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High-Performance Liquid Chromatographic Determination of Free Myxin and Its Reduction Product as Impurities in Cuprimyxin-Containing Creams

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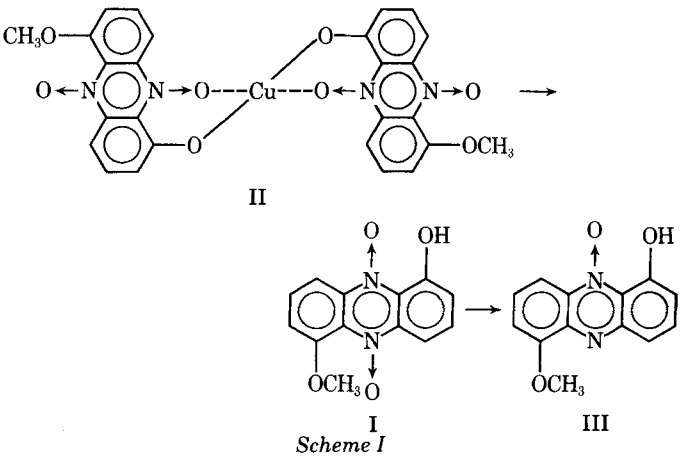
Abstract □ A stability-indicating high-performance liquid chromatographic method was developed that can detect and quantitate low levels of the two most likely breakdown products of cuprimyxin. These degradation compounds, free myxin and its reduction product, can be determined in topical cream preparations in which cuprimyxin is formulated at the 0.5% (w/w) level. The method requires a simple two-step extraction, the addition of an internal standard, and chromatography on an amine-bonded column.

Keyphrases □ Myxin—high-performance liquid chromatographic assay in cuprimyxin-containing creams □ Cuprimyxin—high-performance liquid chromatographic determination of free myxin and its reduction product □ High-performance liquid chromatography—determination of free myxin and its reduction product in cuprimyxin-containing creams □ Antibacterial agents—high-performance liquid chromatographic determination of myxin and its reduction product in cuprimyxin-containing creams

Myxin (6-methoxy-1-phenazinol 5,10-dioxide) (I) was first isolated from the soil (1) and identified as a phenazine-type product from the broth of a myxobacter (2). Its correct structure was established by Weigele and Leimgruber (3), and it was realized in its most effective antimicrobial form as the cupric complex (II) (4, 5). When applied topically, II was found to have considerable antibacterial, antiyeast, and antifungal activity in veterinary applications without the irritation side effects experienced with I (6). It was formulated in a cream and tested *in vitro* against Gram-positive and Gram-negative bacterial and fungal pathogens (7) and yeast infections (8), *in vivo* as a cream for otic and ophthalmic infections (9), and as a suspension with hydrocortisone acetate for the treatment of otitis (10). The apparent biological mechanism of action involves alteration of the invading bacterial DNA template (11).

BACKGROUND

A polarographic investigation found the electrochemical behavior of I to be a function of pH, reducing to 6-methoxy-1-phenazinol at pH < 3, to the anionic species 6-methoxyphenazinol at pH > 9, and to the intermediate 6-methoxy-1-phenazinol 10-oxide (III) at pH 3–9 (12). Compound III can be further reduced by intramolecular hydrogen bonding to 6-methoxy-1-phenazinol. The major degradation product of I in acid media and in pH 3–9 buffer solutions was found to be III[1]. Although the copper complex of II is readily dissociated in acid media to form I (Scheme I), pharmaceutical creams containing excess copper ions minimize dissociation when formulated at pH 5.7–6.2 (13).



Scheme I

The rapid conversion of II to I was used to assay for II by measurement of the amount of I found spectrophotometrically[2] and by TLC (14). Microbiological assay methods for determining II directly from seeded agar plates also were reported[3].

The determination of I and III as probable impurities in formulations involving II is difficult for two reasons: (*a*) the relative ease of conversion of II to its free form (I), and (*b*) the inability to differentiate I and III spectrophotometrically. A separation technique that minimizes dissociation of the copper complex is essential. This paper describes the development of a high-performance liquid chromatographic (HPLC) method that meets these requirements.

