

# Biological and Chemical Detections in Adsorbent Layer for Monitoring Microbial Production of Primycin

Antal Szabó, Attila Kónya, Valéria Széll, György Máté, and Balázs Erdélyi\*

IVAX Drug Research Institute Ltd., Berliini u. 47-49, 1049 Budapest, Hungary

## Abstract

Primycin is a macrolide antibiotic complex produced in microbiological fermentation processes. The microbial production of primycin requires an in-process analytical method suitable for monitoring the level of the active agents. In this paper, a method fulfilling the described requirement is presented. This method consists of a simple, efficacious extraction step, an instrumental sample application followed by a high-performance thin-layer chromatographic separation in relatively short time and a quantitative chromatogram evaluation. A dipping technique, in a solution containing sulfuric acid followed by heating at 120°C, is used for chromogen formation, resulting in an absorption maximum at 290 nm. A progress diagram of the fermentation obtained by this technique is compared with one obtained by a microbiological agar diffusion method. The bioautographic evaluation of the active spots in the chromatogram are also presented. By our TLC method, the group of the active primycin components in the fermentation broth and by-products formed during the fermentation can be well separated. The relative intensities of the different TLC spots provide some information on the formation of the active components.

## Introduction

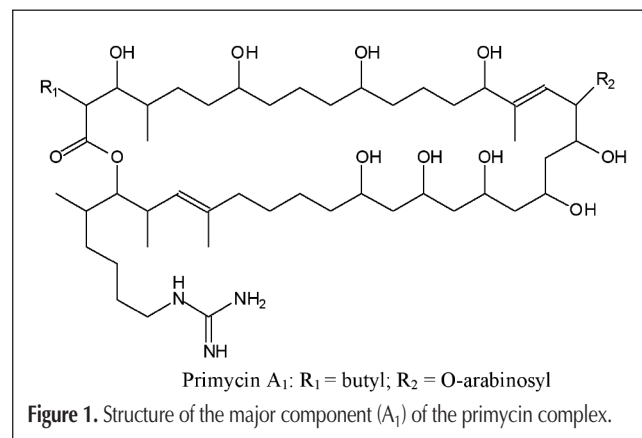
Primycin is a macrolide antibiotic complex produced by an actinomycete strain found in the intestinal tract of the wax moth (*Galleria melonella*). It was also isolated from cultures of *Streptomyces primycini* (1), and later, this strain was reclassified as *Micromonospora galeriensis* (2), and since 1984 this strain has been regarded as *Saccharomonospora galeriensis* (3). The antibacterial spectrum of primycin (4) and the mode of its action (5) were also investigated. Primycin had been marketed in Hungary as superficial alcoholic gel for the treatment of first-degree burn and other injuries and contaminations on the skin (6–9).

A described high-performance thin-layer chromatography (HPTLC) separation revealed three main sets of spots (A, B, and C) along with some minor compounds, where the retention

factor ( $R_f$ ) values in a set were extremely close (10). The constitution of primycin was revealed by nuclear magnetic resonance (11–14), and the composition of the nine main components ( $A_{1-3}$ ,  $B_{1-3}$ , and  $C_{1-3}$ ) by MS methods (10). Figure 1 represents the structure of  $A_1$  synthesized in the greatest amount among the components. The structures of the eight important minor components differ from the  $A_1$  in the  $R_1$  and  $R_2$  (pentyl, hexyl, and -OH, -H, respectively) substituents. In the course of time primycin was produced at different fermentation scales (15,16). The development of the fermentation process of primycin is running in our fermentation pilot plant, in up to 1000 L fermentor. An appropriate in-process analytical method for monitoring the primycin concentration during the biosyntheses was necessary to develop. Till now, only agar diffusion technique was applied for tracking the biosynthesis of primycin.

Bioautographic techniques are also widely used to monitor biosynthetic processes (17,18). Nevertheless, these methods are laborous and time consuming. Rapid results are necessary to intervene in a biosynthetic process at the appropriate moment (feeding/substrate completion and harvesting).

