ELSEVIER

Journal of Chromatography A, 829 (1998) 161–166

JOURNAL OF CHROMATOGRAPHY A

# Liquid chromatographic method for separation of lincomycin from its related substances

J.A. Orwa, F. Bosmans, S. Depuydt, E. Roets, J. Hoogmartens*

*Katholieke Universiteit Leuven, Faculteit Farmaceutische Wetenschappen, Laboratorium voor Farmaceutische Chemie en Analyse van Geneesmiddelen, Van Evenstraat 4, B-3000 Leuven, Belgium*

Received 7 July 1998; received in revised form 22 September 1998; accepted 22 September 1998

**Abstract**

A reversed-phase ion-pair liquid chromatographic method with UV detection at 210 nm is described for the separation of lincomycin from 7-epilincomycin and lincomycin B. The method utilizes a base-deactivated Supelcosil LC-ABZ, $C_{12-18}$, 5 μm, 250×4.6 mm I.D. column maintained at 45°C. The mobile phase consists of 2.25% (v/v) acetonitrile, 5% (v/v) phosphate buffer (2.72%, m/v $KH_2PO_4$ adjusted to pH 5.0 with 3.48%, m/v $K_2HPO_4$), 0.067% (v/v) methanesulfonic acid and water to 100%. The flow-rate is 1.0 ml/min. The separation of lincomycin from 7-epilincomycin is only possible on base-deactivated (BDS) columns. Other octadecylsilyl columns examined showed insufficient selectivity. The method was also tested on other BDS columns (Spherisorb S5-ODS-B, 5 μm, Hypersil BDS, $C_{18}$, 5 μm and Supelcosil LC-ABZ, $C_{18}$, 5 μm, all 250×4.6 mm I.D.) and showed good robustness. Robustness was further evaluated by performing a full-factorial design experiment. The method showed good selectivity, repeatability, linearity and sensitivity. It is also suitable for analysis of lincomycin formulations. 

*Keywords:* Pharmaceutical analysis; Lincomycins; Antibiotics

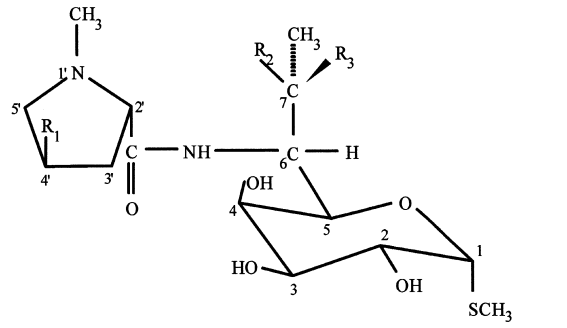
## 1. Introduction

Lincomycin is a medium spectrum antibiotic which is produced by *Streptomyces lincolnensis* [1]. Lincomycin inhibits the growth mainly of Gram-positive bacteria. It is used in both human and veterinary medicine. Common impurities (Fig. 1) in lincomycin bulk drug are lincomycin B and 7-epi-lincomycin, which are formed during biosynthesis [2]. It has not been described in literature that epimerization can also occur in solution.

Microbiological assay methods [3–5], which have originally been used for assay of lincomycin are non-specific and less accurate. Alternative analytical methods used for lincomycin include chemical assay [6], thin-layer chromatography or paper chromatography [1,7] and isotachophoresis [8]. These methods also lack specificity and lincomycin cannot be differentiated from lincomycin B or 7-epilincomycin. Gas chromatographic procedures require elaborate extraction and derivatization steps and are not selective for 7-epilincomycin [2,9–11]. Capillary zone electrophoresis with amperometric detection was recently described for the determination of lincomycin and lincomycin B in bulk drug and pharmaceutical formulations [12]. However, resolution of 7-epilincomycin has not yet been reported.

*Corresponding author. Fax: +32-16-32-3448; E-mail: Jos.Hoogmartens@farm.kuleuven.ac.be

0021-9673/98/$ – see front matter 
PII: S0021-9673(98)00777-8




| | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| Lincomycin B (1) | $CH_3$-$CH_2$- | OH | H |
| Lincomycin (5) | $CH_3$-$CH_2$-$CH_2$- | OH | H |
| 7-Epilincomycin (6) | $CH_3$-$CH_2$-$CH_2$- | H | OH |

Fig. 1. Structures of lincomycin and related compounds.

