Journal of Chromatography, 423 (1987) 189–197
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3896

DETERMINATION OF ERYTHROMYCIN IN HUMAN PLASMA, USING COLUMN LIQUID CHROMATOGRAPHY WITH A POLYMERIC PACKING MATERIAL, ALKALINE MOBILE PHASE AND AMPEROMETRIC DETECTION

LARS-GÖRAN NILSSON*, BIRGIT WALLDORF and OTTO PAULSEN

Department of Clinical Pharmacology, University Hospital of Lund, S-221 85 Lund (Sweden)

(Received July 8th, 1987)

SUMMARY

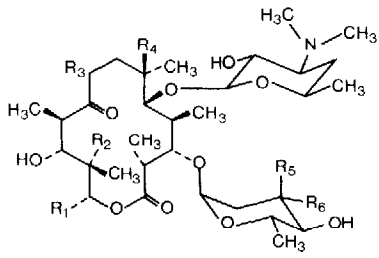
A method based on column liquid chromatography was developed for determination of plasma concentrations of erythromycin. PRP-1, a polymeric type of packing material suitable for chromatography and amperometric detection at high pH, was used. The effect of pH on the column performance and on the electrochemical response was studied. A pH of ca. 10 was found to be optimal. After extraction with *tert.*-butyl methyl ether, plasma concentrations down to 0.2 μmol/l could be measured, using automated sample injection. Oleandomycin was used as internal standard. The method was used for determination of plasma concentrations in a pharmacokinetic study under steady-state conditions.

INTRODUCTION

Erythromycin (Fig. 1) is the most widely used of the macrolide antibiotics. It has a broad antimicrobial spectrum and is used for a variety of infectious diseases. However, its absorption after oral administration is highly variable, and resulting plasma concentrations are unpredictable. One important reason for the variability is the instability of erythromycin base in the acid environment of the stomach. To overcome this, erythromycin is usually administered as an ester, as an enteric coated preparation or encapsuled in acid-resistant microgranules. The esters serve as prodrugs and are hydrolysed gradually after absorption to the free base, the only antibacterially active form.

Most pharmacokinetic data have been obtained using microbiological assays. These assays are, however, of limited specificity. Furthermore, during incubation hydrolysis of erythromycin esters occurs [1], thus leading to an overestimation of the active base unless special procedures are applied [2]. Some methods based

0378-4347/87/$03.50



	R_1	R_2	R_3	R_4	R_5	R_6
ERYTHROMYCIN	CH_2CH_3	OH	--CH_3	OH	OCH_3	CH_3
OLEANDOMYCIN	CH_3	H		H	H	OCH_3

Fig. 1. Molecular structures of erythromycin and oleandomycin.

on column liquid chromatography (LC) have been developed for determination of erythromycin concentrations in biological fluids. They all include reversed-phase chromatography with the use of conventional silica-based C_{18} columns. Different detection principles have been used, e.g. fluorescense [1], UV absorption at 200 nm [3] and coulometric detection [4,5]. Tsuji [1] used a complex procedure with post-column ion-pairing with a fluorescent reagent followed by on-line extraction and detection of the ion-pair. The method is said to be sensitive down to 0.01 μg/ml (0.014 μmol/l) but no data are given on the precision for concentrations below 0.6 μg/ml. Erythromycin has a very weak UV absorbance and only wavelengths of ca. 200 nm can be used for detection at sufficient sensitivity. These low wavelengths are mostly associated with large interferences from co-extracted species. In the method of Stubbs et al. [3], where sample work-up includes a solid-phase extraction with a final phase separation step, a sensitivity of 0.25 μg/ml (0.34 μmol/l) is obtained using a 2-ml sample.

Simpler procedures using electrochemical detection (ED) with equal or better sensitivity have been described [4,5]. In both reports the ESA 5100 A Coulochem detector was used in a dual-electrode mode. 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 for 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 experienced by Duthu [5] with a glassy carbon electrode and amperometric detection.

The use of non-ionic poly(styrene–divinylbenzene) copolymeric stationary phases (e.g. PRP-1) for the separation of erythromycin and related substances was studied by Kibwage et al. [6]. They examined the effect of various parameters on the separation but restricted their study of pH effects to values between 5.5 and 8, although these columns are stable up to pH 12.

In this work we examined the effect of a more alkaline pH on the retention behaviour for erythromycin on the PRP-1 column. We also studied the effect of a combination of this stationary phase and amperometric detection for erythromycin. Oleandomycin, another macrolide antibiotic (Fig. 1),was included as an internal standard in the plasma assay.