EXPERIMENTAL

Apparatus—A constant-flow solvent delivery system[4] was connected to a loop injector[5]. An amine-bonded silica column[6] was coupled to a spectrophotometric detector[7] set at 280 nm. A 10-mv recorder[8] was set at a chart speed of 50 cm/hr.

Reagents and Chemicals—Distilled-in-glass grade ethyl acetate[9] and heptane[9] and reagent grade acetic acid[10] were used. Samples and

[1] B. Z. Senkowski and J. E. Heveran, Hoffmann–La Roche Inc., Nutley, NJ 07110, 1971, unpublished data.

[2] M. Araujo, W. J. Mergens, and M. Osadca, Hoffmann–La Roche Inc., Nutley, NJ 07110, 1972, unpublished data.

[3] J. A. Bontempo and J. Unowsky, Hoffmann–La Roche Inc., Nutley, NJ 07110, 1969, unpublished data.

[4] Model 6000A, Waters Associates, Milford, Mass.

[5] Model U6K, Waters Associates, Milford, Mass.

[6] Chromegabond NH_2 (10 μm), 30 cm × 4.6 mm i.d., ES Industries, Marlton, N.J.

[7] Spectro Monitor II, LDC, Riviera Beach, Fla.

[8] Model 20, Varian Aerograph, Palo Alto, Calif.

[9] Burdick & Jackson Laboratories, Muskegon, Mich.

[10] J. T. Baker Chemical Co., Phillipsburg, N.J.

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reference materials were obtained from the same source[11], except for the internal standard, 3-acetylindole[12].

Mobile Phase—A mixture of ethyl acetate–heptane–acetic acid (30:69.5:0.5) was prepared and run at a flow rate of 3.0 ml/min.

Reference Standard Stock Solution—Approximately 18 mg of I reference standard and 10 mg of III reference standard were weighed into a 500-ml volumetric flask and then dissolved and diluted to volume with ethyl acetate. A 10-ml aliquot was transferred into a 25-ml volumetric flask, which then was diluted to volume with heptane.

Internal Standard Stock Solution I—Approximately 80 mg of 3-acetylindole was weighed into a 500-ml volumetric flask and then dissolved and diluted to volume with ethyl acetate.

Internal Standard Stock Solution II—A 10-ml aliquot of internal standard stock solution I was transferred into a 25-ml volumetric flask, which then was diluted to volume with heptane.

Working Reference Standard Solution—Equal volumes of the reference standard stock solution and the internal standard stock solution II were pipetted.

Extraction Solution—The internal standard stock solution I (250 ml) was transferred into a 500-ml volumetric flask and diluted to volume with ethyl acetate.

Sample Preparation—Approximately 1.0 g of sample was weighed into a 50-ml centrifuge tube. Then 10 ml of the extraction solution was pipetted into the centrifuge tube, which was stoppered and shaken vigorously by hand until the sample was dispersed in the extracting solvent (~1 min). The tube then was centrifuged, and the liquid was decanted into a 25-ml volumetric flask. The extraction was repeated with 15 ml of a 2:3 ethyl acetate–heptane mixture, and the tube again was centrifuged. The liquid was decanted and combined with the first extraction, and the final volume was adjusted to 25 ml with heptane.

Procedure—The working reference standard solution (50 μl) was injected to determine retention times and relative response ratios. This injection was followed by 50-μl injections of the sample solutions. Peaks were quantitated by measurement of their heights in millimeters.

Calculations—The level of I in the sample was determined by the following:

$$\% \text{ (w/w) I in sample} = \frac{(H_I)(C_A)(25)(100)}{(H_A)(W)(RR)} \qquad \text{(Eq. 1)}$$

where H_I and H_A are the respective peak heights of I and 3-acetylindole in the sample solution, C_A is the 3-acetylindole concentration in the sample solution, W is the sample weight (milligrams), RR is the relative response ratio, 25 is the dilution factor, and 100 is the conversion to percent.

