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A rapid HPLC-UV method for the quantification of erythromycin in dermatological preparations

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According to the USP erythromycin determination in finished oral products as well as in some topical formulations is mainly carried out via microbiological assays. However, these assays are known for their long incubation periods, lack of precision and low sensitivity. In literature one HPLC method for the quantification of erythromycin in creams is described, which depends on electrochemical detection, but HPLC electrochemical detection has not emerged as popular choice in routine analysis. Furthermore two other HPLC-UV methods are described for the isolation of erythromycin from gels and creams involving tedious and time consuming extraction steps, the reason why they are not suited to be applied in routine analysis. This paper describes a new HPLC-UV method for the determination of erythromycin in creams, which implies a much easier extraction procedure than that cited in literature to date, based solely on the solubilization of erythromycin followed by freezing the cream matrix. Validation experiments confirmed the precision and accuracy of the method. Good linearity of the assay was found over the investigated concentration range of 70–130% (corresponding to 0.77–1.43 g of erythromycin A in 100 g cream base). The coefficient of correlation resulting from unweighted linear regression was 0.9998, allowing a one-point calibration in routine analysis. By the implementation of an internal standard in the quantification of erythromycin an improved precision could be achieved in routine analysis. This new analytical method yields cleaner extracts and allows a higher throughput, saving costs, solvents and time and can be thus recommended to all laboratories.

1. Introduction

In spite of the wide offer of industrially manufactured products, dispensing of erythromycin topical preparations is still considered to be an indispensable part of external dermatotherapy. In order to prevent incompatibilities of erythromycin with other formulation components (Bagdi and Staudacher 2000) and to prevent its decomposition at pH values lower than 8, standardized extemporaneous preparations, which are based on rationally justifiable recommendations in relevant publications, as the "Neue Rezeptur Formularium (NRF)" [New Prescription Formulary] available in Germany, are recommended. These standardized formulations like the "Hydrophilic Erythromycin Creme 1%", the composition of which is shown in Table 1, can be prepared in pharmacies without any difficulties and there is a minor risk of poor galenic quality (NRF 2005).

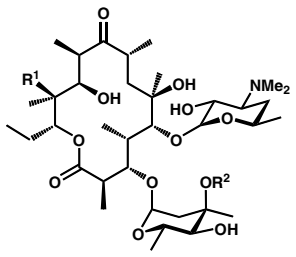
The main component of commercially available erythromycin is erythromycin A (European Pharmacopoeia 2005). In addition to small amounts of the similarly effective related components erythromycin B and C, not exceeding 5%, negligible traces of erythromycin D and F may be found.

In general, erythromycin in drug products is determined by means of microbiological assays measuring the growth inhibition of *Staphylococcus aureus* (USP monographs and European Pharmacopoea monographs). This applies also for the determination in topical formulations as mentioned in the USP monographs "Erythromycin topical gel" and "Erythromycin topical solution" (United States Pharmacopoeia 2006a, 2006b). Although being used very often for the determination of erythromycin, microbiological assays are known for their long incubation periods, lack of precision and low sensitivity because of interference with related substances also contributing to biological ac-

Table 1: Composition of the "Hydrophilic Erythromycin Cream 1%" according to the NRF (Neues Rezeptur Formularium)

Constituents	1%
Erythromycin (microfine pulverized)	1.1 g
Basiscreme DAC*	50.0 g
Citric acid-solution 0.5%	8.0 g
Propylenglycol	10.0 g
Purified water	ad 100.0 g

* 100 g of Basiscreme DAC is composed of 4.0 g glycerol monostearate 60, 6.0 g cetylalcohol, 7.5 g middlechain triglycerides, 25.5 g white vaseline, 7.0 g macrogol-20-glycerolmonostearate, 10.0 g propylenglycole and 40.0 g purified water



