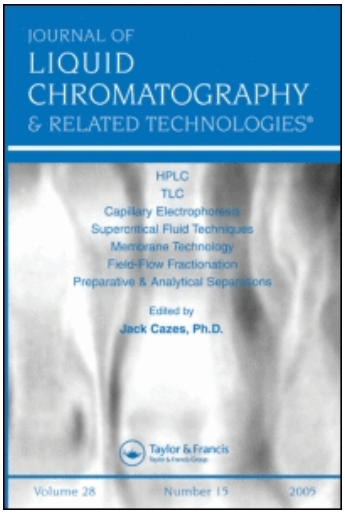


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# Simultaneous Determination of Fusidic Acid, m- and p-Hydroxybenzoates and Butylhydroxyanisole by TLC with Densitometric Detection in UV

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# Simultaneous Determination of Fusidic Acid, m- and p-Hydroxybenzoates and Butylhydroxyanisole by TLC with Densitometric Detection in UV

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**Abstract:** A thin-layer chromatography (TLC)-densitometric method has been developed for the identification and quantification of fusidic acid (FA) ($R_F = 0.53$), methyl hydroxybenzoate (MHB) ($R_F = 0.64$), propyl hydroxybenzoate (PHB) ($R_F = 0.72$), and butylated hydroxyanisol (BHA) ($R_F = 0.77$), which are the components of a dermatological ointment. To separate these constituents, *n*-hexane-ethyl acetate-glacial acetic acid (6 : 3 : 1, v/v/v) has been used as the mobile phase and silica gel 60 $F_{254}$ TLC plates as the stationary phase. To detect the spots on chromatograms, densitometric measurements at three different wavelengths were carried out, i.e., 240 nm (FA), 260 nm (MHB, PHB), and 290 nm (BHA), leading to increased selectivity and decreased interferences of the peaks. The results of the model drug examination were characterized by good precision, accuracy, and high sensitivity.

**Keywords:** Fusidic acid, Densitometry, TLC, Analysis of pharmaceuticals, Preservative agents

## INTRODUCTION

Fusidic acid (FA) is an antibiotic of steroidal structure, isolated from the mold of the fungus *Fusidium coccineum*.[1,2] It is a weak acid of pKa = 5.7 that occurs in serum and tissues in the ionized form.[3,4]

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It is used in pharmacotherapy in the form of the acid or sodium salt. It has bacteriostatic action on Gram-positive bacteria, in particular on pencillin-resistant staphylococcus.[5]

To determine FA, microbiological,[5,6] spectrophotometric,[7] colorimetric analysis based on coordination compounds with iron,[7] and titration methods with potentiometric determination of the equivalence point[8] have been used. Reversed-phase liquid chromatography with spectrophotometric and fluorescence detection was employed in the analysis of drugs and body fluids.[8–10]

Nuclear magnetic resonance techniques[11] were used in the analysis of FA complexes, with $\beta$- and $\gamma$-cyclodextrins.

Methyl hydroxybenzoate (MHB) and propyl hydroxybenzoate (PHB), in an ointment, were determined simultaneously with bacitracin, polymyxin, and neomycin by thin-layer chromatography (TLC) with densitometric detection by using two different mobile phases for appropriate constituents.[12] In pharmacopoeal monographs, UV spectrophotometry[13] is recommended for determining butylhydroxyanisole (BHA).

An advantageous effect of FA in treatment of staphylococcal infections, including those caused by penicillin-resistant strains, results in wide medical use in the form of an ointment.[14,15] In dermatological creams, FA occurs with other constituents added as preservatives (e.g., MHB, PHB, and BHA antioxidants). Coexistence of several constituents of similar physicochemical properties and highly diversified concentrations in cream base causes analytical problems in its identification and quantitative determination. In such cases, the methods described in pharmacopoeias usually fail.