EXPERIMENTAL

Materials

Erythromycin base was kindly supplied by Astra (Södertälje, Sweden). Oleandomycin phosphate (95%) was obtained from Sigma (St. Louis, MO, U.S.A.), and was further purified by LC in order to remove a component that appeared as a peak with a retention time close to erythromycin on the LC system used in this work. LC solvents were normal HPLC grade from Rathburn (Walkerburn, U.K.). *tert.*-Butyl methyl ether was "zur Rückstandsanalyse" (M 1995) from Merck (Darmstadt, F.R.G.), and all other reagents were analytical grade from Merck. Water was purified by a Milli-Q/Organex-Q system (Millipore, Neu Isenburg, F.R.G.). MF-1 centrifugal microfilters (Bioanalytical Systems, West Lafayette, IN, U.S.A.) were used with RC-60, 1-μm membranes for filtration of plasma extracts before injecting on the LC column. PRP-1 columns (15 cm$\times$4.1 mm I.D., 5 μm) were obtained from Hamilton (Reno, NV, U.S.A.). Three columns were used (serial numbers 344, 459 and 716) with performance varying between 59 000 and 116 000 theoretical plates per metre (N/m) according to the manufacturer's test.

LC instrumentation

The LC equipment consisted of an SP 8700XR LC pump, an SP 8780XR autosampler (both from Spectra-Physics, Darmstadt-Kranickstein, F.R.G.), an LC-4 amperometric detector (Bioanalytical Systems) and an SP 4270 integrator (Spectra-Physics). The column was thermostated using a Universal thermostat (Mikrolab, Aarhus, Denmark). The amperometric detector was equipped with a TL-5 thin-layer cell with a glassy carbon electrode and an RE-1 Ag/AgCl reference electrode. The applied oxidative potential was 1.0–1.1 V vs. the reference electrode unless otherwise stated.

Mobile phase

Mobile phases were prepared by mixing potassium phosphate buffers with acetonitrile in various proportions. The phosphate buffers, mostly 50 mM, were adjusted to appropriate pH by mixing isomolar solutions of potassium dihydrogenphosphate and potassium hydrogenphosphate or by adding potassium hydroxide. When comparing retention and column efficiency at various pH values, the ionic strength was adjusted to 150 mM at need, using sodium sulphate. Likewise the ionic strength in the resulting solution was held constant when mobile phases with different proportions of acetonitrile were compared. The buffers were filtered before mixing with acetonitrile and the final mobile phase was

deaerated by helium degassing. The flow-rate was always 0.7 ml/min and the resulting back-pressure was 100–150 bar.

Extraction of erythromycin from plasma

Plasma samples (1.0 ml) were made slightly alkaline with saturated potassium carbonate (pH 9.8). After addition of oleandomycin as internal standard, extraction from plasma was made with *tert.*-butyl methyl ether (5 ml) by gentle rocking for 15 min. After a short centrifugation to separate the phases, the ether layers were transferred to new test-tubes and dried with sodium sulphate. The ether was finally evaporated under a gentle stream of nitrogen and the residue dissolved in 125 μl of water–acetonitrile (70:30, v/v). The resulting solutions were mostly turbid and for that reason filtered before injection of 25 μl on the LC column.

RESULTS AND DISCUSSION

PRP-1 column

When testing these columns, the manufacturer used the peak shape of phenol under conditions where phenol is almost unretained, thus obtaining a high performance of more than 50 000 plates per metre. Using the cumene peak in the same accompanying test chromatograms, the column efficiencies of the three columns used in this work were only 17 500–20 500 N/m (k=1.5–1.7). More strongly retained peaks showed even less separation efficiency. We also found a high flow-rate dependence. Increasing the flow-rate from 0.5 to 1.0 ml/min decreased the number of plates by ca. 25%.

LC separation

Chromatographic performance for erythromycin is generally poor. By measurements in chromatograms from earlier LC methods [1,3–5], column efficiency was estimated at 300–1000 theoretical plates. In one paper a log-linear relation was found between column temperature and HETP, resulting in an almost three-fold increase in the number of plates when increasing the column temperature from 25 to 70 °C [7]. With the PRP-1 column, we found that column efficiency was largely dependent on the pH of the mobile phase (Fig. 2). When the retention was held fairly constant by varying the acetonitrile content, the efficiency was at a minimum at a pH of ca. 8 and then increased when the pH of the mobile phase was increased. Peak symmetry was similarly affected by pH. The three columns used varied in their ability to give small bandwidths for the macrolides. The one with the best test results also showed the best performance for erythromycin.

Increasing the temperature from 25 to 50 °C had a positive effect on separation efficiency, but since the electrochemical detection was negatively influenced by high temperatures, columns were normally thermostated at 25 °C.