Erythromycin	Formula	M_r	R1	R2
A	$C_{37}H_{67}NO_{13}$	734	OH	CH_3
B	$C_{37}H_{67}NO_{12}$	718	H	CH_3
C	$C_{36}H_{65}NO_{13}$	720	OH	H

tivity. Other methods for the determination of erythromycin in creams are based on HPLC with electrochemical detection as described in the USP monographs "Erythromycin ointment" (United States Pharmacopoeia 2006c) and "Erythromycin ophthalmic ointment" (United States Pharmacopoeia 2006d). However, HPLC electrochemical detection has not emerged as popular choice in routine analysis as electrochemical detection is very sensitive to a variety of factors like the pH value and the salt concentration in the eluent system. In addition, the electrode response may drift with time, requiring more frequent calibration with standards, which significantly prolongates the analysis time in routine application. Moreover, these methods described in the USP are very tedious, time consuming and susceptible to many handling errors, since the isolation of the active ingredient from the formulation matrix is achieved by multiple extraction steps.

In literature some HPLC methods have been published for the determination of erythromycin using column switching (Cachet et al. 1991) as well as for the quantification of erythromycin salts ethylsuccinate and estolate (Cachet et al. 1992a, 1992b), however only two methods are described for the isolation of erythromycin from topical formulations. Dehouck et al. (2003) report the extraction of erythromycin from a gel and Paesen et al. (1998) describe the isolation of erythromycin from hydrophilic creams in a study on erythromycin stability. Both methods depend on a mixture of organic solvents in the extraction of erythromycin from the formulation matrix which cost solvent, effort and time and moreover often lead to the formation of emulsions which may effect the purity of the extract.

In view of handling a high throughput of samples in an adequate time, an easier extraction procedure and a much more rapid determination of erythromycin than that cited in literature to date is required. This paper describes a new HPLC method for the determination of erythromycin with UV detection, which implies for the first time an internal standard for quantification and a much easier extraction procedure based on the solubilization of erythromycin followed by freezing the cream matrix.

2. Investigations, results and discussion

A new HPLC-UV method based on a more rapid and easier extraction procedure has been developed for the determination of erythromycin in the "Hydrophilic Erythromycin Creme 1%" mentioned in the NRF.

By simply adding methanol to the cream, erythromycin is dissolved completely, whereas the bulk of the cream matrix ingredients congeal in the methanolic solution after cooling the mixture to −4 °C, allowing thus its easy separation by centrifugation. An aliquot of the clear solution may be then immediately subjected to HPLC-UV analysis.

Until now no internal standard has been characterized for the quantification of erythromycin. In view of compensating for a potential loss during sample preparation and better handling of a large number of samples in routine analysis, an internal standard is urgently needed. Since roxithromycin is structurally very similar to erythromycin it was tested for its suitability as internal standard. Results of the recovery study have shown that roxithromycin does not influence the behaviour of erythromycin present in the cream. Furthermore it is stable and well separated from all erythromycin components and elutes with a relative retention time of 0.53 (with respect to erythromycin A), possessing no interfering signals. By the implementation of an internal standard in the quantification of erythromycin several advantages, which are, among other things, reflected by an improved precision, could be achieved in routine analysis.

As shown in Fig. 1 the investigation of specificity showed, that the method is suitable to discriminate between the