Bearing in mind the problems mentioned above, the aim of this work was to establish chromatographic conditions for separating FA, MHB, PHB, and BHA with the application of ultraviolet (UV) densitometry for identification and quantitative determination. Suitability of this method for routine analysis has been proven in our earlier research studies.[12,16]



## EXPERIMENTAL

### Apparatus

Densitometer: Camag (Muttenz, Switzerland) TLC Scanner 3 with winCats 13.4 software.

Sample applicator: Linomat IV (Camag).

Computer: PC Pentium MMX, 16 MB RAM, Hewlett-Packard LaserJet 6L printer and software (Microsoft Office 2000 Premium, Statistica 5.1 edition '97).

TLC plates: 10 × 10 cm (cut from 20 × 20 cm precoated TLC sheets of silica gel 60 $F_{254}$ on aluminium; Art. 1.05554; Merck Darmstadt, Germany).

Chromatographic chamber: 18 × 9 × 18 cm (Sigma–Aldrich, St. Louis, MO USA, Cat. No. Z20, 415-3).

## Standard Substances

FA: Cat. No. F0756, (Sigma-Aldrich);
MHB: Cat. No. H5501, (Sigma-Aldrich);
PHB: Cat. No. P5835,(Sigma-Aldrich);
BHA: Cat. No. B1253, (Sigma-Aldrich).

The substances met requirements specified in the European Pharmacopeia.[17]

## Model Product

Fusacid cream consisted of FA 2.0 g, MHB 0.18 g, PHB 0.02 g, BHA 0.02 g, and vehicle up to 100.0 g. The preparation was made according to The British Pharmacopoeia.[18]



## Placebo

Placebo of composition identical with that of model product, containing cream base, solubilizers, and emulsifiers and no constituents under investigation.

## Solutions

Standard Solutions: Standard solutions were prepared in 10.0 mL of methanol by dissolving weighed amounts of constituents comparable to its model product concentrations. For this purpose, the following amounts were weighed up to 0.01 mg approximately: 50 mg FA, 4.5 mg MHB, 0.5 mg PHB, and 0.5 mg BHA. Directly before analysis, the solutions were diluted to obtain the following concentrations: 1.25 mg/mL FA, 0.072 mg/mL MHB, 0.0125 mg/mL PHB, and 0.0125 mg/mL BHA.

Model product solution: Approximately 2.5 g of ointment was weighed up to 0.001 g into a graduated flask connected to a reflux condenser, and 10 mL of methanol was added and heated in a water bath at 70°C for 20 min. The suspension was cooled to 20°C and kept at +4°C for 30 min., and then filtered through a filter paper of medium hardness. Four weighed amounts were prepared for analysis and two determinations were carried out for each of them.

Placebo solution: It was prepared as model product solution by weighing 2.5 g of vehicle.

Solvents: n-hexane, (Merck); methanol, ethyl acetate (Chempur, Piekary Śląskie, Poland); glacial acetic acid (POCh, Gliwice, Poland); all analytical grade.

## Chromatographic-Densitometric Analysis

From 2.5 to 20 μL of appropriate solutions and mixtures were applied onto 10 × 10 cm TLC plates. Chromatograms were developed by using the following mobile phase: hexane-ethyl acetate-glacial acetic acid (6 : 3 : 1, v/v/v) up to a height of 9 cm. The chromatograms were developed for 35 min. The dried chromatograms were viewed under a UV lamp at 254 nm and 366 nm and then subjected to densitometric analysis. The chromatograms were scanned and peak areas were recorded at the three wavelengths selected from absorption spectra recorded directly from chromatograms. In addition to characteristic absorption spectra, the $R_F$ values were used for constituent identification.

Based on the results of these studies, the determination procedure was developed and validation of the method was carried out.[19]



## Determination

10 μL of the examined solution and 10 μL of standard solutions were applied in bands of 8 mm width onto a silica gel coated aluminium plate. Chromatograms were developed in the mobile phase *n*-hexane-ethyl acetate-glacial acetic acid (6 : 3 : 1, v/v/v) up to a height of 9 cm and dried at room temperature for 15 min. The peak areas were recorded at the following wavelengths: 240 nm for FA, 260 nm for MHB and PHB, and 290 nm for BHA. The constituents were identified by comparing the retention coefficient values and spectra recorded in the range 200 nm to 400 nm for the examined and standard constituents. The concentrations of the examined constituents were calculated by comparing corresponding peak areas recorded for the model product and standard solutions.