A TLC method was elaborated to quantitate a guanidinium group containing bioactive compounds such as streptomycin, dihydrostreptomycin, and primycin without post derivatization under extremely short wavelength (19). Under these conditions relatively high background appeared even when using



\* Author to whom correspondence should be addressed: email b.erdelyi@gmail.com

prewashed plates. Furthermore, focus adjustment was laborious by these invisible spots. Avoiding these disadvantages of the direct evaluation method, our attention was focused on the visualization techniques. Sakaguchi reaction used for visualization of primycin (20) was not suitable for quantitative analysis on account of its insufficient reproducibility and the high background.

Chromogen formation was chosen for the visualization of primycin using sulfuric acid solution with the aim of a considerable diminution of the undesired high background. The analytical development of sample preparation, TLC separation, derivatization, and quantitative evaluation is the subject of this paper. The visualized spots were compared with the Sakaguchi positive ones. Furthermore, the developed TLC plates were evaluated by a bioautographic method to determine the biologically active spots.

## Experimental

### Solvents and TLC plates

Primycin working standard was isolated from fermentation broth and qualified in our institute. All solvents used in the experiments were of analytical grade. Precoated polyester sheet (20 × 20 cm, Macherey-Nagel, Polygram SIL G, Germany) was applied without prewashing.

### Preparation of the samples

Two milliliters of thoroughly shaken fermentation broth was homogenized with a 4-mL mixture of butanol–methanol (1:1 v/v). After sedimentation, the clear supernatant was used in subsequent steps.

### Application, development, visualization, and evaluation

#### Quantifying the primycin concentration in the fermentation broth

Samples from the supernatants (3–3  $\mu\text{L}$ ) and 1, 2, 3, and 5  $\mu\text{L}$  of a solution of working standard (1000  $\mu\text{g}/\text{mL}$  in a mixture of 1-butanol–methanol–water; 1:1:1, v/v) were applied to the sheet as 2-mm bands, with a distance of 8 mm between the bands, by means of a CAMAG Linomat IV (Switzerland) sample applicator.

Plates were developed in a CAMAG twin-trough chamber previously saturated with the vapour of the mobile phase. The mobile phase consisted of chloroform–carbon tetrachloride–35% (v/v) formic acid (40:10:7, v/v). The migration distance was 160–180 mm, taking approximately 35 min. After drying the plate, it was immersed in sulfuric acid solution [10 mL cc. sulfuric acid was added to 200 mL refrigerated mixture of carbon tetrachloride–1-propanol (1:1, v/v); it was then heated at 108°C for 8 min in an electric oven].

The visualized chromatogram was evaluated by means of the computer controlled CAMAG TLC Scanner II in reflectance mode at 290 nm. The primycin concentration in the fermentation broth was obtained from a four-point calibration graph obtained by plotting the peak areas against of the primycin working standard.

### Identifying the peaks obtained by means of sulfuric acid

Both the samples from fermentation broth and working standards were prepared as previously described. 10, 25, 50, 100  $\mu\text{L}$  were applied as 25 mm bands. After development, the bands were cut into two 10-mm and one 5-mm wide strips. One 10-mm wide strip was visualized as described earlier; the other 10-mm wide strip was visualized by modified Sakaguchi reaction as described in the literature (13,14). The 5-mm wide strip was used for bioautography as described earlier. The spots obtained by sulfuric acid visualization were compared with the Sakaguchi positive spots and to the inhibition zone in the bioautogram.

