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DETERMINATION OF NEOMYCIN IN THE FORM OF NEOMYCIN DERIVATIVE WITH DABSYL CHLORIDE BY THIN LAYER CHROMATOGRAPHY AND DENSITOMETRY

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Abstract: A thin layer chromatographic–densitometric method has been developed for identification and quantitative determination of neomycin derivative with dabsyl chloride. The analysis of antibiotic was achieved on the silica gel TLC plates with fluorescent indicator with n-butanol – 2-butanone – 25% ammonia – water (10 : 6 : 2 : 2, v/v/v/v) as the mobile phase. The densitometric measurements were made at 460 nm. Under these conditions good separation of chosen aminoglycoside antibiotic from reagent used to make a complex was obtained. The method is characterized by high sensitivity, LOD from 0.1953 µg per band and LOQ from 0.5918 µg per band, wide linearity range from 0.5918 to 2.1960 µg per band for neomycin. The precision of the method was good; RSD varied from 1.17 to 2.05%. Satisfactory results of validation of the method were also confirmed by determination of selected antibiotic in pharmaceutical commercial preparation. The results obtained by TLC–densitometric method were compared with those obtained by spectrophotometric method.

Keywords: pharmaceutical research, neomycin, dabsyl chloride, TLC, densitometry

Neomycin is an aminoglycoside antibiotic that is produced naturally by the actinomycete bacterium *Streptomyces fradiae via* the fermentation process. It has bactericidal properties against Gram negative bacteria and partial also against Gram positive bacteria. Neomycin in a sulfate form is a common aminoglycoside indicated for treatment of gastrointestinal infections. Neomycin sulfate is mainly composed of the two isometric components neomycins B and C. The component B has higher antibiotic activity than component C (1). Neomycin is mostly administered in the form of powder, aerosols, creams and also drops and ointments often in complex preparations with bacitracin, dexamethasone, polymyxin, gramicidin, hydrocortisone, fluocinolone, etc. (2, 3, 4).

There are several methods described for the determination of neomycin in pharmaceutical preparations: colorimetric determination (5), fluorimetric determination (6), near-infrared spectroscopy (7) and spectrophotometric determination (8, 9). Some of the most popular methods are chromatographic methods: liquid chromatography with varied methods of detection like HPLC-PED (pulsed electrochemical detection) (10, 11), HPLC-ELSD (Evaporative Light Scatering Detection) (12), HPAE-IPAD (High-performance anion-exchange chromatography with integrated pulsed amperometric detection) (13) and thin layer chromatography (14, 15).

Pendela et al. described the analysis of ear drops containing neomycin sulfate, polymyxin B sulfate and dexamethasone sodium phosphate using liquid chromatography with pulsed electrochemical detection on a gold electrode (10). The similar study was performed to a formulation, which contained neomycin sulfate, polymyxin B sulfate and gramicidin (11). HPLC-ELSD method was applied successfully for the determination of neomycin and sulfate in raw materials, pharmaceutical formulations (powder, aerosols and creams) and medicated animal feeds (12). HPAE-IPAD was used to determine neomycin B and its impurities. The method was shown to be rugged for the intended application of neomycin identity, purity and assay (13). A thin layer chromatography with densitometric detection for simultaneous identification and quantitative determination of neomycin sulfate, polymyxin B sulfate, zinc

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bacitracin and methyl and propyl hydroxybenzoate in ophthalmic ointment was described by Krzek et al. (14). Hubicka et al. described the analysis of amikacin, gentamicin, kanamicin, neomycin, netilmycin and tobramycin in pharmaceutical preparations (tobramycin ampoules, amikacin vials, tablets containing 250 mg of neomycin and eye drops containing 3 mg/mL of gentamicin) (15). The densitometric measurements were made after detection with a 0.2% ninhydrin solution in ethanol (14, 15).

Anionic capillary isotachophoresis (cITP) with conductometric detection was used for indirect determination of neomycin trisulfate as sulfate anion in pharmaceutical preparations (16, 17). Capillary elektrophoresis with direct (18) and indirect (19, 20) UV detection for simultaneous determination of neomycin sulfate and such active substances as hydrocortisone, polymyxin in various pharmaceutical preparations have been reported (18–20).

Since 1975, dabsyl chloride (DBS) has been used for identification of N-terminal amino acid in polypeptide chain during analysis of compounds containing amine group (21). The first step in the process is condensation of DBS with polypeptide through N-terminal amino acid. The second step is hydrolysis in acidic environment that results in a product – sulfonamide derivative. The final third step is chromatographic identification of obtained product. This reaction is highly sensitive (10^{-9}–10^{-10} mol/L). For example, biogenic amines, bisoprolol, labetalol, and propafenone as dabsyl derivatives were determined (22–25).