Liquid chromatography (LC) methods have been described for quantitative determination of lincomycin [13–17]. Analysis of lincomycin in fermentation beers by reversed-phase ion-pair LC on octylsilica gel with UV detection at 214 nm resolved lincomycin from lincomycin B [13]. Methods for quantitation of lincomycin residues in milk and tissues by reversed-phase LC on a $C_{18}$ column and using UV detection [14] or in tissues by ion-pair reversed-phase LC with electrochemical detection [17] were only selective for lincomycin. The current USP [16] method for lincomycin hydrochloride utilizes reversed-phase LC at pH 6 on a $C_8$ column with UV detection at 210 nm. This USP method is not selective for 7-epilincomycin. In this study, a simple, selective LC method capable of resolving lincomycin from lincomycin B, 7-epilincomycin and other related substances of unknown identity is described.

## 2. Experimental

### *2.1. Reagents and samples*

Acetonitrile Grade S was from Rathburn (Walkerburn, UK). Potassium dihydrogenphosphate, dipotassiumhydrogen phosphate and methanesulfonic acid (MSA) were from Merck (Darmstadt, Germany). Water was distilled twice from glass apparatus. Commercial samples of Linco-Spectin injection (I), Lincocin injection (II), Lincocin tablets (III), Linco-Spectin soluble powder (IV) as well as reference samples of lincomycin hydrochloride, lincomycin B hydrochloride and 7-epilincomycin hydrochloride were obtained from Pharmacia and Upjohn (Kalamazoo, MI, USA).

### *2.2. LC apparatus and operating conditions*

The isocratic LC system consisted of an L-6200 intelligent pump (Merck–Hitachi, Darmstadt, Germany), a Merck–Hitachi Model 655A-40 autosampler set to inject 20 μl, an electronic integrator HP 3396 Series II (Hewlett-Packard, Avondale, PA, USA) and a Merck–Hitachi Model L-4000 variable-wavelength UV detector set at 210 nm. Base-deactivated (BDS) columns (250×4.6 mm I.D.), Supelcosil LC-ABZ $C_{12-18}$, 5 μm (Supelco, Bellefonte, PA, USA), Spherisorb S5-ODS-B, 5 μm (Phase Separations, UK) and Hypersil BDS $C_{18}$, 5 μm (Shandon, Runcorn, UK) were used. Other columns (250×4.6 mm I.D.) were packed in the laboratory with Hypersil $C_{18}$, 5 μm (Shandon) or Rsil $C_{18}$ LL (Alltech, Avondale, PA, USA). The column temperature was maintained at 45°C in a water bath controlled by means of a Julabo EM thermostat (Julabo, Seelbach, Germany).

### *2.3. Analytical procedure*

Lincomycin hydrochloride bulk drug or reference standard solution was prepared at a concentration of 2.0 mg/ml in water. The related substances were each dissolved at a concentration of 0.2 mg/ml in water and used for spiking the bulk drug in order to identify the various impurities. The test solution for tablets was prepared by grinding 20 tablets into fine powder and extracting an amount equivalent to 200.0 mg with 100.0 ml of water. The resulting solution was centrifuged for 10 min at 2900 *g*. The test solution for soluble powder was prepared by dissolving an amount of powder equivalent to 200.0 mg in 100.0 ml of water. The test solutions for the injections were prepared by dilution with water to obtain 2.0 mg/ml solutions.

## 3. Results and discussion

### 3.1. Development of chromatographic method and robustness

In developing this procedure the most difficult compounds to separate were the epimers, lincomycin and 7-epilincomycin. They differ structurally only in the configuration of the hydroxyl and hydrogen in the 7 position (Fig. 1). The methods described in the USP [16] for lincomycin hydrochloride and for clindamycin hydrochloride and the Ph. Eur. [11] method for clindamycin phosphate ester were examined for their selectivity on lincomycin and related substances. For these three methods examined using octadecylsilyl silica gel, lincomycin was always separated from lincomycin B. However, sufficient separation of 7-epilincomycin could not be obtained. Modification of the ion-pair LC method with refractive index detection described for clindamycin hydrochloride [16], by changing the composition of the mobile phase and using UV detection, did not achieve separation of the epimers, lincomycin and 7-epilincomycin. The epimers were not separated completely on a $C_{18}$ column using a mobile phase containing acetonitrile, phosphate buffer pH 6.0 and MSA as the ion-pair. Other ion-pairing reagents examined were sodium dioctylsulfosuccinate, sodium octanesulfonate and sodium pentanesulfonate, but the epimers were still not separated completely. Baseline resolution of the epimers was finally achieved only on BDS columns. Chromatography was optimized using Dry Lab software (LC Resources, Berlin, Germany). The reproducibility of the selectivity was examined on different BDS $C_{18}$ stationary phases (Table 1). Baseline resolution of lincomycin from its major impurities was obtained on all the columns examined indicating robustness of the method. Fig. 2 shows a chromatogram of lincomycin bulk drug spiked with 7- epilincomycin. Lincomycin was resolved from 7-epilincomycin and lincomycin B. Other impurities of unknown identity were also resolved. Further work was carried out with Supelcosil LC-ABZ, $C_{12-18}$, 5 μm, 250×4.6 mm I.D. column because it gave the best resolution.