Erythromycin retention was found to increase when the mobile phase was made alkaline (Fig. 3). This was expected because erythromycin is a weak base with a pK_a of ca. 8.8. However, above pH 9, the retention unexpectedly decreased for two of the three columns used. As for silica-based reversed-phase columns, the

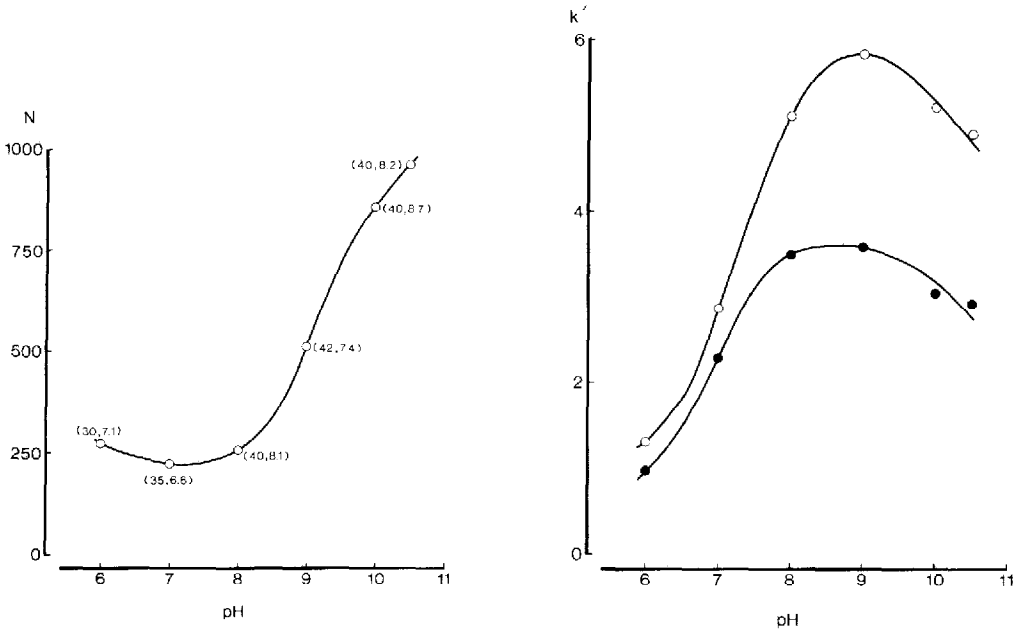


Fig. 2. Effect of pH on PRP-1 column plate number (N) for erythromycin. Numbers in brackets are the percentage of acetonitrile in the mobile phase and the resulting capacity factor, k', for each determination; N is calculated using the peak width at half height.

Fig. 3. Effect of pH on retention for erythromycin (○) and oleandomycin (●) on a PRP-1 column (5 μm, 15 cm×4.1 mm I.D.). Mobile phase: 50 mM potassium phosphate at various pH, adjusted to 150 mM with sodium sulphate–water–acetonitrile (50:5:45).

retention could be varied by varying the amount of organic modifier, e.g. acetonitrile, and it was also influenced by the ionic strength of the mobile phase (Table I).

Electrochemical detection

In the two earlier studies in which ED was employed [4,5], the ESA 5100 A Coulochem detector was used in the oxidative screen mode. In our work, we found

TABLE I

EFFECT OF IONIC STRENGTH ON RETENTION AND PLATE NUMBER FOR ERYTHROMYCIN AND OLEANDOMYCIN

Column, PRP-1, 5 μm, 15 cm×4.1 mm I.D.; mobile phase, phosphate buffer pH 9.5–acetonitrile (60:40, v/v); flow-rate, 0.7 ml/min; column temperature 25 °C.

Phosphate concentration (mM)	k'		N (erythromycin)
	Erythromycin	Oleandomycin	
10	8.7	4.9	550
25	7.3	4.3	700
50	6.8	4.0	850
100	6.1	3.7	850