The quantitative determination of bacitracin after condensation reaction with DBS was presented by Krzek and Piotrowska. Modification in the method of N-terminal amino acid determination with the use of DBS was done by exclusion of hydrolysis and establishing reaction conditions to enable direct spectrophotometric estimation of the product of DBS reaction with the antibiotic (26).

In this paper a new TLC–densitometric method was developed for the simultaneous identification and quantitative determination of neomycin derivative with dabsyl chloride (NDC), directly without the requirement of chromatogram visualization. The developed method was validated and used for the quantitative determination of neomycin in pharmaceutical preparation.

EXPERIMENTAL

Reagents

Acetone, sodium carbonate, sodium bicarbonate, 25% ammonia, butanol, 2-butanone were of analytical grade and were purchased either from POCh (Gliwice, Poland) or Chempur (Piekary Śląskie, Poland).

Dabsyl chloride (DBS) – assay = 97.5% (AT) no. 39068 (Fluka, Chemie AG Buchs, Switzerland). Solutions of DBS in acetone were prepared at concentrations 0.3238 mg/mL and 0.1619 mg/mL.

Standard solutions and substance

The following pharmaceutical raw material of neomycin sulfate was used: 25 g PPH Galfarm – no. 011209. Neomycin met the requirements described in a monograph in Polish Pharmacopoeia (FP VIII) within the scope of identity, purity and biological activity. Standard solutions in water were prepared at concentrations from 1.8603 to 0.1970 mg/mL calculated on neomycin.

Pharmaceutical preparation and solution

Neomycinum – tablets (250 mg neomycin sulfate, macrogol 4000, sodium starch glycolate type A, talc, magnesium stearate, saccharose about 260 mg in one tablet). Polfa Tarchomin S.A., Poland, series No. 1010904.

A solutions of Neomycinum – to prepare sample solution from powdered mass of 20 tablets, 50.10 mg corresponding to 15.52 mg of neomycin was accurately weighed. Then, 15.0 mL of water was added into the sample and the solution was shaken for 15 min. Then, the solution was filled up with water to 25.0 mL. The suspension was filtered directly into a 25.0 mL volumetric flask through qualitative filter paper. For testing by spectrophotometric method, the solution of neomycin at a concentration of 1.8600 mg/mL was prepared similarly.

TLC method

Preparation of neomycin derivative with dabsyl chloride (NDC)

A product of reaction was formed when 0.5 mL of neomycin solution was mixed with 1.0 mL of DBS (0.1619 mg/mL) and 0.2 mL of carbonate buffer (pH = 9.0). The samples and blank solution were heated up in water bath at 70°C for 15 min, and then cooled down and adjusted with acetone to the final volume of 5.0 mL. A blank solution containing identical volume of carbonate buffer (pH = 9.00) and DBS concentration as in the study sample was prepared.

TLC analysis

TLC was performed on 10 × 12 cm TLC plates cut from 20 × 20 cm aluminium foil-backed plates precoated with silica gel 60 F_{254} (Merck, Germany;

#1.05548). Solutions of the NDC obtained for standard solutions and for preparation solution (30 µL) were applied to the plates as 0.8 cm bands, 1.0 cm from the bottom edge, 1.0 cm from the sides and 0.8 cm apart, by use of a Linomat V sample applicator (Camag, Switzerland). Chromatograms were developed to a distance of 12 cm with n-butanol – 2-butanone – 25% ammonia – water (10 : 6 : 2 : 2, v/v/v/v) as a mobile phase in a chromatographic chamber (18 × 9 × 18 cm Sigma-Aldrich, USA, #Z20,415-3). The mobile phase was chosen experimentally by checking different solvent mixtures. Plates were dried at room temperature for one hour. Bands on the chromatograms retain durable yellow color within 24 h. Color of bands contrasts with a white background of chromatogram. Bands were visible and could be used for quantitative densitometric determination. Detection was carried out by Camag TLC Scanner 3 with winCats 1.3.4 software at λ = 460 nm; this wavelength was selected on the basis of the recorded absorption spectra. In addition to the R_F values, identification of analyzed substances were done. Peak areas of NDC obtained for standard solutions and for preparation solution were recorded directly from the chromatograms and were used for quantitative analysis.

Method validation

The method was validated by checking the specificity, linearity, precision, recovery, limits of detection and quantification and also robustness in accordance with ICH guidelines (27).

Specificity

Specificity of the method was assessed by comparing chromatograms of NDC obtained for standard solutions and for preparation solution, chromatogram of blank solution and blank chromatogram.

In obtained chromatograms, the retardation factor (R_F) values of analyzed substances, resolution factor, peak areas, peak purity and spot color were taken into account. Resolution factor (R_s) was calculated according to the formula:

R_s = 2 × (distance between the centers of two adjacent spots) / (sum of the two spots in the direction of development).