### Bioautographic method

The bioautographic detection of primycin was performed by the agar diffusion method. Agar medium signed H8 [0.5% peptone, 0.3% Lab Lemco powder (Difco), 1.2% agar in distilled water, pH was adjusted to 8.0] was prepared and sterilized at 121°C for 25 min. The medium was cooled to 55–60°C, and supplemented with 2% NaCl (from 20% steril solution) and  $5 \times 10^5/\text{mL}$  *Bacillus subtilis* ATCC 6633 (from a suspension

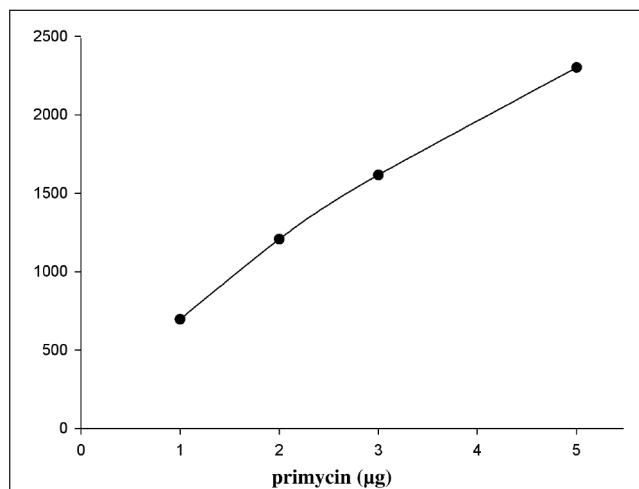


Figure 2. Calibration graph. Reflectance area versus concentration of primycin after visualization with sulfuric acid solution.

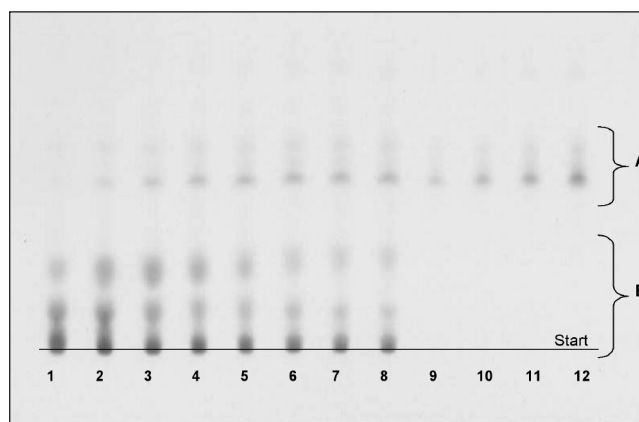


Figure 3. Chromatogram of fermentation samples at light (from 66 h to 213 h, from line 1 to 8, respectively) with a range of the working standard (line 9–12): area of spots with biological activity (A) and region of inactive spots arising from the fermentation broth (B).

containing  $10^7$ /mL bacteria). Agar plate (30 × 30 cm) was poured from the agar medium described earlier. The solidified agar plate was stored at 4°C for 1–2 h before use.

For the determination of the primycin concentration of the fermentation broth holes with 10-mm diameter were prepared in the agar plate. Fermentation broth samples were diluted tenfold with *n*-butanol–ethanol–distilled water 1:1:2 (v/v) buffer and mixed thoroughly. The diluted samples were kept at room temperature for 1 h, then they were further diluted with 65mM potassium phosphate buffer pH 8.0–ethanol 80:20 (v/v). Diluted samples (100 µL) were pipetted into the holes of the agar plates.

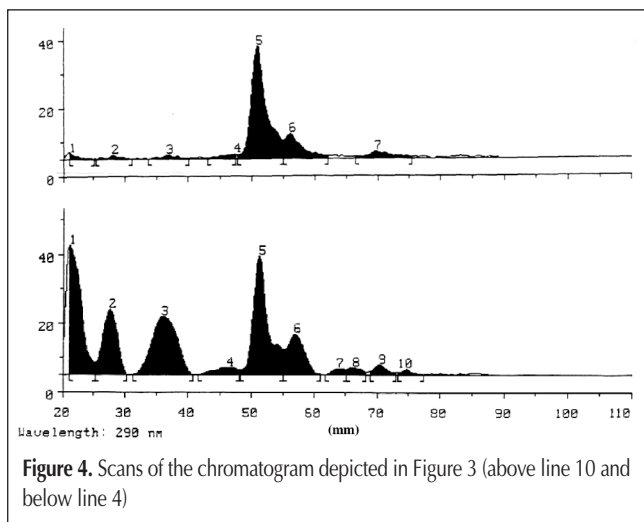


Figure 4. Scans of the chromatogram depicted in Figure 3 (above line 10 and below line 4)