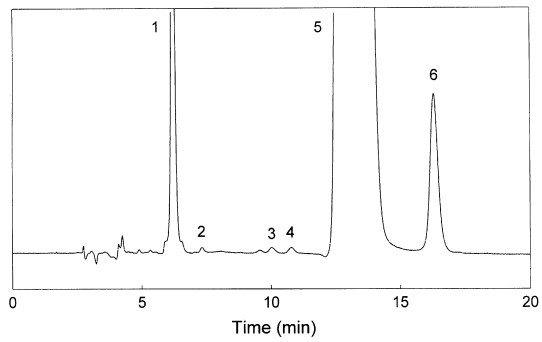



Fig. 2. Chromatogram of lincomycin bulk drug spiked with 7-epilincomycin. Conditions: Supelcosil LC-ABZ, $C_{12-18}$, 5 μm, 250 mm×4.6 mm I.D. column maintained at 45°C; mobile phase, 3.5% (v/v) acetonitrile, 5% (v/v) phosphate buffer (2.72%, m/v $KH_2PO_4$ adjusted to pH 5.0 with 3.48%, m/v $K_2HPO_4$), 0.067% (v/v) methanesulphonic acid, water to 100% at a flow-rate of 1.0 ml/min. 1, Lincomycin B; 2, unknown; 3, unknown; 4, unknown; 5, lincomycin; 6, 7-epilincomycin.

The robustness of the method was further evaluated by performing a full-factorial design experiment. The set up of the full-factorial design, together with the analysis of the measured response variables and multivariate regression calculation, were sup-

Table 1
Chromatoraphic parameters on different BDS columns

| Column | Stationary phase (250×4.6 mm ID) | Acetonitrile in mobile phase (%) | Symmetry[a] | Resolution[b] | Theoretical plates[a] | $k'$[a] |
|---|---|---|---|---|---|---|
| A | Supelcosil ABZ $C_{12-18}$, 5 μm | 2.25 | 1.2 | 3.2 | 4170 | 3.1 |
| B | Supelcosil ABZ $C_{18}$, 5 μm | 5.25 | 1.5 | 2.4 | 4050 | 3.0 |
| C | Spherisorb S5 ODS-B | 3.75 | 1.3 | 3.1 | 5110 | 3.4 |
| D | Hypersil BDS $C_{18}$, 5 μm | 6.75 | 1.5 | 2.5 | 5600 | 6.6 |

[a] Calculated for lincomycin peak according to Ph.Eur method [11].
[b] Resolution between lincomycin and 7-epilincomycin.

ported by the statistical graphic software system STATGRAPHICS Version 6.0 (Manugistics, Rockville, MD, USA). The influence of each of the three chromatographic parameters that governed the separation process was examined by applying a full-factorial design at two levels. This involved $2^3=8$ different experimental measurements, combining the three parameters examined at two previously fixed extreme levels of each parameter. One central level was included in the design and so nine measurements were performed as well as duplicate experiments. The chromatographic parameters examined as variables were the concentration of acetonitrile, the concentration of MSA and the pH of the mobile phase. The values of the design are given in Table 2. The estimated effects of the three chromatographic parameters with their second order interactions on the selectivity between lincomycin B and lincomycin ($\alpha$ 1–5) and between lincomycin and 7-epilincomycin ($\alpha$ 5–6) as response variables are presented on the standardized pareto charts in Fig. 3. The bars are displayed in order of the size of the effects, with the largest effects on top. The charts include a vertical line at the critical *t*-value for an $\alpha$ of 0.05. Effects for which the bars are smaller than the critical *t*-value are considered as not significant. Effects may be positive or negative. A positive effect means an increase in selectivity with an increase in chromatographic parameter variable while a negative effect means a decrease in selectivity with an increase in chromatographic parameter variable.