Linearity

The calibration plot for the method was constructed by analysis of seven solutions containing different concentrations of neomycin in the range 0.1970 – 0.7320 mg/mL after reaction with dabsyl chloride. Preparation of samples working solutions were described before. Further analytical procedure was described in "TLC analysis". Linearity was assessed in triplicate on the basis of the relationship between mean peak area and amount of neomycin in micrograms per band. Linearity was reported as regression equations, correlation coefficients (r) and determination coefficient (r^2).

The limit of detection (LOD) and quantification (LOQ)

Solutions of neomycin in the form of derivative with DBS (0.1220 – 0.3660 mg/mL) were applied on the plates. LOD and LOQ were calculated on the basis of the slope (a) of the calibration line and the standard error of the estimate (S_e), using formulas:

$$LOD = 3.3\ S_e / a \text{ and } LOQ = 10\ S_e / a.$$

Precision

The repeatability of the method was determined by analysis of five replicates of NDC obtained for standard solutions from individual weighings. Study was done for three concentration levels, 50% (0.5918 µg per band of NDC), 100% (1.0980 µg per band of NDC) and 150% (1.6470 µg per band of NDC). Intermediate precision was obtained by analysis of solutions at the same concentration by a different analyst who performed the analysis over a period of one week.

Accuracy

Accuracy was assessed by determination of the recovery (%) of the drug. Precisely known weighed amounts of the neomycin standard (from 80 to 120% of the declared content) were added to preparation solutions and percentage recovery was calculated on the basis of the determined amounts of added neomycin standard under conditions of developed method in relation to the amount weighed. For each level three determinations were done.

Robustness

Under conditions of the developed method, comparison of results with change of stationary phase from TLC (Merck, Germany) to TLC (polyester sheets precoated with silica gel 60 F_{254} Macherey-Nagel, Germany; #805023) plates was done.

The impact of small changes of 25% ammonia in the composition of the mobile phase (± 5%) for chromatographic separation was checked.

Determination of neomycin in pharmaceutical preparation

Determination of neomycin in tested tablets was carried out according to earlier described proce-

dure. The amount of 0.9150 μg per band of NDC obtained for standard solution and preparation solution were applied onto TLC plates for the determination of neomycin content. For each determination three measurements were performed and the mean value was taken for calculations.

Spectrophotometric method

In order to compare the test results obtained by TLC method for the determination of neomycin, spectrophotometric method was performed (26). The method was validated in accordance with ICH guidelines (27). The obtained results were used for statistical evaluation.

Formation of neomycin derivative with dabsyl chloride

A product of reaction was formed when 0.2 mL of neomycin solution (0.6100 mg/mL) in water was mixed with 1.2 mL of DBS solution (0.3238 mg/mL) and 0.2 mL of carbonate buffer (pH = 9.0), heated up at 70°C for 15 min, then cooled down to 25°C, 0.5 mL of distilled water added and adjusted with acetone to the final volume of 5.0 mL. A reference material containing identical volume of carbonate buffer (pH = 9.00) and DBS concentration as in the studied sample was prepared for each individual sample. Absorbance at 492 nm was measured by use of a UV/Vis spectrophotometer (Varian Cary 100 Conc.) in relation to appropriate reference material.

Determination of neomycin in pharmaceutical preparation

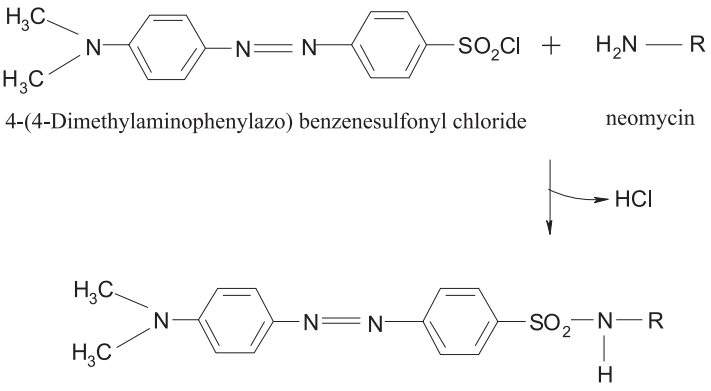
Determination of neomycin in tested tablets after formation of neomycin derivative with dabsyl chloride was carried out according to earlier described procedure. For this purpose, a series of six solutions containing DBS with neomycin preparation solution at a concentration of 1.8600 mg/mL were prepared. Then, the content of neomycin in pharmaceutical preparation was calculated by comparing appropriate values of absorbance recorded at λ_{max} = 492 nm for standard and sample solutions.

