CHAPTER I

INTRODUCTION

Erythromycin is a widely used macrolide antibiotic produced by fermentation of *Streptomyces erythreus*. The main component is erythromycin A, but some related substances such as erythromycin B, C, D, E and F are present in small amounts (Glasby, 1979 ; Martin, *et al*, 1982).

Erythromycin A - F are composed of a 14-membered lactone ring (erythronolide), aminosugar (D-desosamine) and neutral branch-chain sugar (L-cladinose or L-mycarose). The sugars are normally attached via glycoside linkage to the lactone ring, however, in erythromycin E, L-cladinose is attached by an ortho ester coupling (Majer *et al.*, 1977; Florey, 1979 ; Martin *et al.*, 1982 ; The Merck Index, 1989). Structures are shown in Fig. 1.

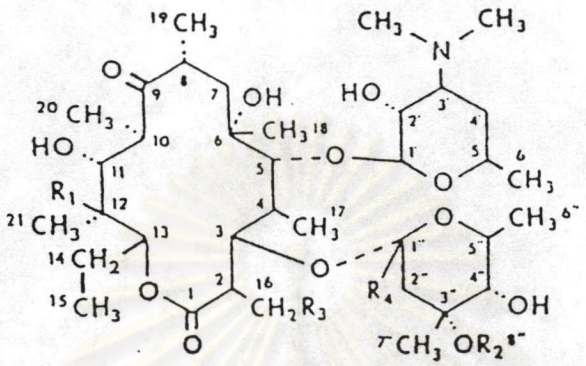


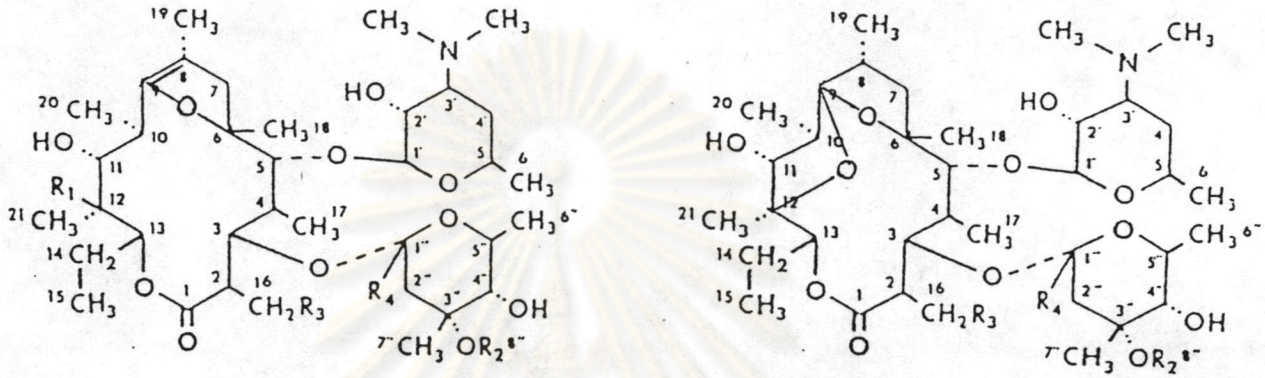
Fig. 1 Structures of erythromycins

	R_1	R_2	R_3	R_4
Erythromycin A	OH	CH_3	H	H
Erythromycin B	H	CH_3	H	H
Erythromycin C	OH	H	H	H
Erythromycin D	H	H	H	H
Erythromycin E	OH	CH_3	—O—	
Erythromycin F	OH	CH_3	OH	H

Erythromycin A is the major component, it is active against most gram-positive and some gram-negative bacteria including *Neisseria* spp., *Haemophilus influenzae* and *Bordetella pertussis*, against spirochaetes and some rickettsias and chlamydias (Martindale, The Extra Pharmacopoeia, 1989). Various amount of other components was found. Kibwage, Janssen *et al* (1985) reported that

erythromycin B, C and D were found about 7.2, 2.4 and 0.6 % respectively. Kibwage *et al* (1985) reported that the antibacterial activity of erythromycin B against gram-positive bacteria was similar to erythromycin A. The antibacterial activity of erythromycin C and D was lower for most gram-positive and gram-negative microorganisms. Tsuji and Kane (1982) reported that the activity of erythromycin B and C against *Staphylococcus aureus* (ATCC 6538P) was less than erythromycin A. They found that the potency of erythromycin A, B and C were 1000, 787 and 487 μg/mg respectively. Martin *et al* (1982) reported that the activity of erythromycin E was about equal to that of erythromycin F or 2-8 fold less active than erythromycin A.

Under mildly acidic conditions and during storage, erythromycins rapidly degraded via intramolecular dehydration to form the 8, 9 - anhydro - 6, 9 - hemiketal (enol ether) and the anhydro - 6, 9 ; 9, 12 - spiroketal (spiroketal) compounds. Structures are shown in Fig. 2 a and b respectively.