From these experiments, it is observed that the selectivity between the critical pair, lincomycin and 7-epilincomycin is principally influenced by the percentage of MSA in the mobile phase (Fig. 3b). The selectivity decreases with increase of the ion-pair concentration in the mobile phase. With further increase in the ion-pairing reagent in the mobile phase, the retention of these more non-polar components, lincomycin and 7-epilincomycin becomes longer and they coelute. The ion-pair is however necessary for resolution and peak symmetry. The concentration of acetonitrile and the chromatographic parameter interaction between acetonitrile and MSA have less important but significant effect on the selectivity between the two compounds. The pH and other parameter interactions have no significant influence. The selectivity between lincomycin B and lincomycin is influenced by all the chromatographic parameter and parameter interactions examined (all negative influence). Acetonitrile concentration has the most significant influence on this selectivity. There was however sufficient separation under all the conditions examined. Response surface plots were constructed for lincomycin B, lincomycin and 7-epilincomycin, with capacity factors as a function of the most important chromatographic parameters, the concentrations of MSA and acetonitrile in the mobile phase (Fig. 4). For all the conditions examined there was no overlapping indicating the robustness of the method.

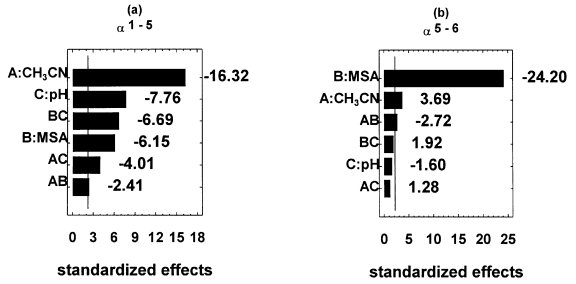



Fig. 3. Standardized pareto charts representing the estimated effects of parameters (*A, B, C*) and parameter interactions (*AB, BC, AC*) on the selectivity between (a) lincomycin B and lincomycin ($\alpha$ 1–5) and (b) lincomycin and 7-epilincomycin ($\alpha$ 5–6).

Table 2
Nominal values corresponding to −1, 0 and +1 levels

| Chromatographic variable | Low value (−1) | Central value (0) | High value (+1) |
|---|---|---|---|
| Methanesulfonate (%) | 0.037 | 0.067 | 0.097 |
| Acetonitrile (%) | 2.00 | 2.25 | 2.50 |
| pH | 4.5 | 5.0 | 5.5 |

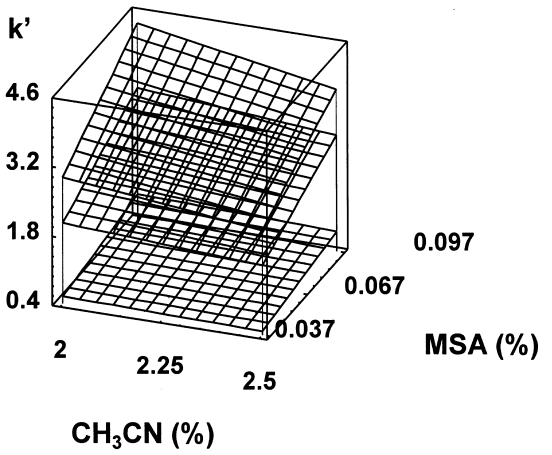



Fig. 4. Estimated response surface plots for lincomycin B (lower plane), lincomycin (middle plane) and 7-epilincomycin (upper plane) constructed with the capacity factors as a function of MSA concentration and acetonitrile concentration in the mobile phase.

### 3.2. Repeatability, linearity and detection limits

The precision of the method was assessed using six replicate injections of a 2.0 mg/ml solution of lincomycin. The relative standard deviation (R.S.D.) of the peak area of the main component was 0.07%. The calibration curve obtained by replicate analysis ($n=3$) of a series of analyte concentrations corresponding to 1.2, 1.6, 2.0, 2.4 and 2.8 mg/ml was subjected to linear regression analysis: $y=1.84+64.11x$, where $y$=peak area$\cdot 10^{-6}$, $x$=concentration in mg/ml; correlation coefficient $r=0.9997$, standard error of estimate $S_{y,x}=0.64$. The limit of quantitation (LOQ) was 0.015% of the nominal content of the formulation, that is, 0.006 μg injected mass ($n=6$; R.S.D.=4.2%). The limit of detection (LOD) with a signal-to-noise ratio of 3 was 0.005%.