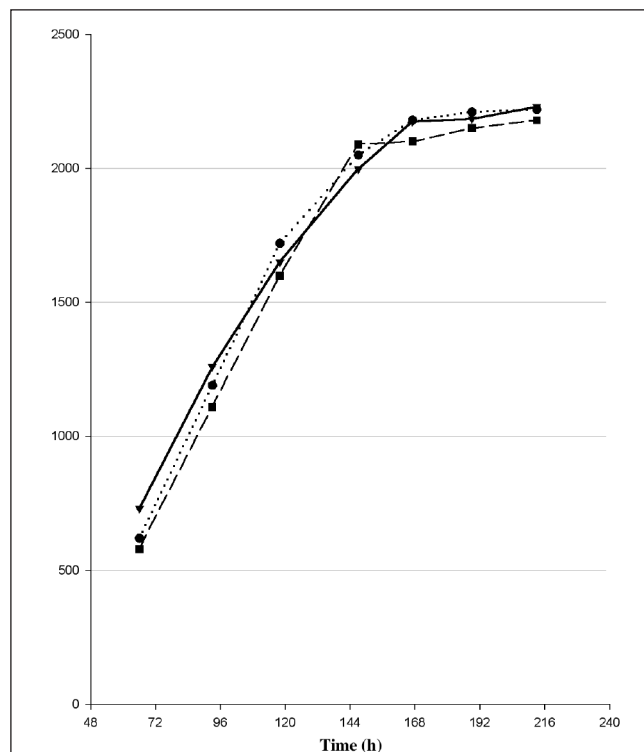


Figure 5. Production of primycin in a fermentation broth. Values obtained by TLC (continuous line), by agar diffusion technique from the original broth (dotted line), and by the same way starting from the extract used for TLC application (dashed line).

Every sample was investigated in quadruplicate in at least three different dilutions. Primycin standard containing solutions (1, 2, and 3 µg/mL) were also pipetted into holes of the agar plate. The plate was kept at 4°C for 2 h, then they were incubated at 37°C for 16–18 h, then the inhibition zones were measured around the holes of the plate. Primycin concentration of the samples were calculated from the calibration curve.

For the bioautographical investigation of TLC, a strip of the sheet was placed on the surface of the solidified agar plate. The plate was kept at 4°C for 1.5 h, then the strip was removed from the surface of the agar. The plate was incubated at 37°C for 16–18 h, then it was evaluated.

## Results and Discussion

A calibration graph was drawn from the reflectance area vs concentrations of the working standard (1, 2, 3 and 5 µg, Figure 2). Deviation from this curve is less than 2% (5 assays).

A chromatogram of samples taken from a fermentor during a biosynthesis process from the 3rd till the 8th day together with a series of working standard is depicted in Figure 3. Sharp separation was achieved after the development between the compounds belonging to the primycin complex and the metabolic compounds (decreasing spot intensities). The

