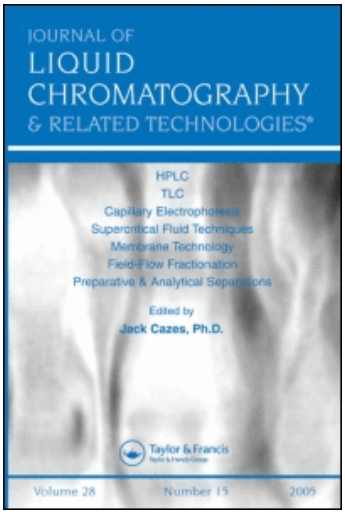

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

TLC SEPARATION AND IDENTIFICATION OF NEOMYCIN SULFATE, POLYMYXIN B SULFATE, AND BACITRACIN ZINC IN OINTMENTS

M. H. M. Sharaf[a]; A. L. Sanchez[a]; P. A. White[a]; R. G. Manning[a]
[a] Research and Development Laboratory, The United States Pharmacopeial Convention, Inc., Rockville, MD, U.S.A.

Online publication date: 05 September 2002

To cite this Article Sharaf, M. H. M. , Sanchez, A. L. , White, P. A. and Manning, R. G.(2002) 'TLC SEPARATION AND IDENTIFICATION OF NEOMYCIN SULFATE, POLYMYXIN B SULFATE, AND BACITRACIN ZINC IN OINTMENTS', Journal of Liquid Chromatography & Related Technologies, 25: 6, 927 — 935
To link to this Article: DOI: 10.1081/JLC-120003270
URL: http://dx.doi.org/10.1081/JLC-120003270

J. LIQ. CHROM. & REL. TECHNOL., 25(6), 927–935 (2002)

TLC SEPARATION AND IDENTIFICATION OF NEOMYCIN SULFATE, POLYMYXIN B SULFATE, AND BACITRACIN ZINC IN OINTMENTS

M. H. M. Sharaf,* A. L. Sanchez, P. A. White, and R. G. Manning

Research and Development Laboratory, The United States Pharmacopeial Convention, Inc., Rockville, MD 20852, USA

ABSTRACT

The development of a thin-layer chromatography method for the separation and identification of polymyxin B sulfate, neomycin sulfate, and bacitracin zinc in ointments, either alone or in combinations, is described.

The ointments were dispersed in chloroform and the components of interest were extracted into an aqueous 0.1 *N* hydrochloric acid layer. The stationary phase was silica gel G and the mobile phase consisted of a mixture of methanol, ethanol, methylene chloride, ammonium hydroxide, and water (3 : 3 : 2 : 2 : 1.5, v/v) or methanol, isopropanol, methylene chloride, ammonium hydroxide, and water (4 : 2 : 2 : 2 : 1.5, v/v). Detection was performed by spraying with a 0.2% solution of ninhydrin in 1-butanol.

*Corresponding author.

A number of commercial samples were analyzed using the described method. Our method worked on all brands tested, except one brand of the three-component ointment (Brand A), which contained interfering excipients. A sample clean-up step was developed to remove the interferences.

The method described was compared to the official methods described in the United States Pharmacopeia-National Formulary (USP24-NF19). Our method offered much better separation and well-defined spots compared to the official methods.

INTRODUCTION

Bacitracin is a basic polypeptide antibiotic produced by an organism of the *licheniformis* group of *Bacillus subtilis* (1). Commercial bacitracin is a mixture of at least nine closely related compounds and is stabilized by reaction with zinc or methylene disalicylate (2).

Neomycin is an aminoglycoside antibiotic produced by *Streptomyces fradiae*. It is mainly composed of neomycin B and its stereoisomer, neomycin C. The anti-microbial potency of neomycin C is lower than that of neomycin B. Small amount of other constituents may also present in commercial samples (3).

Polymyxin B is a member of closely related basic cyclic polypeptide antibiotics. Polymyxins are produced by *Bacillus polymyxa* (4).

Ointments containing one or more of the three antibiotics are used topically against a wide range of gram-positive and gram-negative bacteria. The ointments are generally petrolatum based. Other ingredients are sometimes added, such as cocoa butter, vegetable oils, and vitamin E. Ointments are sometimes augmented with additional medications; these include antibiotics such as gramicidin, antibacterial, such as sulfacetamide sodium, steroidal anti-inflammatory, such as hydrocortisone acetate, dexamethasone sodium phosphate, and methylprednisilone acetate, or local anesthetic, such as lidocaine.

Thin-layer chromatography offers a quick, affordable, and easy technique for the separation and identification of components of interest in different matrices.