The level of I as II was determined by:

$$\% \text{ (w/w) I as II} = \frac{(\% \text{ I in sample})(1.12)(100)}{(0.5)} \qquad \text{(Eq. 2)}$$

where 1.12 is the factor to convert I to II, 0.5 is the percent of II in the sample, and 100 is the conversion to percent.

The level of III was calculated in the same manner as I except that the 1.12 factor was omitted.

RESULTS AND DISCUSSION

A representative chromatogram showing the elution of III, I, and the internal standard (IV) at 4, 7, and 10.5 min, respectively, is presented in Fig. 1. Table I lists data obtained on several lots of cream containing 0.5% II by weight. The data reflect double extractions of several samples encompassing a wide range of storage time.

Ethyl acetate was selected as the extracting solvent because of the high solubility of I and III and the very low solubility of II in it. In addition, ethyl acetate contributed only slightly to the dissociation of the copper complex. Conversely, solvents such as hexane and heptane incompletely dissolved I at the level required for preparation of the reference standard. Solvents such as methylene chloride, chloroform, and water produced dissociation of the complex, resulting in substantial amounts of I being formed for each extraction. Furthermore, ethyl acetate was complementary to heptane as a mobile phase in that it helped achieve reasonable retention times on the HPLC column.

Dissociation of the copper complex, facilitated by the nature of the cream, was singularly the most difficult aspect in developing the analytical method. For example, II was maintained as a 50% aqueous paste since it is subject to violent decomposition in the dry state (15). Investigations showed that extracts of the paste, even after several days of standing in contact with the paste, resulted in little I formation. Yet creams to which water was added as an extraction aid produced extensive amounts of I when extracted. It is believed that water in the presence of another ingredient in the cream, perhaps acid, causes this degradation.

Table I—Levels of I and III in Creams Containing 0.5% II

Sample Lot	Age of Sample, months	I as Percentage of Cream	I as Percentage of II	III as Percentage of Cream	III as Percentage of II
A	60	0.0055	1.2	0.015	3.0
B	52	0.0025	0.5	0.005	1.0
C	52	0.0072	1.6	0.038	7.5
D	47	0.0058	1.3	0.03	6.0
E	45	0.0055	1.2	0.036	7.1
F	25	0.0017	0.4	0.004	0.7
G	23	0.0045	1.0	0.002	0.4
H	16	0.0018	0.4	0.002	0.4
I	4	0.0025	0.5	0.007	1.4
J	3	0.0023	0.5	0.002	0.3
K	2	0.0038	0.9	0.001	0.2

Some dissociation of the complex was observed regardless of the solvent chosen to extract the cream. The extent was about the same for hexane as for ethyl acetate. A study[2] that included hexane, methanol, and ether found the stability of II in hexane to be the greatest. Of interest is the apparent equilibrium established so that a finite and consistent amount of I is always formed with each additional extraction. The level of I generated thus can be related to the extent of dissociation.

The destruction of the complex, at least within the framework of the cream matrix, is both time and heat dependent and, to a certain degree, water dependent. Therefore, it is necessary to proceed swiftly during and after extractions.

Allowing the extract to contact the paste for any length of time increases the likelihood of dissociation. Furthermore, conversion of I to III accelerates in solution. Similarly, the introduction of heat during the extraction, even from mechanical shaking or ultrasonication, resulted in an increased formation of I. Although the effect of water has already been cited, no difference was obtained for extractions that incorporated sodium sulfate. Therefore, its use is not obligatory. Extraction data are shown in Table II.