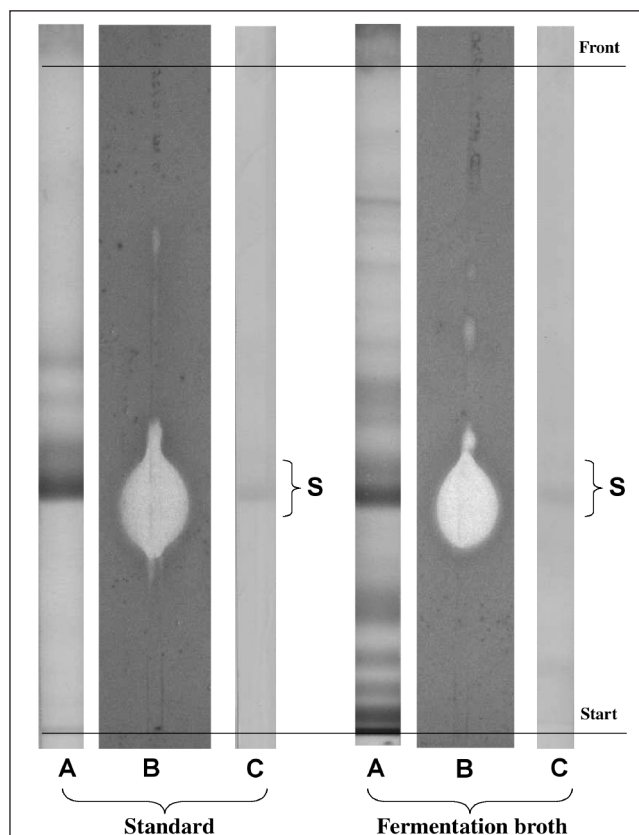


Figure 6. Chromatogram strips visualized by sulfuric acid (A) compared to the bioautograms (B) obtained by lying a developed sheet on the surface of the agar before visualization and to the chromatogram strips visualized by modified Sakaguchi reaction (Sakaguchi positive peaks are signed by an S) (C).

identification of these metabolic compounds as well as a setting up for a probably theory about guanidinium-containing (Sakaguchi positive) and sulfuric acid-positive spots connected to the primycin biosynthesis are under investigation.

The quantitative evaluation of the primycin concentration during a fermentation as well as the drawing of the calibration curve are based on the scans. One scan obtained from a fermentation sample and another one from a solution of working standard are presented in Figure 4.

Integration of the areas between the marked parts are used for quantitation. Correspondence between the values obtained by TLC and the agar-diffusion technique is presented in Figure 5. Good correlations were obtained, as shown in Figure 5. The efficacy of the extraction of the fermentation broth was proven by parallel measurements of biological activity of the original fermentation broth and of the extracted solution. The values obtained from these samples were in good correlation, verifying the completeness of the extraction in these experiments.

An attempt was made to identify the biologically active and guanidinium-containing spots in the chromatogram visualized by sulfuric acid (Figure 6). The modified Sakaguchi reaction is highly selective to the guanidinium group. The Sakaguchi positive spots are marked by an S in Figure 6. The same eluent system was used in the chromatogram strips depicted in Figure 6 as in Figure 3.

A simple, rapid quantitative TLC method was developed suitable for replacing the laborious agar diffusion technique, which was used till now for monitoring the primycin biosynthesis during the fermentation process.

## Conclusion

The TLC method described proved to be adaptable for monitoring primycin biosynthesis during the fermentation and down-stream processes as well. The main features of the described methods are: (i) simple evaluation; (ii) frequent sampling from the fermentation broth is possible due to the fast and simple analytical method; (iii) selectivity is proved to be satisfactory; (iv) quantitative results are appropriate for get information about the fermentation processes. The reported method cannot satisfy the demands of a method for qualitative control of primycin complex.

Based on these features, this method is able to replace the laborous and costly microbiological agar diffusion technique.

## Acknowledgments

Authors are to thank Dr. L. Ila for giving the working standard used in these experiments.