We observed poor separation in the official (*USP24-NF19*) TLC *Identification B* test between bacitracin zinc (BZ) and neomycin sulfate (N) when the Neomycin and Polymyxin B Sulfates and Bacitracin Zinc Ointment (three-component ointment) was tested (5). Although polymyxin B sulfate (PB) was adequately separated in the same test, it tailed badly.

The BZ and PB spots were marginally separated in the official (*USP24-NF19*) TLC *Identification* test when the Bacitracin Zinc and Polymyxin B Sulfate

Ointment (two-component ointment) was examined (6). In addition, the polymyxin B sulfate spot tailed badly.

The results of the official tests were even less acceptable and anomalous when ointments with complicated bases were analyzed. The added excipients interfered with the identification of the components of interest.

In this paper, we report a rapid and reliable TLC method for the extraction and simultaneous identification of BZ, PB, and N in ointments. Our method works well for different commercially available petrolatum-based two-component and three-component ointments and on different brands of silica gel G plates. For formulations with complicated matrices, an easy and quick sample preparation is also reported.

EXPERIMENTAL

Chemicals

USP Bacitracin Zinc Lot M-1, USP Polymyxin B Sulfate Lot K, and USP Neomycin Sulfate Lot L-1 Reference Standards (USP Reference Standards Distribution, Rockville, MD, USA); acetone, isopropanol, methanol, and methylene chloride (Burdick and Jackson, Muskegon, MI, USA); hydrochloric acid, ammonium hydroxide, and sulfuric acid (Fisher, Pittsburgh, PA, USA); ninhydrin and 1-butanol (J.T. Baker, Phillipsburg, NJ, USA); picric acid saturated aqueous solution (1.2% w/v) (VWR, South Plainfield, NJ, USA); ethanol (Quantum Chemical Company, Cincinnati, OH, USA); Milli-Q® water (Millipore Corporation, Bedford, MA, USA). Silica gel G TLC plates, 20×20 cm with a layer thickness of 0.25 mm and without fluorescence indicator were obtained from EM Science (Gibbstown, NJ, USA), J.T. Baker (Phillipsburg, NJ, USA), and Analtech (Newark, DE, USA).

Method

Standard Solutions

Standard solutions of BZ, PB, and N were prepared by dissolving 7.3-, 1.3-, and 4.6-mg portions of USP Bacitracin Zinc, Polymyxin B Sulfate, and Neomycin Sulfate Reference Standards, respectively, in 1-mL aliquots of 0.1 *N* aqueous hydrochloric acid solution. A standard solution containing a mixture of BZ, PB, and N was prepared by dissolving 7.4-, 0.8-, and 4.5-mg portions of USP Bacitracin Zinc, Polymyxin B Sulfate, and Neomycin Sulfate Reference Standards, respectively. in a 1-mL aliquot of 0.1 *N* aqueous hydrochloric acid solution.

Sample Solutions

Extraction Conditions for the Three-Component Ointments: For All the Tested Brands Except Brand A

A portion of the ointment equivalent to about 500 USP Bacitracin Units (about 1.25 g) was transferred into a 15-mL centrifuge tube. A 4-mL aliquot of chloroform was added and the mixture was vortexed or shaken until the ointment was completely dispersed. A 1-mL aliquot of 0.1 *N* hydrochloric acid was added. The mixture was vortexed for 4 minutes, centrifuged for 10 minutes, and the clear supernatant was used for the TLC procedure.

Extraction Conditions for the Three-Component Ointments: For Brand A

A portion of the ointment equivalent to about 500 USP Bacitracin Units (about 1.25 g) was transferred into a 15-mL centrifuge tube. A 4-mL aliquot of chloroform was added and the mixture was vortexed or shaken until the ointment was completely dispersed. A 1-mL aliquot of 0.1 *N* hydrochloric acid was added. The mixture was vortexed for 4 minutes, centrifuged for 10 minutes, and the clear supernatant was transferred to a 15-mL centrifuge tube with care taken not to withdraw the emulsion interface layer. A 10-mL aliquot of saturated aqueous picric acid solution (1.2%, w/v) was added, the tube was vortexed for 1 minute, centrifuged for 10 minutes, and the supernatant discarded. The residue was rinsed with 1-mL aliquots of water until no yellow color was observed and the washings discarded. The residue was dried under a stream of nitrogen at 50°C, dissolved in 1 mL of acetone, and 1-mL aliquot of freshly prepared 1% sulfuric acid in acetone was added. The mixture was shaken, centrifuged for 5 minutes, and the supernatant discarded. The residue was rinsed with a 1-mL aliquot of acetone, the tube centrifuged briefly, and the washing discarded. The washing step was repeated until no yellow color was observed. The residue was dried under a stream of nitrogen at 50°C, dissolved in 0.5 mL of 0.1 *N* hydrochloric acid, and the solution was used for the TLC procedure.

