amount of phosphoric acid in distilled water. Stock 0.2 *M* K_2HPO_4 buffer (pH 9) was prepared by dissolving a calculated amount of potassium phosphate dibasic in distilled water and adjusting the pH with dilute phosphoric acid. The 0.067 *M* K_2HPO_4 buffer (pH 8) was prepared by dissolving a calculated amount of potassium phosphate dibasic in distilled water and adjusting with dilute phosphoric acid to pH 8.

Chromatographic Conditions

The chromatographic system consisted of a Shimadzu LC10AD pump, a Shimadzu SIL-10AD autosampler with cooling tray set at 4°C (RMS

Table 1. Chromatographic Conditions for Erythromycin (Methods A and B)

Method	Parameter	Condition
A	Column	Astec C18 Polymeric, 5 μm, 1000 Å, 4.6 × 250 mm
	Mobile phase	0.02 *M* K_2HPO_4 buffer pH 9: acetonitrile (60:40)
	Flow rate	~1.0 mL/min
	Detector	205 nm
	Injection volume	30 μL
	Column temperature	~50 °C
B	Column	Polymer Laboratories PLRP-S, PS-DVB, 8 μm, 1000 Å, 4.6 × 250 mm
	Mobile phase	0.01 *M* K_2HPO_4 buffer, pH 9: *t*-butanol: acetonitrile (80:17:3)
	Flow rate	~2.0 mL/min
	Detector	205 nm
	Injection volume	50 μL
	Column temperature	~70 °C

Lauda RM6), a Shimadzu column oven CTO-10AC, a Shimadzu degasser DGU3A, a Shimadzu detector SPD-10A (205nm), a Dell Dimension XPS90 computer, and Shimadzu Class-VP software. The chromatographic conditions for both methods are outlined in Table 1. Prior to use, the mobile phase was filtered through a Millipore filtration apparatus using nylon 0.45-μm, 47-mm filters (Alltech).

Standard Preparation

Erythromycin standard was prepared by weighing 120 mg of erythromycin base reference standard, dissolving in 10 mL of methanol, and then diluting with buffer (pH 8) to 25 mL. A blank solution was prepared by diluting 10 mL of methanol with pH 8 buffer to 25 mL. A chromatographic system check sample was prepared by weighing 120 mg of erythromycin base check sample, dissolving in 10 mL of methanol, and diluting with buffer (pH 8) to volume. Standards were allowed to cool to room temperature before making final volumetric adjustments.

Sample Preparation

Five EryTab 250-mg tablets were placed in 100 mL of methanol, shaken for 40 min, and then diluted with buffer (pH 8) to 250 mL. The samples were left to cool to room temperature for a minimum of 1 h before making final volumetric adjustments. The samples were then left to stand for ~10 min at room temperature to allow excipients to settle out. About 2 mL of sample solution were prefiltered through a 0.45-μm filter attached to a plastic disposable syringe into a Pall Filtron Microsep 10K centrifuge filter. This prefiltration step minimized particulates and centrifuge time. To prevent any possible methanol evaporation from the filter cartridge, the adjoining portion of the filter assembly was sealed with a thin strip of Parafilm®. The solution was centrifuged for ~30 min at high speed. Thirty minutes of centrifuge time produced sufficient sample to fill about one third of a standard HPLC vial. If desired, however, small volume inserts (e.g., Alltech 100—μL limited volume inserts, stock number 95202 or equivalent), may be used to help to reduce centrifuge time by ~15 min. Some solvent evaporation may take place depending on the heat produced by the centrifuge; therefore, the sample solution from the bottom section of the plastic filter unit must be carefully withdrawn and used to rinse the inside of the bottom filter unit to collect any condensed methanol. The blank solution was also filtered through the molecular weight filter in the same manner as the sample preparation.

RESULTS AND DISCUSSION

Sample Preparation Procedure

A shake time of 40 min was found to be sufficient to disintegrate the coated tablets and produce maximum recoveries of erythromycin A, B, and C. The Pall Filtron Microsep 10K Molecular Weight Cut Off (MWCO) filtration centrifuge step was introduced to remove interferences due to HP-55, one of the enteric coating excipients. Attempts to selectively remove the polymer from the sample preparation through adjustment of pH or using different solvents were unsuccessful because of the high instability of erythromycin at low pH and the fact that both erythromycin and the polymer are more soluble in organic solvents than aqueous solutions. Selective polymer removal efforts therefore focused on size exclusion via mo-

lecular weight filtration. It was found that a 10K molecular weight filter removes most of the polymer with a molecular weight of ~130 K and allows erythromycin (MW 789) and related compounds to pass through.