## References

1. T. Vályi-Nagy, J. Uri, and I. Szilágyi. Primycin, a New Antibiotic. *Nature* **174**: 1105–1106 (1954).
2. I. M. Szabó, M. Marton, G. Kulcsár, and I. Buti. Taxonomy of Primycin Producing Actinomycetes. *Acta Microbiol. Acad. Sci. Hung.* **23**: 371–76 (1976).
3. A. J. McCarthy. *Bergey Manual of Systematic Bacteriology*, N.R. Krieg and J.G. Holt, Eds. Williams and Wilkins, Baltimore, MD, 1984, pp. 2402–2404.
4. J. V. Uri. Antibacterial Activity of primycin against multiple strains of gram-positive bacteria. *Acta Microbiol. Hung.* **33**: 141–46 (1986)
5. I. Horváth, M. Kramer, P.I. Bauer, and K.G. Büki. The mode of action of primycin. *Arch. Microbiol.* **121**: 135–39 (1979).
6. C. Mészáros and K. Vezekényi. Use of ebrimycin gel in dermatology. *Ther. Hung.* **35**: 136–39 (1987).
7. J. Bíró and V. Várkonyi. Ebrimycin gel in the treatment of pyoderma and bacterial secondary infections. *Ther. Hung.* **35**: 136–39 (1987).
8. G. Bálint. Favourable observations with ebrimycin gel in the outpatient department of surgery. *Ther. Hung.* **35**: 140–42 (1987).
9. T. Papp, L. Ménesi, and I. Szalai. Experiences in the ebrimycin gel treatment of burns. *Ther. Hung.* **38**: 125–28 (1990).
10. J. Frank, G. Dékány, I. Pelczer, and J. W. ApSimon. The composition of primycin. *Tetrahedron Lett.* **28**: 2759–62 (1987).
11. J. Aberhart, T. Fehr, R.C. Jain, P. de Mayo, O. Motl, L. Baczynskyj, D.E. Gracey, D.B. MacLean, and I. Szilágyi. Primycin. *J. Am. Chem. Soc.*, **92**: 5816–17 (1970).
12. J. Aberhart, R.C. Jain, T. Fehr, P. de Mayo, and I. Szilágyi. The constitution of primycin. Part I. Characterisation, functional groups, and degradation to the secoprimycins. *J. Chem Soc. Perkin Trans 4*: 816–26 (1974)
13. D.E. Fergus Gracey, L. Baczynskyj, T.I. Martin, and D.B. MacLean. The constitution of primycin. Part II. The mass spectra of the secoprimycins. *J. Chem Soc. Perkin Trans 4*: 827–36 (1974)
14. T. Fehr, R.C. Jain, P. de Mayo, O. Motl, I. Szilágyi, L. Baczynskyj, D.E. Fergus Gracey, H.L. Holland, and D.B. MacLean. The constitution of primycin. Part III. Degradation of methylated primycin, and the structure of primycin. *J. Chem Soc. Perkin Trans 4*: 836–47 (1974)
15. T. Vályi-Nagy and G. Kulcsár. Fermentation of primycin. *Acta Biol. Acad. Sci. Hung.* **20**: 127–40 (1969).
16. T. Vályi-Nagy. Process for producing primycin. U.S. Patent 3498884 (1970).
17. T. Kokubun, N.C. Veitch, P.D. Bridge, and M.S.J. Simmonds. Dihydroisocoumarins and a tetralone from *Cytospora eucalypticola*. *Phytochem.* **62**: 779–82 (2003).
18. K.D. Burkhead, D.A. Schisler, and P.J. Slininger. Bioautography shows antibiotic production by soil bacterial isolates antagonistic to fungal dry rot of potatoes. *Soil Biol. Biochem.* **27**: 1611–16 (1995).
19. A. Szabó, B. Erdélyi, J. Salát, and G. Máté. Densitometric Determination of Some Bioactive Guanidinium Compounds Without Post-Derivatization. *J. Planar. Chromatogr.* **18**: 203–206. (2005).
20. I. Szilágyi, E. Mincsovics, and G. Kulcsár. Separation of the Homologous Components (A<sub>1</sub>, A<sub>3</sub> and B) of Primycin by Thin-Layer Chromatography. *J. Chromatogr.* **295**: 141–51 (1984).

Manuscript received December 22, 2005;  
revision received September 18, 2006.