Extraction Condition for the Two-Component Ointments

The tested brand was formulated using a simple white petrolatum base. A portion of the ointment equivalent to about 500 USP Bacitracin Units (about 1.0 g) was transferred into a 15-mL centrifuge tube. A 4-mL aliquot of chloroform was added and the mixture was vortexed or shaken until the ointment

was completely dispersed. A 1-mL aliquot of 0.1 *N* hydrochloric acid was added. The mixture was vortexed for 4 minutes, centrifuged for 10 minutes, and the clear supernatant was used for the TLC procedure.

Thin-Layer Chromatography

TLC was performed on 20×20 cm silica gel pre-coated plates (EM Science, Analtech, and J.T. Baker). Ten micro-liters of the standard solutions and sample solutions prepared using different ointments, were spotted manually on each of the three brands of TLC plates. Ascending chromatography was performed in pre-saturated chambers at 25°C. The mobile phase composed of either a mixture of methanol : ethanol : methylene chloride : ammonium hydroxide : water (3 : 3 : 2 : 2 : 1.5, v/v) or a mixture of methanol : isopropanol : methylene chloride : ammonium hydroxide : water (4 : 2 : 2 : 2 : 1.5, v/v). The migration distance was about 10 cm. The plates were dried in an oven at 105°C for 10 minutes, sprayed evenly with a 0.2% w/v solution of ninhydrin in 1-butanol, heated at 105°C for approximately 5 minutes to produce colored spots, examined, and photographed under white light.

RESULTS AND DISCUSSION

Systematic studies were made in search of efficient solvent systems for the separation of the three antibiotics: BZ, PB, and N. These studies resulted in the development of two solvent systems. A mixture of methanol, ethanol, methylene chloride, ammonium hydroxide, and water (3 : 3 : 2 : 2 : 1.5, v/v), and a mixture of methanol, isopropanol, methylene chloride, ammonium hydroxide, and water (4 : 2 : 2 : 2 : 1.5, v/v).

Our solvent systems were able to separate a mixture of USP Neomycin Sulfate, Polymyxin B Sulfate, and Bacitracin Zinc Reference Standards on three different brands of silica gel G TLC plates. The spots corresponding to the three antibiotics were well separated and did not tail on any of the plates.

We came up with a sample preparation that was based on the "test solution" preparation in the *Identification* tests in the official USP *Neomycin and Polymyxin B Sulfates and Bacitracin Zinc Ointment* and *Bacitracin Zinc and Polymyxin B Sulfate Ointment* monographs (5,6). This involved dispersing a portion of the ointment in chloroform, followed by extracting the components of interest into an aliquot of aqueous 0.1 *N* hydrochloric acid. The mixture was centrifuged, and the clear supernatant liquid was used as the sample solution.

Other less toxic, water-immiscible, organic solvents of comparable polarities were tried instead of chloroform. Aqueous 0.1 *N* hydrochloric acid

extracts obtained by shaking suspensions of the ointments in ethyl acetate showed the same TLC pattern as those obtained when chloroform was used. However, one of the chemists performing the tests found it more difficult to disperse some brands of the ointments in ethyl acetate. Heterogeneous suspensions were obtained and clumps were observed. We selected to use chloroform and we decided to use one fifth the amounts mentioned in the official methods.

Our method was then used to separate and identify the components of interest in commercially obtained two-component and three-component ointments. Five different brands of the three-component ointment were tested in addition to one brand of the two-component ointment. Two series of silica gel plates were used. Each series is composed of one plate from each of the three brands of silica gel plates and was developed in one of our solvent systems.

Our method worked well on all brands of TLC plates and in both solvent systems. With the exception of Brand A, the spots corresponding to the antibiotics of interest were well separated with no tailing and exhibited the same TLC characteristics and R_f values as those in the standard solutions. Table 1 lists the R_f values of the spots corresponding to the three antibiotics in both solvent systems and on different TLC plates.

The results of Brand A extract were unsatisfactory. The most problematic was the polymyxin spot. A faint ill-defined spot at an R_f similar to that of the polymyxin standard was visible only on the Analtech plates. The polymyxin spot was not visible on the other two brands (EM Sciences and J.T. Baker). On some plates, the sample neomycin spot was seen at an R_f higher than that of the standard neomycin spot. The plates showed UV-active spots when examined under UV light before spraying. This was not expected given the structures of the components of interest.