Removal of the coating interference provided a better baseline for peak integration in addition to the prevention of peak masking. Figure 3 shows the beneficial effect of filtration on the sample preparation chromatogram for method A. A similar effect is seen for method B.

This method of sample preparation has a significant advantage over the current BP method for enteric-coated erythromycin tablets, which requires the manual removal of each individual tablet coat with a sharp blade. Pall Filtron Microsep 30K filters can also be used, although reduction of baseline interference was less. ChromTech 5K filters were found to be unsuitable because of the high retention of drug and poor sample volume production.

Prefiltration through a 0.45-μm filter is necessary to reduce particulates and centrifuge time through the smaller pore size of the MWCO filter to ~30 min. Excessive centrifuge time is unwanted because of the heat produced in the centrifuge. The filter units should be removed from the centrifuge as soon as the spin time is completed. The sample solution is transferred to an HPLC vial and stored at 4°C to minimize erythromycin degradation. Gelman nylon Acrodisc 0.45-μm filters were selected for the prefiltration step due to good sample compatibility.

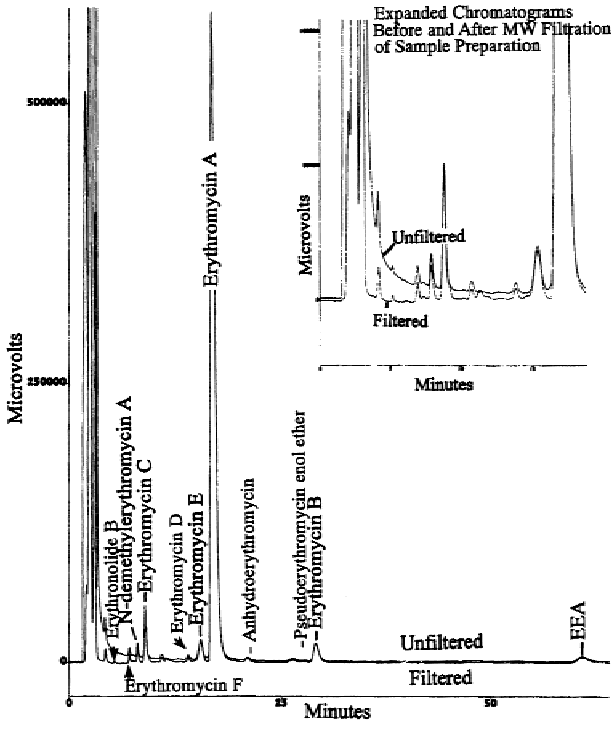


Figure 3. Chromatogram showing the effect of MWCO filtration on EryTab® 250-mg tablets (method A).

Chromatographic Conditions

The greatest difficulty encountered when running the BP based assay (method B) was the consistent preparation of the mobile phase. One of the components, *t*-butanol, is a solid at room temperature and must be melted to a liquid at a consistent temperature to ensure consistent mixing volumes. The retention time of erythromycin A varies greatly with small changes in percent of *t*-butanol. The retention of EEA is even more dramatically affected, potentially leading to carryover of peaks to the next chromatogram. A related factor contributing to changes in retention times was the temperature of the laboratory. It was noticed during overnight runs that the retention time would decrease, when presumably the temperature of the laboratory dropped, and the *t*-butanol would collect at the bottom of the mobile phase reservoir. This situation was remedied by gently stirring the mobile phase continuously to ensure good mixing and a more homogeneous mobile phase. Retention times then remained more constant during longer analysis runs. Improved chromatography was also produced when a 3-ft section of coiled 0.01-in. i.d. stainless steel tubing was attached to the column inlet inside the column oven. The tubing helps to equilibrate the temperature of the mobile phase before it reaches the column to 70°C and improves peak shape. Another point noted in terms of HPLC hardware is that the BP method (B) requires a column temperature of 70°C which cannot be reached by many standard HPLC internal column ovens, therefore, an external oven must often be used.

The preferred chromatographic conditions, therefore, included the use of the Astec polymeric column (method A: Table 1).

Quantitation and Accuracy

Using ultraviolet (UV) detection at 205 nm, the detector response was demonstrated to be linear over the working sample concentration range, or anticipated maximum expected concentration, for

both methods. Method A linearity is shown in Table 2. Based on these results, the values for potency and related substances were calculated using erythromycin base external standard.

Accuracy was determined by monitoring the recovery of erythromycin A and related substances added to coated placebo EryTab 250-mg tablets. Ery A, Ery B, Ery C, NDM, ANH, and EEA were added in various amounts to placebo tablets, and samples were analyzed as detailed in the sample preparation procedure. Recovery was within ±3% in all cases.