## Validation of the Method

### Specificity of the Method

Specificity of the method was determined by comparing chromatograms obtained for sample solutions, placebo, and standard solution mixtures: 5 μL to 20 μL of appropriate solutions were applied onto the chromatographic plates.

### Linearity

To determine the linearity range for individual constituents, from 2.5 μL to 25 μL of standard solution mixtures were applied onto chromatographic plates.

### Limit of Detection and Determination

The limits of detection and determination were considered together, as chromatograms showed no other peaks except those of the constituents under investigation. Decreasing volumes of standard solutions from 10 μL to 1 μL were applied to the plates.

### Recovery

The recovery was computed as a ratio of constituent concentrations determined in the model product to their weighed amounts.

### Precision

The precision of the method was determined as the degree of consistency between the peak areas recorded for individual constituents. For this purpose, 10 μL of appropriate standard solutions were applied onto the plates (Table 1); 6 measurements were made for each constituent. The results of validation, along with statistical analysis, are presented in Table 1.



## RESULTS AND DISCUSSION

Searching for general-purpose analytical methods enabling not only determination of active constituents but also coexistent preservatives or stabilizers is reasonable for widely understood drug quality control. A number of additives can perform assigned roles when they are used in appropriate concentrations, and this is why it is necessary to determine them.

This paper presents an attempt to solve this problem by identification and quantitative determination of FA, MHB, PHB, and BHA in a dermatological ointment. It was found under experimentally determined conditions that, by using the mobile phase n-hexane-ethyl acetate-glacial acetic acid (6 : 3 : 1, v/v/v) and $TLC_{F254}$ plates, separation was sufficiently good and enabled visual chromatogram inspection with a UV lamp and guaranteed well developed peaks to be obtained for densitometric analysis, despite minor differences in $R_F$ values.

An analysis of absorption spectra has indicated significant differences in absorption maximum locations for individual constituents (Figure 1). Only MHB and PHB had the same absorption maximum that coincided with the FA absorption minimum and partly that of BHA. The absorption maxima of FA and BHA occured at MHB and PHB absorption minima. These findings were used in scanning and recording the peak areas to obtain the results presented in Figures 2, 3, and 4.

***Table 1.*** Validation of the method[a]

| Parameter | FA | MHB | PHB | BHA |
|---|---|---|---|---|
| Wavelength, nm | 240 | 260 | 260 | 290 |
| $R_F$ | 0.53 | 0.64 | 0.72 | 0.77 |
| Quantitation and detection limits, ng | 10 | 10 | 6 | 18 |
| Linearity range, μg/mL | 39.05–625 | 4.05–110 | 1.25–14 | 7.5–17 |
| Regression coefficients[b] | a = 10.79 | a = 114.71 | a = 158.62 | a = 31.53 |
| | b = 103.85 ± 130.70 | b = 936.58 ± 538.78 | b = 133.96 ± 66.332 | b = −44.00 ± 18.501 |
| $S_d$ | $S_a$ = 0.274 | $S_a$ = 5.664 | $S_a$ = 6.490 | $S_a$ = 2.216 |
| | $S_b$ = 88.480 | $S_b$ = 302.147 | $S_b$ = 50.725 | $S_b$ = 27.004 |
| Correlation coefficients, r | r = 0.99903 | r = 0.99396 | r = 0.99664 | r = 0.99267 |
| Precision, n = 6 | $x_{śr}$ = 20601.65 | $x_{śr}$ = 23202.87 | $x_{śr}$ = 5137.58 | $x_{śr}$ = 1290.38 |
| | Sd = 166.41 | $S_d$ = 204.49 | $S_d$ = 60.24 | $S_d$ = 47.31 |
| | RSD = 0.81% | RSD =0.88% | RSD = 1.17% | RSD = 3.66% |
| | $\mu = x_{śr} \pm 133.15$ | $\mu = x_{śr} \pm 163.62$ | $\mu = x_{śr} \pm 48.21$ | $\mu = x_{śr} \pm 37.85$ |
| Recovery, % | $x_{śr}$ = 102.64 | $x_{śr}$ = 103.81 | $x_{śr}$ = 106.87 | $x_{śr}$ = 98.12 |
| | $S_d$ = 1.68 | $S_d$ = 5.58 | $S_d$ = 10.32 | $S_d$ = 12.23 |
| | RSD =1.63% | RSD = 5.37% | RSD = 9.66% | RSD =12.5% |