We were able to conclude that the failure of our method, in the case of Brand A, was due to the interference of other ingredients in the ointment base. Brand A contains, as indicated on the label, cocoa butter, cottonseed oil, olive oil, sodium pyruvate, and tocopheryl acetate, in addition to the three antibiotics, and a base made with white petrolatum.

Our sample preparation was further extended for Brand A. We introduced a purification step that was able to remove most of the interfering spots. It involved the selective precipitation of the antibiotics of interest as picric acid salts. The antibiotics were then recovered as sulfates using 1% sulfuric acid solution in acetone in which they are not soluble. 0.1 *N* aqueous hydrochloric acid solution of the antibiotic sulfates was then tested on the three brands of silica gel TLC plates using both of our solvent systems. The three antibiotics in the sample solution exhibited the same TLC characteristics and R_f values as those of the standard solutions. The spots were well separated and were not tailing. The results were the same on all plates and in both solvent systems.

Table 1. R_f Values of Bacitracin Zinc, Neomycin Sulfate, and Polymyxin B Sulfate on Different Silica Gel G TLC Plates

	Bacitracin		Polymyxin		Neomycin	
Plate/antibiotic	Solvent I	Solvent II	Solvent I	Solvent II	Solvent I	Solvent II
EM Science	0.76	0.74	0.50	0.46	0.11	0.09
Baker	0.79	0.74	0.48	0.48	0.13	0.09
Analtech	0.81	0.76	0.48	0.48	0.12	0.11

Solvent I: methanol, ethanol, methylene chloride, ammonium hydroxide, and water (3 : 3 : 2 : 2 : 1.5, v/v).
Solvent II: methanol, isopropanol, methylene chloride, ammonium hydroxide, and water (4 : 2 : 2 : 2 : 1.5, v/v).

Residual picric acid was not interfering with the identification and appeared as a yellow spot near the solvent front.

When the picric acid solution was added in 1-mL increments, aggregates that later precipitated appeared upon the addition of the first 1-mL aliquot. This precipitate was rich in bacitracin and polymyxin. The second fraction obtained, using additional 1-mL aliquots of picric acid solution added to the supernatant until no more precipitation was obtained, contained all three antibiotics.

With the exception of an extra spot in the picric acid-treated standard neomycin solution, the treated and untreated standard solutions looked the same when it came to the main spot. This indicated that the picric acid treatment does not alter the standards. It also indicated that there is no need to treat the standards with picric acid.

1% sulfuric acid solution in acetone needs to be freshly prepared (daily). The acetone chars with time.

Robustness

The same results were observed on three brands of silica gel G TLC plates, using different brands of the ointments, and when the method was tried independently by two chemists.

CONCLUSION

A simple and robust method was developed for the simultaneous TLC separation and identification of BZ, PB, and N in ointments. An easy and reliable extraction step is described in addition to a simple and reproducible purification step that is able to extract the components of interest from complicated ointment bases. Our method is superior to the official (USP24-NF19) methods. A proposal to revise the current official TLC Identification tests for Bacitracin Zinc and Polymyxin B Sulfate Ointment and Neomycin and Polymyxin B Sulfates and Bacitracin Zinc Ointment was submitted to the USP Antibiotics and Anti-infective Expert Committee.

ACKNOWLEDGMENT

An article entitled, “Simultaneous identification and quantitative determination of neomycin sulfate, polymyxin B sulfate, zinc bacitracin, and methyl and propyl hydroxybenzoates in ophthalmic ointment by TLC,” by J. Krzek et al.

appeared in the J. Pharm. Biomed. Anal., 24, 629–636 while this manuscript was in preparation.

REFERENCES

1. Martindale. *The Extra Pharmacopeia*, 30th Ed.; Royal Pharmaceutical Society of Great Britain, 1993; 121.
2. Hormazabal, V.; Yndestad, M. Rapid Assay for the Determination of Zinc Bacitracin in Feed by Liquid Chromatography-Mass Spectrometry. J. Liq. Chrom. & Rel. Technol. **2000**, *23* (7), 1083–1088.
3. Martindale. *The Extra Pharmacopeia*, 30th Ed.; Royal Pharmaceutical Society of Great Britain, 1993; 185.
4. Martindale. *The Extra Pharmacopeia*, 30th Ed.; Royal Pharmaceutical Society of Great Britain, 1993; 194.
5. The United States Pharmacopeia – National Formulary (USP 24-NF19); The United States Pharmacopeial Convention, Inc.: Rockville, MD, USA, 2000; 1162.
6. The United States Pharmacopeia – National Formulary (USP 24-NF19); The United States Pharmacopeial Convention, Inc.: Rockville, MD, USA, 2000; 193–194.

Received November 3, 2001
Accepted November 30, 2001
Manuscript 5676