To establish the reproducibility of both methods, the same lot of EryTab 250-mg tablets was analyzed by two different analysts using two different HPLC systems in two separate laboratories. Different gel lots of Aztec packing material were also used. The two analysts determined the potency and related substances by each method. Table 3 shows the potency results from both methods.

Precision results for related substances are shown in Table 4. The total number of related substances is greater using method A; this result is thought to be due to the better resolution capability of this column that allows many small peaks to be easily distinguishable from the baseline and quantified. Similarly, the quantification of the broad, late-eluting EEA is much easier with method A because the peak is often difficult to distinguish from baseline noise using method B. The degradation product, ANH, is identified in method B with a relative retention time of 1.51. However, using method A, this broad peak is resolved into three smaller peaks, one of which corresponds to the retention time of the ANH reference standard. The initial identification of this peak in the sample using method B, therefore, is unlikely to be correct because sensitivity and specificity are not optimal.

Comparison of Liquid Chromatography and Microbiological Methods

Historically, the potency of erythromycin products and drug substance has been determined microbiologically. To confirm that a change in testing procedure would not significantly affect testing results, comparison of methods was necessary. HPLC potency results for both EryTab 250-mg Tablets and Erythromycin Base Filmtabs 250 mg were compared with Content Uniformity and Stability Assay Results, both obtained by microbiological methods.[15] The same lot of tablets was subjected to both testing procedures.

In both cases, microbiological results are equivalent to HPLC results; that is, within twice the %RSD (relative standard deviation) of the method. Erytab® potency: HPLC = 102.1% (SD = 1.20, %RSD = 1.17), Microbiological = 103.8% (SD = 2.48, %RSD = 2.39); Erythromycin Base FilmTab® potency: HPLC = 102.9% (SD = 0.96, %RSD = 0.94), Microbiological = 102.7% (SD = 1.61, %RSD = 1.57%). The HPLC method shows a significantly lower %RSD than the microbiological method, indicating better precision. In addition, the advantage of using the LC method is the information obtained regarding amounts of specific components present in erythromycin products; that is, the separate quantitation of erythromycin A, B, and C and related substances provides maximum information during stability studies.

Stability

Initial studies showed that erythromycin standards degraded ~5% in 24 h when stored at room temperature in pH 8 buffer. The use of a sample cooling tray in the autosampler is therefore critical. Standard and sample preparations were stored at 4° for ~2 days and assayed against

Table 2. Linearity Results for Erythromycin and Related Substances

Method	Compound	Range (μg/mL)	Slope	y-Intercept	Correlation coefficient
A	Ery A	4.8–6000	4580	51801	0.9999
	Ery B	6.0–296	4119	−4398	0.9998
	Ery C	1.2–310	4708	−401	0.9999
	NDM	7.2–180	3059	−4068	1.0000
	ANH	1.8–181	3152	−1381	0.9998
	EEA	1.8–180	28416	−29007	0.9998

Table 3. HPLC Potency for EryTab® 250 mg Tablets[a]

	Method A $n = 8$			Method B $n = 7$		
Parameter	Mean	SD	%RSD	Mean	SD	%RSD
% Ery A	96.1	0.51	0.53	95.3	1.21	1.27
% Ery B	3.04	0.08	2.77	2.74	0.22	7.87
% Ery C	3.32	0.12	3.51	3.54	0.24	6.90
% Ery Total	102.5	0.55	0.53	101.6	1.42	1.40

[a] Same lot of tablets was used for each method.

freshly prepared standard solutions. Standard concentration was reduced from 100 to 98.1% over 30 h at 4°C, and sample potency was reduced from 102.6 to 101.2% over 35 h at 4°C. These results provided sufficient stability data to justify redilution and injection of sample the next day in the event of a failed run.

Ruggedness

Method A is significantly more rugged than method B. Small changes in the organic composition of the mobile phase in method B produced a dramatic effect in the tailing factor (T) and retention time of erythromycin A and EEA. The tailing factor of erythromycin A increased from 1.34 to 1.54 when the percent *t*-butanol was decreased from 17.5% to 16.5% and the retention time of EEA was >100 min. A significant increase in run time produced overlapping peaks for subsequent injections if run times were not corrected for each fresh batch of mobile phase prepared. Increasing the column temperature from 65 to 75°C reduced T from 1.32 to 0.94. although the retention time

Table 4. Summary of HPLC Precision Data for Erythromycin Related Substances in EryTab® 250-mg Tablets Using Methods A and B