[a]c = concentration; a and b =regression coefficients; $S_e$ = standard error of the estimate; $S_a$ = standard deviation of the regression coefficient a; $S_b$ = standard deviation of the regression coefficient b; $\mu$ = confidence interval; $S_d$ = standard deviation; RSD = relative standard deviation.

[b]$P = ac + b \pm S_e$.

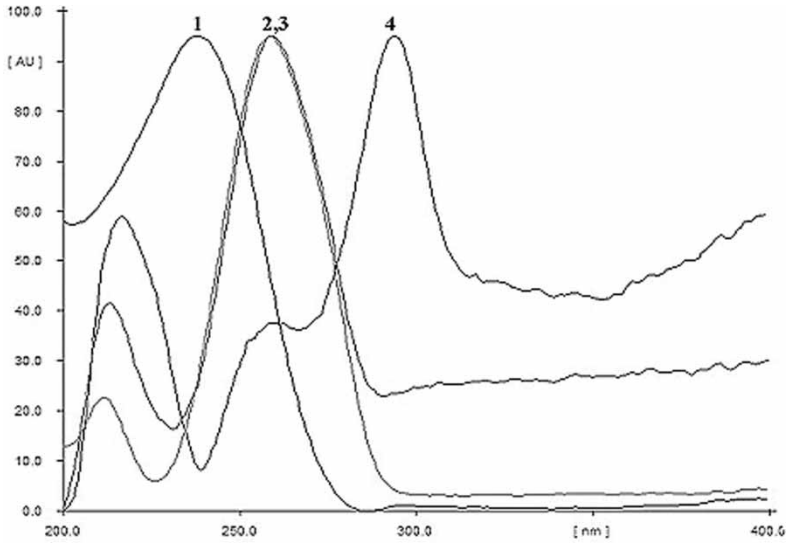


***Figure 1.*** Absorption spectra for FA–1, MHB–2, PHB–3, and BHA–4 recorded from chromatograms.

In densitograms recorded at the maximum absorbance wavelength for a given constituent, the other constituents showed smaller peak areas (Figures 2 and 3) or remained undetectable (e.g., FA at 290 nm; Figure 4). It seems that the proposed method of densitometric recording at appropriate

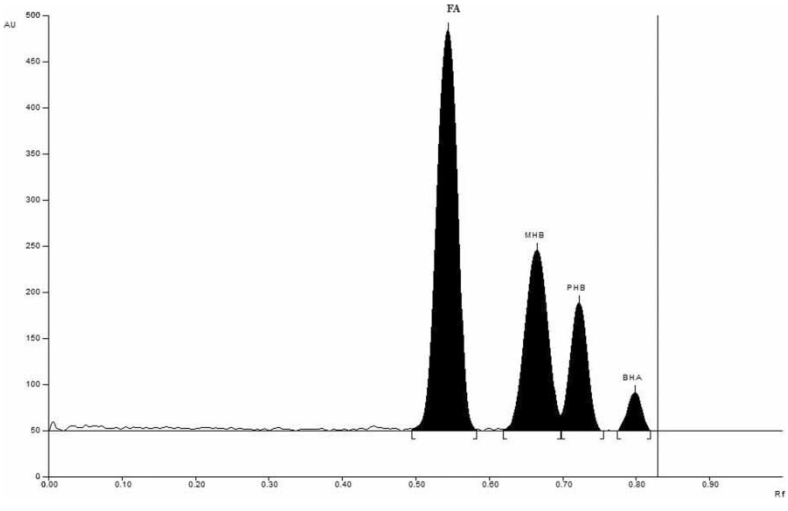


***Figure 2.*** Example densitogram registred from chromatogram of model mixture containing FA, MHB, PHB, and BHA at 240 nm.