(a) (b)

Fig. 2

	R_1	R_2	R_3	R_4
(a) Structure of enol ether				
8, 9-Anhydroerythromycin A-6, 9-hemiketal (Erythromycin A enol ether)	OH	CH_3	H	H
(b) Structures of spiroketals				
Erythromycin A-6, 9 ; 9, 12-spiroketal (Anhydroerythromycin A)	-	CH_3	H	H
Erythromycin C-6, 9 ; 9, 12-spiroketal (Anhydroerythromycin C)	-	H	H	H

Only erythromycin A enol ether was found, although erythromycin B enol ether could have been formed under the same conditions, none was isolated. Two spiroketals were found, i.e., anhydroerythromycin A and anhydroerythromycin C. Erythromycin B and erythromycin D cannot form spiroketals because they lack a hydroxyl group at the C12 position (Kibwage, Janssen, *et al.*, 1985). Tsuji and Goetz (1978) and Atkins, Herbert and Jones (1986) also reported that anhydroerythromycin A and erythromycin A enol ether, which were formed under mildly acidic conditions, were found.

Kibwage *et al* (1985) reported that the MICs of acid degradation products i.e. the enol ether, the anhydro-derivative were much higher and did not contribute to the antibacterial activity.

Majer (1981) found that the 8,9-anhydroerythromycin A 6,9 hemiketal, isolated from urine samples, induced in vitro resistance to the parent drug in the test strain *Staphylococcus aureus* RN 1389, in agreement with previously reported by Allen (1977) that erythromycin was the potent inducer in resistance to *Staphylococcus aureus* 1206. Therefore, it is necessary to determine the amount of the degradation product.

Current official methods of assay for erythromycin in raw material and its dosage forms involve the use of

microbiological method (BP,1988; USP, 1990). This method allow the determination of total erythromycin content as the erythromycin base equivalent. Since the microbiological assay cannot differentiate between active and inactive moieties, the presence of any related degradation products in raw material or dosage forms of erythromycin cannot be determined. So it cannot be used in stability study for analysis of each species. In addition, this method is lack of specificity and involve a time-consuming step.

Kibwage *et al* (1985) reported that the precision of an antibiotic assayed by microbiological method was lower for substances which contain related compounds. Variations occured which were due to the method used (diffusion or turbidimetry) and the microorganism and culture conditions which were applied. They concluded that the precision of the assay of erythromycin was influenced by the presence of related substances, which might be present in rather large amounts.

In recent years a greater awareness of the problems of poor specific assay in antibiotics where drugs are partially degraded, metabolized or when other antibiotics may also be present, together with a requirement for more rapid techniques, has encouraged the investigation of other methods (Hugo and Russell, 1992)

Various methods have been reported for the assay of erythromycin products as follows:

1. Polarimetry

Li *et al.* (1991) reported the determination of erythromycin tablets by measuring the optical rotation of the ethanol solution.

2. Ultraviolet spectrophotometry

Siedlanowska - Krowczynska and Knapczyk (1986) reported that the ultraviolet spectrophotometry can be determined erythromycin base in enteric coated tablets by hydrolysing in an alkaline condition and measure the absorption at 236 nm.

3. Colourimetry

Mostly colourimetry studies were based on the ion-pair dye complex formation. Regosz, *et al.* (1982) reported the dye complex formation of erythromycin in pharmaceutical preparations with bromophenol blue at pH 4.2.

Dabrowska, *et al.*(1984) and Regosz, Dabrowska and Leman (1986) reported the determination of erythromycin in urine and blood from ion-pair complex of bromocresol

purple at pH 5.29.

In 1985, Sulkowska, Budych and Staroscik reported the determination in coated tablets of the ion-pair complex of erythromycin and eriochrome black T at pH 4.

4. Thin-layer chromatography (TLC)

Stahl (1969) described TLC systems for identifying erythromycin on silica gel G. In 1980, Vanderhaeghe and Kerremans reported the separation and identification of the components of erythromycin and various macrolides on silanised silica gel plates with methanol - water - ammonium acetate pH 7.0 (50:20:10).

In 1983, Kibwage, Roets and Hoogmartens developed TLC system for separating erythromycin, its related substances and degradation products on silica gel with diisopropyl ether - methanol - 25% ammonia (75:35:2). However, thin-layer chromatographic method is generally used only in qualitative analysis.

5. Gas chromatography (GC)

Tsuji and Robertson (1971) reported a gas chromatographic method to determine erythromycin and its derivatives, however, this method was not suitable for precise quantitation and it involved derivatization of

erythromycin with silylating agent at 75 ^{o}C for 24 hours which was complicated procedure, moreover, the drawback appeared to be in the instability of the GC column, 3 weeks at 275 ^{o}C.

6. High-performance liquid chromatography (HPLC)

Several high-performance liquid chromatographic methods have been described for the separation and analysis of erythromycin components and their degradation products in raw material and dosage forms. HPLC method meets the requirements of pharmaceutical analysis such as rapid, high specificity and selectivity, good efficiency, suitable sensitivity and appropriate accuracy and precisions. It is especially useful for the assay of those antibiotics metabolized to microbiologically active and inactive metabolites and for difficult mixtures.