Method	RRT	ID	Mean %w/w	SD	%RSD
A[a]	0.34	Unknown	0.08	0.01	18.9
	0.39	Unknown	0.36	0.03	8.56
	0.43	Ery F	0.11	0.02	17.6
	0.46	NDM	0.85	0.06	7.49
	0.63	Unknown	0.29	0.04	12.7
	0.83	Ery D	0.31	0.05	16.4
	0.91	Ery E	2.60	0.14	5.32
	1.28	Unknown	0.23	0.04	16.1
	1.62	PsEEA	0.43	0.06	14.0
	1.69	Unknown	0.14	0.04	30.7
	3.85	EEA	0.15	0.01	5.61
B[b]	0.39	Unknown	0.31	0.03	8.56
	0.44	Unknown	0.21	0.04	18.2
	0.54	NDM	0.79	0.05	6.32
	0.87	Ery E	2.58	0.23	8.91
	1.51	ANH	0.31	0.06	18.3
	1.73	Unknown	—	—	—
	4.45	EEA	—	—	—

[a] Statistical analysis only performed where related substances found at a level of 0.05% or greater in all samples; $n = 8$.
[b] Statistical analysis only performed where related substances found at a level of 0.10% or greater in all samples; $n = 7$.

reduction was not as significant as changing the percent organic composition of the mobile phase. *t*-Butanol is solid at room temperature and can be thawed to room temperature by partially immersing the container in warm water. The temperature of the water, in addition to the final temperature of the liquid *t*-butanol, was found to be a source of variation in run times. This variation can be minimized by keeping temperatures consistent or measuring *t*-butanol by weight. Mobile phase preparation for method B, however, remains relatively difficult.

In contrast, the mobile phase preparation for method A is standard and reproducible. Resolution between NDM and erythromycin C (R) remains constant at ~2.0, when the percent acetonitrile varies by ±3%; that is, small changes in percent organic composition of the mobile phase do not adversely affect resolution of related substances. Increasing pH and temperature to 9.5 and 55°C respectively, produce higher N and R values. The chromatography, in general, is better at higher temperature and high pH, and the conditions chosen represent the highest practical working limits to produce good separation within a reasonable run time.

Application to Other Erythromycin Products

It was found that this method could also be applied to other Abbott erythromycin-containing products. Sample preparations for each product are outlined below:

- Erythromycin Base FilmTab: same as EryTab® with a shake time of 30 min (MW filtration produces a flatter baseline).
- Erythromycin Stearate Tablets (Erythrocin®): five 500-mg tablets were shaken with 200 mL of methanol for 40 min; 200 mL of pH 8 buffer were added to the solution, which was left to cool while the stearic acid precipitated out; the sample was filtered through a 0.45-μm filter (MW filtration not necessary).
- Particle Coated Erythromycin (PCE 333®): seven 333-mg tablets were shaken for 80 min in 100 mL of methanol. The sample preparation is the same as for EryTab® 250 mg, and MW filtration is also beneficial because of the large amount of polymer in the formulation.
- Erythromycin Ethylsuccinate Suspension (EES 200®): 30 mL of suspension was carefully measured into a 50-mL measuring cylinder and left to settle out for ~1 h until air bubbles were reduced to a minimum. Gentle vortexing also helped force the air bubbles to the top of the suspension. The volume was adjusted to exactly 30 mL by removing and adding suspension using a glass pipette. The sample was quantitatively transferred with 100 mL of pH 8 buffer into a 250-mL volumetric flask, 100 mL methanol was added, and then the sample was left at room temperature overnight to allow hydrolysis of the succinic acid. Samples were mechanically shaken next day for 30 min and then diluted with pH 8 buffer to volume. The solutions were filtered through a 0.45-μm filter.

In PCE® tablet and EES® suspension samples, a late eluting peak at ~160 min was observed. This peak is due to an excipient and, if desired, can also be removed using MWCO filtration.

APPLICATION TO ERYTHROMYCIN FERMENTATION BROTHS

Method A can also be modified for the analysis of erythromycin fermentation broths. Chromatographic modifications included lower column temperature (45°C), increased wavelength (210 nm), and increased percent organic (55%) to provide a shorter run time (40 min) with a retention time of ~11 min for erythromycin A and 32 min for EEA.

The erythromycin broth sample was extracted with acetonitrile. A portion of the supernatant was diluted 1:2 with water, vortexed to mix, and filtered through 0.45-μm filters.

CONCLUSIONS

The described HPLC method for determination of erythromycin in enteric-coated tablet formulations yielded improved precision, and more importantly, improved ruggedness compared with the existing BP method. Results obtained with both methods showed good correlation. This method can also be successfully applied to other erythromycin-containing products.

Additional advantages of this method compared with previously published methods for erythromycin and related substances are the use

of an isocratic method with UV detection and increased sensitivity and accuracy.

This method of sample preparation, whereby a molecular weight cut-off filter is used to remove polymeric excipient interference from the chromatogram, may be equally beneficial for other formulations where polymeric interference is problematic.

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