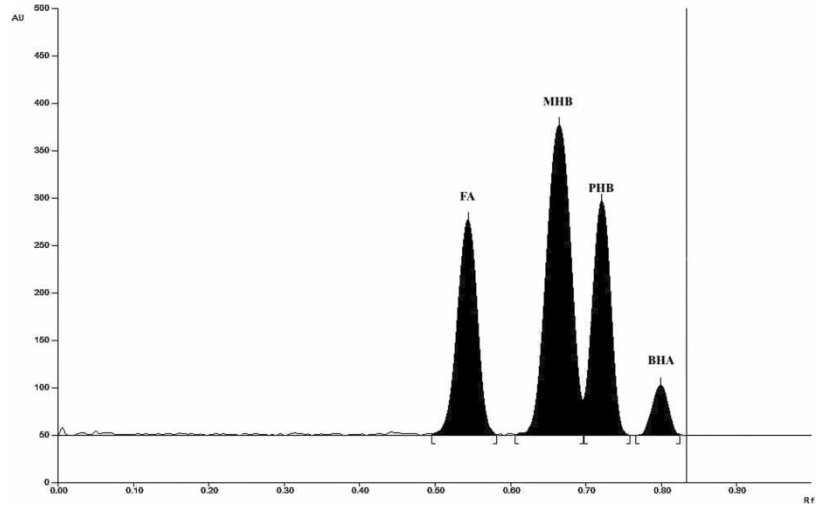


***Figure 3.*** Example densitogram registred from chromatogram of model mixture containing FA, MHB, PHB, and BHA at 260 nm.

absorbance maxima was significant, both in the terms of increased sensitivity and specifity of the method by reducing peak interference when poor spot separation occured in chromatograms.

Good results were also obtained in the analysis of the influence of placebo constituents on densitometric measurements. It was found that no

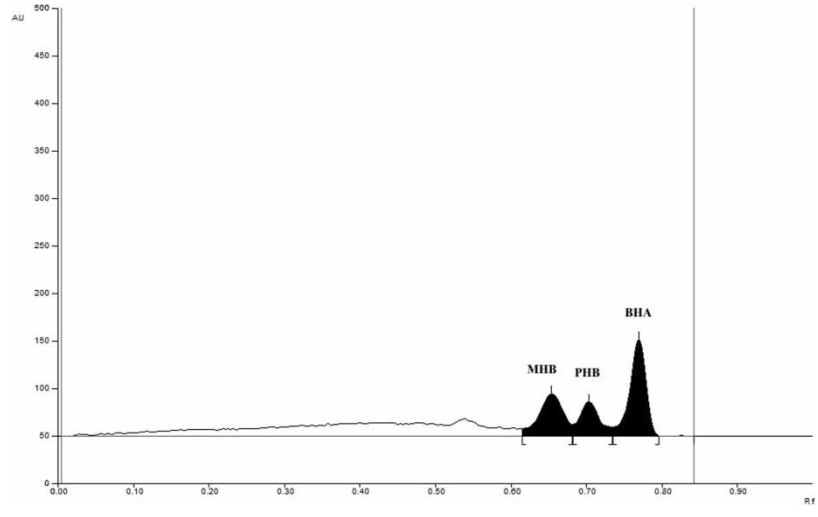


***Figure 4.*** Example densitogram registred from chromatogram of model mixture containing FA, MHB, PHB, and BHA at 290 nm.