### 3.3. Analysis of commercial samples

The method was applied to the assay of different formulations of lincomycin hydrochloride. Lincomycin hydrochloride Ph. Eur. CRS was used as a standard. Replicate injections ($n=3$) of replicate sampling ($n=2$) were carried out in each case. Results of the assay are presented in Table 3. Assay of the samples yields good precision for the major component. The content of the related substances is calculated as lincomycin, using a diluted solution of Ph. Eur. CRS corresponding to 1% of the nominal content. The formulations analyzed have low levels of lincomycin B and 7-epilincomycin. Other impurities of unknown identity were also detected, however, the levels were below the limit of quantification. Lincomycin, being a fermentation product, if not well controlled may have higher amounts of impurities than the amounts obtained in the commercial products analyzed in this study. The amount of impurities may also vary with the origin (manufacturer) of the product. It is therefore useful to have available a selective analytical method with the ability to detect trace contaminants when present in a bulk drug.

## 4. Conclusions

The isocratic LC method presented here is suitable for the separation of lincomycin from its potential

Table 3
Content (%) of lincomycin and related substances[a] in formulations

| Sample (theoretical content) | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| I (50 mg/ml) | 0.42 (2.1) | 0.07 (8) | <LOQ | <LOQ | 99.4 (0.1) | 0.03 (3.1) |
| II (300 mg/ml) | 0.12 (4.2) | <LOQ | <LOD | <LOD | 98.7 (0.4) | 0.03 (5.4) |
| III (200 mg/tablet) | 0.44 (0.7) | 0.02 (25) | <LOQ | <LOQ | 104.1 (0.2) | <LOQ |
| IV (33.3 g/141 g) | 0.07 (7.5) | 0.02 (17) | <LOD | <LOD | 98.3 (0.3) | 0.1 (1.3) |

[a] Related substances calculated as lincomycin.
1, Lincomycin B; 2, unknown (peak 2); 3, unknown (peak 3); 4, unknown (peak 4); 5, lincomycin and 6, 7-epilincomycin.
R.S.D.s are given in parentheses.

impurities. This robust method shows good selectivity, repeatability and linearity. It was applied satisfactorily to the quantitative analysis of commercial lincomycin samples.

## Acknowledgements

J.A.O. thanks the ABOS of the Belgian Government for financial support.

## References

[1] T.E. Eble, in: M.J. Weinstein, G.H Wagman (Eds.), Antibiotics – Isolation, Separation and Purification, Elsevier, Amsterdam, 1978, p. 231.
[2] R.L. Houtman, D.G. Kaiser, A.J. Taraszka, J. Pharm. Sci. 57 (1968) 693.
[3] A.R. Barbiers, A.W. Neff, J. Assoc. Off. Anal. Chem. 59 (1976) 849.
[4] A.W. Neff, R.W. Thomas, J. Assoc. Off. Anal. Chem. 61 (1978) 1107.
[5] G.L. Stahl, J. Assoc. Off. Anal. Chem. 61 (1978) 39.
[6] G.C. Prescott, J. Pharm. Sci. 55 (1966) 423.
[7] G.H. Wagman, M.J. Weinstein (Eds.), Chromatography of Antibiotics, Elsevier, Amsterdam, 1984, p. 215.
[8] H. Klein, R. Teichmann, J. Chromatogr. 250 (1982) 152.
[9] M. Margosis, J. Chromatogr. 37 (1968) 46.
[10] C.H. McMurray, W.J. Blanchflower, D.A. Rice, J. Assoc. Off. Anal. Chem. 67 (1984) 582.
[11] European Pharmacopoeia, Council of Europe, Strasbourg, France, 3rd ed., 1997.
[12] F. Xiaoming, L. Xiaofeng, Y. Jiannong, F. Yuzhi, Anal. Lett. 29 (1996) 1975.
[13] P.A. Asmus, J.B. Landis, C.L. Vila, J. Chromatogr. 264 (1983) 241.
[14] W.A. Moats, J. Agric. Food. Chem. 39 (1991) 1812.
[15] H.Y. Muti, F.H. Al-Hajjar, in: H.G. Brittain (Ed.), Analytical Profiles of Drug Substances and Excipients, Vol. 23, Academic Press, San Diego, CA, 1994, p. 269.
[16] United States Pharmacopeia 23, United States Pharmacopeial Convention, Rockville, MD, 1995.
[17] W. Luo, E.B. Hansen Jr., C.Y.W. Ang, H.C. Thompson Jr., J. Assoc. Off. Anal. Chem. 79 (1996) 839.