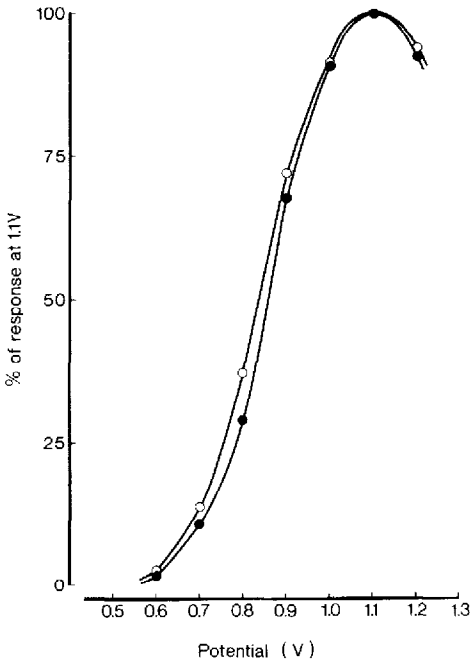


Fig. 4. Hydrodynamic voltammograms for erythromycin (○) and oleandomycin (●). Means of three or four determinations at different times with mobile phases at pH 9–10.

that the single-channel amperometric detector LC-4 from Bioanalytical Systems could be used in connection with LC separations using the PRP-1 column. The hydrodynamic voltammogram obtained (Fig. 4) showed that a working potential of ca. +1.1 V vs. Ag/AgCl was needed for maximum sensitivity. The background current was high at this potential; it also increased with increasing pH, increasing ionic strength and increasing temperature. Under the conditions described for plasma analysis, the background current was ca. 250 nA. In spite of this, a stable baseline could be achieved at a detector sensitivity of 64 nA f.s., where 20 pmol (15 ng) of erythromycin could be detected at a signal-to-noise ratio of 10. A linear response was found up to ca. 2.5 nmol of erythromycin injected. The response at the glassy carbon electrode was furthermore relatively stable from day to day, in contrast to what was found by us as well as by Duthu [5] with silica-based LC columns and mobile phases at pH of ca. 7.

Plasma workup

The extraction from plasma with *tert.*-butyl methyl ether was found to give good recoveries of erythromycin and oleandomycin (84 and 85%, respectively). No interfering peaks were found in chromatograms from erythromycin-free plasma samples (Fig. 5). A late peak (retention time ca.35 min), which delayed the next injection, was found to originate from one source of ether (Merck 818109,"zur Synthese"). It was not found with the present ether.

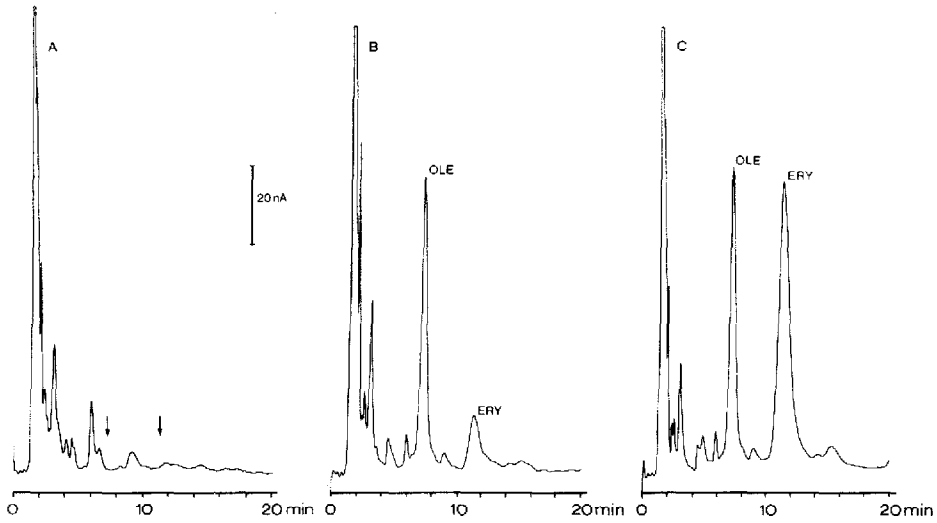


Fig. 5. Chromatograms of extracted plasma samples. (A) Blank plasma sample; the arrows indicate the retention times for oleandomycin and erythromycin, respectively. (B) Plasma from a subject on Ery-Max 500 mg twice daily; the sample was taken immediately before the morning dose with oleandomycin added as internal standard; measured concentration, 0.74 μM. (C) Plasma from the same subject 3 h after dose; measured concentration, 4.6 μM. Chromatographic conditions: column, PRP-1, 5 μm, 15 cm×4.1 mm I.D.; mobile phase, potassium phosphate (50 mM, pH 9.5)–acetonitrile (60:40, v/v); flow-rate, 0.7 ml/min; column pressure, 155 bar; detector potential, +1.10 V vs. Ag/AgCl; detector offset, 250 nA. Peaks: OLE = oleandomycin; ERY = erythromycin.

Stability

In a separate experiment, both erythromycin and oleandomycin were found to be degraded progressively with increasing pH in aqueous buffer (ca. 10% in 24 h at pH 10). Thus, raising the pH above 10 during the extraction and reconstitution with a solution at high pH (e.g. mobile phase) are not recommended. However, using the procedure described in this paper, no significant degradation was found to occur during sample work-up or during the chromatographic separation.