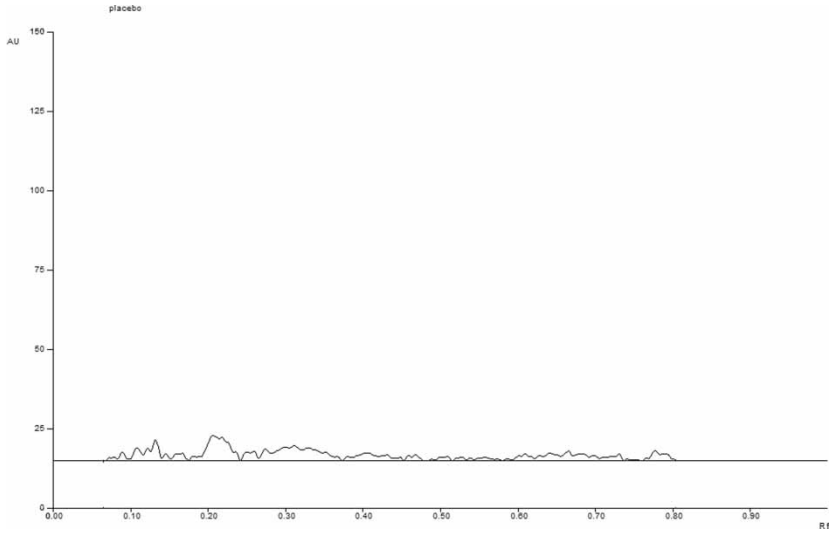


***Figure 5.*** Example densitogram registred from chromatogram of placebo extract at 290 nm.



peaks with $R_F$ values specific for the investigated constituents were observed.

An example of a densitogram recorded at 290 nm (Figure 5) confirms that there were no interferences from placebo constituents, thus enabling the conclusion that the developed method satisfies the specifity acceptance criteria.

The results of validation listed in Table 1 confirm the quality of the method, which featured good separation as evidenced by computed $R_F$ values of FA, MHB, PHB, and BHA (Table 1). The sensitivity of the method was very high; the limits of detection and determination ranged from 6 ng to 18 ng, depending on the constituent under consideration.

The precision of the determination was very good; relative standard deviation (RSD) values for FA, MHB, and PHB were close to 1%, and 3.66% for BHA. The linearity of the method has been confirmed over wide concentration ranges (Table 1).

The accuracy of the method, as determined by using the model product, was very high and ranged from 98.12% to 111.87% for individual constituents. The high recovery confirmed the good efficiency of constituent extraction from the ointment base with methanol (Table 2).

However, it should be noted that the computed average concentrations of constituents in the model product (Table 2) were comparable to declared values, but the precision of determination for MHB, PHB, and BHA was much worse, compared to that of analysis of standard solutions. It seems that such discrepancies are caused by analyte preparation and considerably

*Table 2.* Determination of FA, MHB, PHB and BHA in model ointment by TLC-densitometric determination (n = 8)

| Analyzed constituent | Content range (%) | Mean $x_{śr}$ | $S_d$[a] | RSD[a] (%) |
|---|---|---|---|---|
| FA | 2.010–2.089 | 2.050 | 0.030 | 1.6 |
| MHB | 0.173–0.202 | 0.186 | 0.010 | 5.3 |
| PHB | 0.019–0.025 | 0.022 | 0.002 | 11.6 |
| BHA | 0.016–0.023 | 0.019 | 0.002 | 12.4 |

[a]$S_d$ = standard deviation, RSD = relative standard deviation.

lower concentration of mentioned constituents compared to those of FA, which was determined with much higher precision.

An advantage of the developed method is short time of analysis (not exceeding 1 h) and the possibility of the simultaneous identification and determination of FA, MHB, PHB, and BHA.



## CONCLUSIONS

Based on the results presented above, one can conclude that the developed method satisfies criteria related to simultaneous identification and determination of FA, MHB, PHB, and BHA.

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