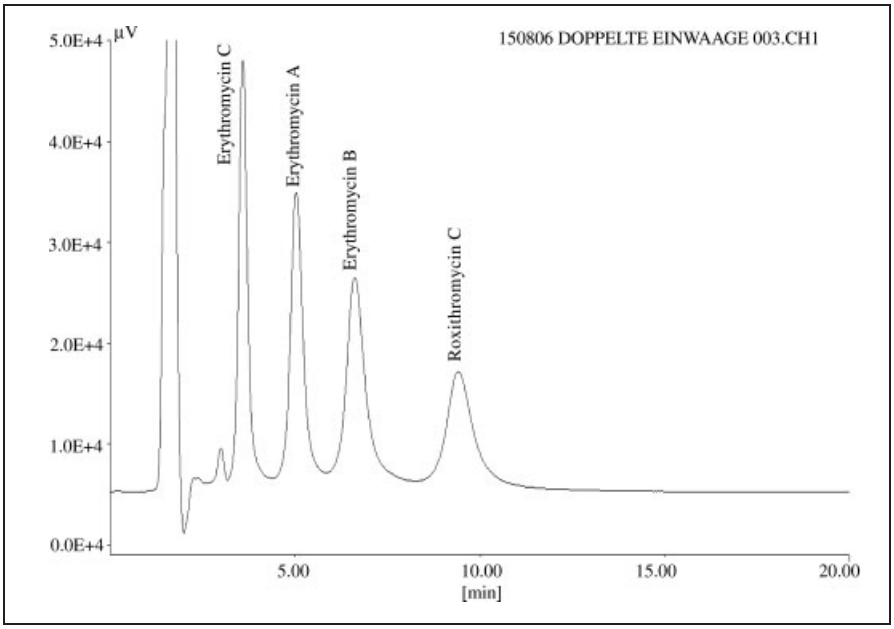


Fig. 1:
Chromatogram of the standard solution containing erythromycin A (510 µg/ml; $R_t = 5.0$ min), erythromycin B (510 µg/ml; $R_t = 6.7$ min), erythromycin C (510 µg/ml; $R_t = 3.6$ min), and roxithromycin (150 µg/ml; $R_t = 9.3$ min)

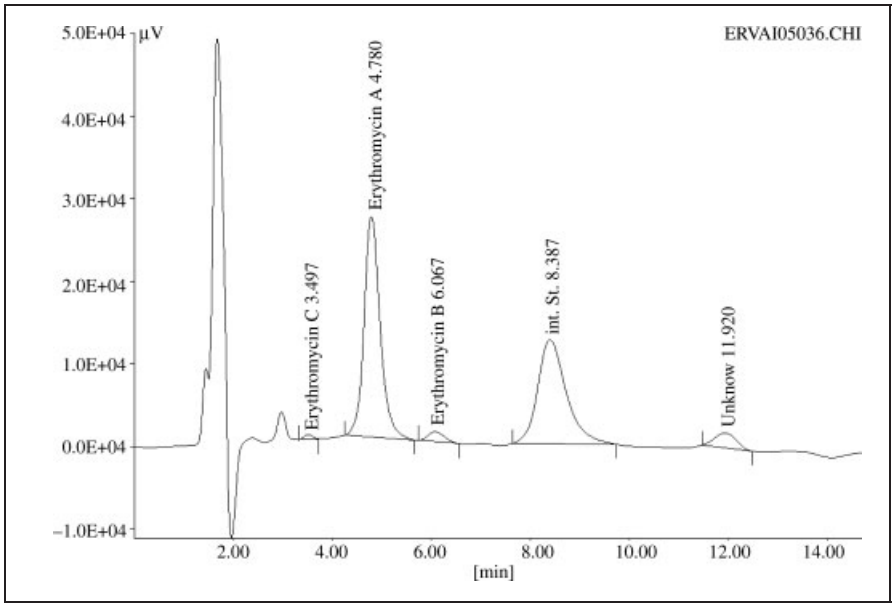


Fig. 2:
Representative chromatogram of a sample solution obtained after processing "Hydrophilic Erythromycin Cream 1%"

internal standard roxithromycin and the compounds erythromycin A, B and C. No interfering signals neither from the cream matrix nor from the solvent could be detected at the retention times of roxithromycin and erythromycin. A chromatogram of a sample solution obtained after processing the cream is illustrated in Fig. 2.

Good linearity of the assay was found over the investigated concentration range of 70–130% (corresponding to 0.77 g–1.43 g of erythromycin A in 100 g cream base). The coefficient of correlation resulting from unweighted linear regression was 0.9998. The relative deviations of the calculated standard concentrations from their nominal values lay between −0.8 and 0.8% and did not exceed 2% as demanded by international guidelines (EMEA 1995, 1997), thus allowing the use of a one-point calibration in routine analysis. A detailed summary of the validation results is given in Table 2.

The stability assays showed no considerable degradation of erythromycin in the standard and sample solutions after storage in the autosampler at 10 °C for 24 h. However a much higher recovery of 105.2% was obtained after storing erythromycin standard solution in the autosampler at 10 °C for 48 h, which may be attributed to the evaporation of methanol. Consequently fresh standard solutions must be prepared daily before use.