It was demonstrated[13] that II is more stable than I, except under acidic conditions whereupon the complex is irreversibly dissociated. The instability of I was observed in the reference solution, both visually, as a color change, and chromatographically, as a gradual diminishing of peak size over 1 day. If necessary, it may be better to prepare a new standard

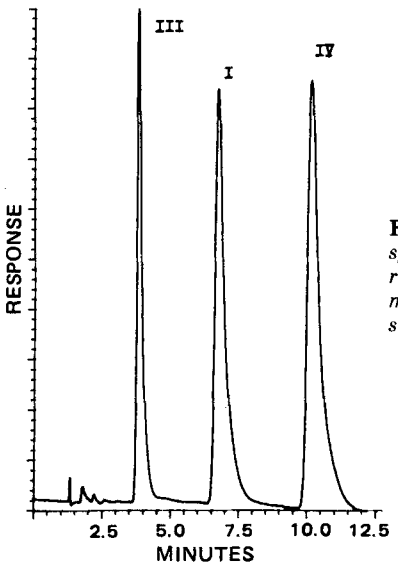

Figure 1—*Chromatogram of a synthetic mixture of the myxin reduction product (III), free myxin (I), and the internal standard (IV).*

[11] Hoffmann–La Roche Inc., Nutley, N.J.
[12] Aldrich Chemical Co., Milwaukee, Wis.

[13] C. Burke and J. E. Heveran, Hoffmann–La Roche Inc., Nutley, NJ 07110, 1971, unpublished data.

Table II—Extraction Results of I as Percentage of II

Extract	Percentage I
Cream Extracts, Ultrasonication and Mechanical Shaking	
1	0.9
2	0.5
3	0.5
4	0.5
5[a]	1.2
Cream Extracts, Manual Shaking	
1	1.0
2	0.2
3	0.4
4	0.1
5	0.1
6	0.1
7	0.1
8[b]	1.2
Cream Extracts, Sodium Sulfate	
1	1.0
2	0.3
3	0.2
Cream Extracts, Water Dispersed	
1	4.1
2	4.8
3	6.4
4	6.2
II Paste	
1	0.2
2	<0.1
3	<0.1
4	<0.1
5[c]	0.2

[a] Overnight standing of extract in contact with cream. [b] Overweekend standing of extract in contact with cream. [c] Overweekend standing of extract in contact with paste.

than to rely on a reduced value for reference response. The small amount (0.5%) of acetic acid added to the mobile phase to suppress solute ionization was not a factor and did not appear to convert any extracted II significantly.

Linearity studies performed on both I and III showed each to be completely linear within the concentration range selected, 0.0008–0.008 and 0.008–0.03 mg/ml, respectively. For I, the y intercept was −0.07, the slope was 0.996, and the correlation coefficient was 1.000. For III, these values were 1.89, 1.49, and 0.999, respectively. An amount of I equivalent to 1.5% of II was added to 1 g of sample, which then was extracted. The recovery was 97.6%.

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Mechanistic Studies on Transcorneal Permeation of Fluorometholone

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Abstract □ The mechanism of corneal fluorometholone penetration was studied using albino rabbits, and the apparent rate and extent of steroid accumulation in the various cell layers of the cornea and aqueous humor were determined for normal and abraded eyes. The results are compared and contrasted to the mechanism previously reported for pilocarpine. Fluorometholone readily penetrates the intact corneal epithelium and accumulates in the hydrophilic stromal layers of the cornea. The kinetic profile is similar to that of pilocarpine and is largely a result of the precorneal dynamic processes. Pharmacokinetic parameters for each tissue were determined to establish an overall mechanism for corneal permeation of the steroid.

Keyphrases □ Fluorometholone—mechanistic studies on transcorneal permeation, compared with pilocarpine, rabbits □ Corneal permeation—transport mechanism, fluorometholone, compared with pilocarpine, rabbits □ Pharmacokinetics—fluorometholone compared with pilocarpine, mechanistic studies on transcorneal permeation, rabbits

The ocular penetration of steroids has been widely studied (1–8), but with emphasis primarily on quantitating specific tissue levels of drug rather than on establishing the mechanism for drug movement through the cornea. Previous work with pilocarpine defined the role of various corneal tissue layers in the permeation of a drug with both water and oil solubility (9), and the techniques can be used to study a representative steroid with low water solubility

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