Performance characteristics

Standard curves were constructed by extracting blank plasma samples fortified with known amounts of erythromycin (0.20–10 μM). These standard curves seemed to deviate slightly from linearity. Therefore, a "low" standard curve (0.2–2 μM) was used to determine low erythromycin concentrations ($y = 0.250x + 0.005$; $r = 0.9997$; $n = 4$ means from 13 consecutive standard curves; y = peak-height ratio of erythromycin to oleandomycin and x = erythromycin concentration), whereas for concentrations above 1 μM a complete standard curve was used ($y = 0.260x - 0.005$; $r = 0.9999$; $n = 6$ means from 13 consecutive standard curves).

Accuracy and precision, estimated by including control samples regularly during a two-month period are presented in Table II.

The separation from potential metabolites in plasma could not be checked owing to the lack of appropriate standards. According to Kibwage et al. [6] and others, oleandomycin could be expected to be poorly resolved from N-demethyl-

TABLE II

ESTIMATES OF ACCURACY AND PRECISION FOR PLASMA ANALYSIS OF ERYTHROMYCIN

Added concentration (μM)	Found concentration (μM)	Intra-assay precision★ (%)	Inter-assay precision★★ (%)
0.60	0.64	6.5 (12)	8.7 (27)
1.51	1.52	3.3 (12)	4.9 (27)
6.03	6.17	5.2 (10)	5.4 (25)

★Coefficient of variation estimated from duplicates; number of duplicate runs in parentheses.
★★Coefficient of variation estimated from all analyses over a two-month period; number of control samples in parentheses.

erythromycin and anhydro-N-demethylerythromycin. However, no significant concentrations of these metabolites in human plasma have been reported. Furthermore, according to Duthu [5], these N-demethylated metabolites are not detectable by ED.

Applications

This method for determination of erythromycin concentrations in plasma was used in a study of the potential interaction between erythromycin (Ery-Max®, Astra, Sweden) and theophyllamine (Aco, Sweden) in ten healthy volunteers [8]. Under steady-state conditions (500 mg of erythromycin base twice daily), the plasma concentration immediately before the morning dose was 1.03 μM (median; interquartile range: 0.81–1.82 μM) and the maximum concentration during the dosing interval was 7.02 μM (median; interquartile range: 5.87–8.22 μM). Examples of chromatograms are given in Fig. 5.

CONCLUSIONS

In LC assays for erythromycin and oleandomycin an alkaline mobile phase is advantageous because of improved chromatographic performance. In combination with ED, a compromise is necessary as far as the mobile phase pH and applied working potential are concerned. Using a mobile phase at pH 9.5–10 and a potential of 1.0–1.1 V vs. Ag/AgCl, acceptable background current and detector noise were obtained, and detection of these macrolides at the picomole level was possible. Detector stability was such that automatic, overnight analysis could be performed.

The PRP-1 column shows for erythromycin a performance comparable with that of the generally superior NovapakC_{18} column used by Stubbs et al. [3]. The larger pH-range available also increases the possibility of selectively affecting the separation. The development of non-silica-based packing materials more efficient than the one used in this work will further increase the usefulness of alkaline eluents for LC separations of erythromycin and similar substances.

ACKNOWLEDGEMENTS

The authors thank Astra (Södertälje, Sweden) for financial support and Dr. Lars-Erik Edholm for valuable discussion when preparing the manuscript.

REFERENCES

1 K. Tsuji, J. Chromatogr., 158 (1978) 337.
2 G.J. Yakatan,W.J. Poynor, S.A. Breeding, C.E.Lankford, S.V. Dighe, A.N. Martin and J.T. Doluisio, J. Clin. Pharmacol., 20 (1980) 625.
3 C. Stubbs, J.M. Haigh and I. Kanfer, J. Pharm. Sci., 74 (1985) 1126.
4 M.-L. Chen and W.L. Chiou, J. Chromatogr., 278 (1983) 91.
5 G.S. Duthu, J.Liq. Chromatogr., 7 (1984) 1023.
6 I.O. Kibwage, E. Roets, J. Hoogmartens and H. Vanderhaeghe, J. Chromatogr., 330 (1985) 275.
7 K. Tsuji and J.F. Goetz, J. Chromatogr., 157 (1978) 185.
8 O. Paulsen, P. Höglund, L.-G. Nilsson and H.-I. Bengtsson, Eur. J. Clin. Pharmacol., 32 (1987) 493.