Studying the robustness of the method revealed, varying the extraction time from 10 to 8 and 12 min in the shaking water bath did not affect the recovery of erythromycin which was 100.6% after 8 min and 101.0% after 12 min. On the other hand special attention should be turned to variations in the composition of the mobile phase. Whereas increasing the buffer fraction (acetonitrile : buffer (48/52, v/v)) was well tolerated, increasing the acetonitrile fraction (acetonitrile : buffer (52/48, v/v)) worsened the resolution of erythromycin A, B and C on the one hand and the separation of erythromycin from the cream matrix components on the other hand and affected the recovery of erythromycin too, exceeding the allowed tolerance range of 100 ± 2%. Based on these results the method cannot be considered robust with regard to any increase in the acetonitrile fraction.

In contrast, the analytical method may be considered robust with regard to changes in the flow rate. Modifying the flow rate from 1.5 ml/min to 1.3 and 1.7 ml/min yielded a recovery of erythromycin of 101.4 and 100.6%, respectively. Also the system suitability test met the requirements of ± 2% with variation coefficients of 0.25% at 1.3 ml/min and 0.84% at 1.7 ml/min. Furthermore changing the lot of the PolymerX column did not affect the robustness of the analytical method.

Comparing the peak area ratios of erythromycin A and B as well as erythromycin A and C in standard erythromycin A solution with those in sample solutions obtained after processing, ratios of 31.1 and 30.4 for erythromycin A/B and ratios of 462.2 and 459.8 for erythromycin A/C in standard and sample solutions, respectively were determined, indicating that also erythromycin B and C may be completely extracted from the cream matrix.

Finally the response factors (peak area/concentration of the standard solution) of the minor components B and C with respect to erythromycin A were determined. In case of quantifying erythromycin B and C via erythromycin A in routine analysis, a correction factor of 1.0855 for erythromycin B and 1.0224 for erythromycin C should be taken into consideration.

Based on these results it may be concluded that this novel HPLC-UV method provides an excellent and simple method for the extraction and quantification of erythromycin in "Hydrophilic Erythromycin Creme". Precision and accuracy as major control parameters for the whole validation procedure met the international acceptance criteria for analytical method validation. Furthermore this method may

Table 2: Summary of the validation results for the quantification of "Hydrophilic Erythroymycin Creme 1%"

Validation parameter	Results (mean values)
System suitability test	CV = 0.9%
Accuracy	Recovery: 100.6% (CV = 1.2%)
Precision	CV = 1.1%
Intermediate precision	CV = 0.64%
Stability of erythromycin standard solution at 5 ± 3 °C for 24 h	Recovery: 98.2%
Stability of erythromycin standard solution in the autosampler at 10 °C for 24 h	Recovery: 98.3%
Stability of erythromycin standard solution in the autosampler at 10 °C for 48 h	Recovery: 105.2%
Stability of erythromycin sample solution in the autosampler at 10 °C for 24 h	Recovery: 99.0%

be applied also to individual extemporaneous erythromycin formulations composed solely of erythromycin in "Basiscreme DAC", as could be shown by means of cross validation. The simple extraction procedure based on the solubilization of erythromycin followed by freezing the cream matrix provides very clean extracts, allowing thus a higher throughput and more easier and rapid determination of erythromycin in topical cream formulations, making this method an ideal analytical alternative to be implied in routine analysis.

3. Experimental

3.1. Materials

For the preparation of the standardized "Hydrophilic Erythromycin Creme 1%" erythromycin as well as Basiscreme DAC, propylenglycole and citric acid were purchased from Fagron (Barsbüttel, Germany). Distilled water was produced in the Central Laboratory.
For the purpose of method validation erythromycin A CRS, erythromycin B CRS and erythromycin C CRS were obtained from Promochem (Wesel, Germany). Roxithromycin, used as internal standard, was purchased from Sigma (Rödermark, Germany).
Acetonitrile, methanol, monobasic potassium phosphate, phosphoric acid 85% and tertiary butanol of analytical grade were obtaind from Merck (Darmstadt, Germany).