Hash (1975) reported chromatographic conditions for separating anhydroerythromycin from erythromycin using a normal phase silica gel column, with chloroform as mobile phase and refractive index detection. Since the instability of normal phase column, this method was not widely used.

For reversed phase, C_8 and C_{18} silica-based reversed phase column were widely reported in several papers. However, mostly researchers have found that the retention

of erythromycin on these columns was too strong, which a tailing peak and poor resolution was obtained. Tsuji (1978) ; Tsuji and Kane (1982) required a column temperature of 70 ^{o}C to achieve satisfactory results, but column temperature usually do not exceed 50 ^{o}C due to adverse effects of column lifetime (Swarbrick and Boylan, 1990). Cachet, Quintens *et al.* (1991) improved the separation of erythromycin by using an aged C_{18} packing columns. The separation was improved due to the loss of metal impurities which may affect silanol group activity. However, the aged column is uncontrollable, so it is not practical to select the appropriate one for suitable separation.

In 1985, Kibwage, Roets *et al.* reported chromatographic condition using columns of poly (styrene-divinylbenzene) co-polymers packing materials with mobile phase of higher pH (8.0) at 60 ^{o}C, however the obtained chromatogram was broad, poor resolution and long analysis time i.e. the retention time of the last peak (erythromycin A enol ether) was 60 minutes. The improved separation of erythromycin using poly (styrene-divinylbenzene) column by heating the column at 70 ^{o}C were reported by Paesen, Roets and Hoogmartens (1991) and Hoogmartens (1992).

The selection of an appropriate detection system for HPLC analysis depends upon the sensitivity required by

the assay. In pharmacokinetic and bioavailability studies, fluorometric and electrochemical detection are chosen because of its highly sensitive for analysis in biological fluids, however, for raw material and pharmaceutical dosage forms, these methods are not practical. For fluorometric detection, a complex post-column derivatization procedure was used (Tsuji, 1978). In addition, fluorescence quenching may result in a nonlinear relationship between solute mass and detector response. (Swarbrick and Boylan, 1990).

For electrochemical detection, the instability of the baseline often leads to quantitation problems. It has to leave the mobile phase at least 12 hour recycling through the electrochemical cell to reach a constant background current (Grgurinovich and Matthews, 1988). In addition, the background current increased with increasing pH, increasing ionic strength and increasing temperature. The electrochemical response is probably due to the tertiary amino group in the erythromycin molecule, which further has to be unprotonated to be oxidizable. Thus, to avoid a post column adjustment, the pH of the mobile phase should be maintained around or above the pK_a of erythromycin (8.8). However, dissolution of silica-based stationary phases increases progressively at pH above 7, leading to short column life and probably also to the poor detector stability with a glassy carbon electrode (Nilsson, Walldorf and Paulsen, 1987).

For raw material and pharmaceutical dosage forms, ultraviolet detector is commonly used. The UV detector is suited to lengthy automated analyses especially when performed by less experienced technical staff. Several HPLC methods using this detection system have been published (Cachet, Kibwage, *et al.*, 1987; Geria, Hong and Daly, 1987; Stubbs and Kanfer, 1990; Cachet *et al.*,1992 ; Cachet, Lannoo *et al.*,1992).

Although erythromycin has a weak UV absorption, the suitable sensitivity will be obtained at the selected optimum wavelength. Wavelength of about 200 nm can be used for detection at sufficient sensitivity, but low wavelength is mostly associated with large interferences from other species and also with unstable baseline from UV cut-off of the solvents. These problems can be eliminated by using longer wavelength such as at 288 nm (Geria, Hong and Daly, 1987), but the absorption is very weak. It can be detected only major component i.e. erythromycin A, but other components and degradation products cannot be detected because of small amount presence and low molar absorptivity.

For relating erythromycin content obtained by the HPLC method with the microbiological assay, Tsuji and Robertson (1971), Tsuji and Goetz (1978) and Tsuji and Kane (1982) used the correction factors for erythromycin A, B and C to express the results of HPLC in terms of antimicrobial bioequivalency. However, Kibwage *et al.*

(1985) suggested that the use of a single conversion factor from chemical assay to potency seemed questionable. They examined the antibacterial activities of these different substances against a series of microorganisms and found that, in microbiological assays, the potencies of these products probably were different, depending on microorganisms and technique used.

The purpose of this study is to develop a HPLC method for determining erythromycin, its related substances and principal degradation products in raw material and pharmaceutical dosage forms by using simple routine instruments and under conditions ensuring a good selectivity of the system. The selectivity can be improved by an appropriate selection of the stationary phase and by the optimization of the mobile phase compositions in order to obtain sufficient separation at moderate pH and ambient temperature. Ultraviolet detector, the most widely used detector, is used and the optimum wavelength is selected for reasonable sensitivity. Method validation is necessary performed to prove the suitability of the obtained chromatographic conditions. Finally the results obtained from the HPLC method comparing with the current official microbiological assay method are studied. This study will be useful for assay of erythromycin and erythromycin derivatives by using HPLC method which is rapid, precise, accurate, specific and stability-indicating method.