3.2. HPLC conditions

The eluent was prepared by mixing 50 ml 0.2 M (35 g/l) monobasic potassium phosphate solution (adjusted to pH 8.0 with phosphoric acid) with 400 ml distilled water, 165 ml tertiary butanol and 30 ml acetonitrile and was than completed to volume (1000 ml) with distilled water. This buffer was than mixed with acetonitrile (1 : 1, v/v) followed by sonication for 5 min.
For all investigations an isocratic HPLC system was used consisting of an injector, HPLC pump, ultraviolet detector and autosampler (Jasco, Groß-Umstadt, Germany). The chromatographic separation was carried out using a polymerX column (4.6 mm × 25.0 cm, 7 μm) (Phenomenex, Aschaffenburg, Germany) at a constant temperature of 75 °C with a flow rate of 1.5 ml/min and an injection volume of 50 μL. Additionally a guard column was used. Detection was carried out at a wavelength of 215 nm. The running time did not exceed 20 min. During the whole analysis the autosampler was kept cool at a constant temperature of 10 °C.

3.3. Erythromycin sample and standard preparation

The standardized formulation "Hydrophilic Erythromycin Cream 1%" was prepared manually and mixed intensively to reach homogeneity.
Erythromycin standard stock solution was prepared by dissolving 55.6 mg erythromycin A reference standard in methanol to reach a concentration of 1000 μg/mL. Than the erythromycin standard stock solution was diluted with methanol to yield concentrations ranging from 334.9 to 616.5 μg/ml erythromycin A corresponding to 70–130% of the declared content. The internal standard solution was prepared by dissolving 61.5 mg roxithromycin reference standard in methanol to reach a concentration of 150 μg/mL. Finally 10 mL of the reference standard solution were mixed with 10 mL of the respective erythromycin standard solution.
The erythromycin samples were prepared by weighing 1 g "Hydrophilic Erythromycin Cream" into a flask. After adding 10 mL of internal standard solution and 10 mL of methanol, the suspension was shaken vigorously in a shaking water bath at 45 °C for 10 min. After cooling in ice water for 10 min an aliquote of 2 mL was centrifuged for 10 min at 3500 rpm and −4 °C. Finally 1 mL of that clear solution was transferred to an HPLC vial for analysis.

3.4. Method validation

In order to prove the suitability of the developed analytical method, validation was performed on the example of "Hydrophilc Erythromycin Cream 1%" according to the current guidelines for method validation (EMEA 1995, 1997).
The specificity of the method was verified by comparing the retention times of the internal standard roxithromycin and the erythromycin components A, B and C. Furthermore the chromatograms of methanol and placebo were compared with the chromatogram of the standard solution containing roxithromycin and erythromycin A, B and C.
Linearity was checked across the range of 70–130% of the declared erythromycin content at 7 concentration levels. The correlation coefficient of the calculated regression curve and the bias of the resulting concentrations from their nominal values (accuracy) were chosen as parameters to verify the linearity of the analytical method. The analyte concentration was evaluated using the internal standard method. Since erythromycin A represents the major component of commercially available erythromycin, it was chosen for quantification. The standard curve was calculated from the peak area ratios of erythromycin A/internal standard and the nominal analyte concentrations using unweighted linear regression. Moreover, it was checked weather comparable peak area ratios were obtained for erythromycin A, B and C in standard and sample solutions, respectively.
The accuracy was assessed by determining the recovery of erythromycin A on the basis of three concentration levels (70%, 100% and 130%) covering the specified range, each injected twice. In order to evaluate the precision of the analytical method, six independently weighed samples of "Hydrophilc Erythromycin Cream 1.0%" were processed and the concentration of erythromycin A as well as the variation coefficient of the individual recoveries were calculated. For the estimation of the intermediate precision the recoveries and variation coefficients for erythromycin A obtained after processing six samples of the hydrophilic cream by two analysts, were compared respectively and the mean variation coefficient obtained by the two analysts was calculated.
For carrying out the system suitability test the variation coefficient of a sixfold determination of a 100% erythromycin standard solution was calculated, which should not exceed ± 2%.
Stability tests of erythromycin A in the standard solutions as well as in the sample solutions were performed for the 100% concentration level after storage in the autosampler at 10 °C for 24 h. Furthermore the stability of erythromycin was assessed in the standard solution after storing in the autosampler at 10 °C for 24 h and in the refrigerator at 5 ± 3 °C for 24 h.

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