RESULTS AND DISCUSSION

Reaction of free amine groups with DBS was used for the development of a new TLC method for the determination of neomycin in pharmaceutical preparation. In the first stage of studies, conditions for the separation of NDC and DBS were established (Fig. 1).

The mobile phase n-butanol – 2-butanone – 25% ammonia – water (10 : 6 : 2 : 2, v/v/v/v) enables good resolution of analyzed substances. NDC on TLC chromatogram appeared as a compact yellow band, which contrasts with the white background of the plate and its R_F value was 0.22. The NDC bands were stable after application to the plate for 24 h. Next to the NDC, band of DBS (R_F 0.53) appeared in chromatogram and didn't interfere with the band of tested complex (Fig. 2).

The application of densitometric detection demonstrated that peaks in the chromatograms were well resolved, symmetrical and easy to identify and determine. Registration of peak areas for NDC was carried out at λ = 460 nm. The analytical wavelength chosen for densitometric registration corresponded to absorption maximum for NDC.



4-(4-Dimethylaminophenylazo) benzenesulfonyl chloride neomycin

4-(4-Dimethylaminophenylazo) benzenesulfonyl neomycin

Figure 1. Scheme of synthesis of neomycin derivative

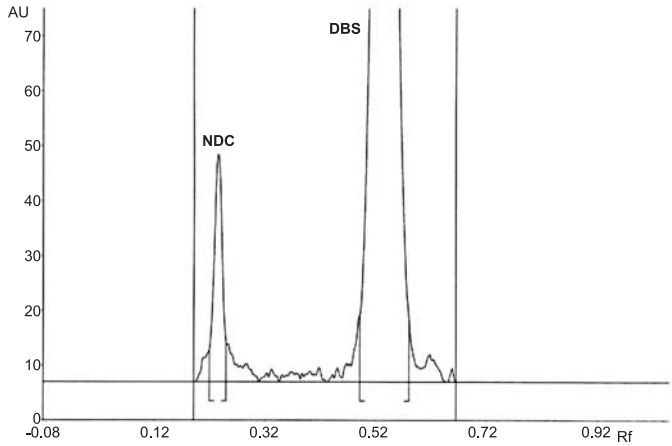


Figure 2. Densitogram of neomycin derivative with dabsyl chloride (NDC) and dabsyl chloride (DBS)

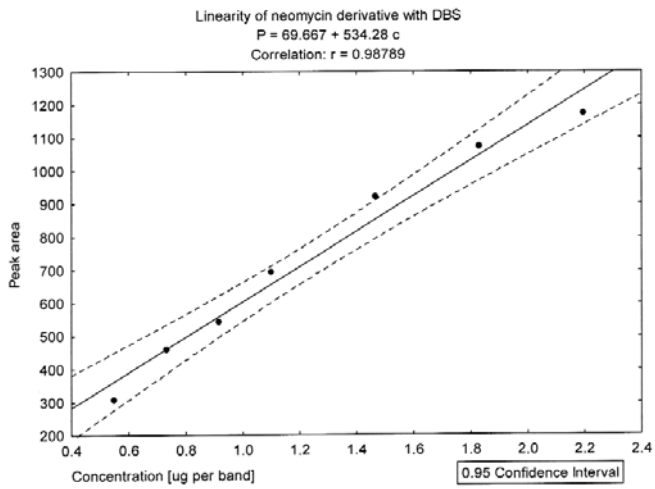


Figure 3. Linear relationship between peak area and concentration of NDC.

In the available literature there were some papers describing the determination of neomycin by thin-layer chromatography, where densitometric detection of neomycin was performed after previously spraying with a ninhydrin solution (14, 15). Application of ninhydrin required complete evaporation of ammonia (mobile phase component) from the stationary phase by heating plates at 100°C for about 1.5 h. The research presented in this paper revealed that the developed method for the determination of neomycin in the form of color derivative with DBS may be an alternative to time consuming method using ninhydrin solution for the visualization of chromatograms.

For estimation of reliability of the developed method according to ICH recommendations, the following parameters were determined: specificity, linearity, limits of detection and quantitation, recovery and robustness (27).

The developed method was specific against the studied components. There are no peaks in chromatogram recorded for blank solution, chromatograms of NDC obtained for standard solutions and for preparation solution and blank chromatogram where studied components occur. Good correlation between VIS spectra acquired from the standard and the pharmaceutical preparation indicated that NDC spot was free of any interference that

Table 1. The results of validation of TLC and spectrophotometric method.

Parameter	TLC – densitometry	VIS spectrophotometry
R_F	0.20 ± 0.03 –	
Limit of detection	0.1953 µg per band	$1.48 \cdot 10^{-3}$ mg/mL
Limit of quantitation	0.59 µg per band	$4.48 \cdot 10^{-3}$ mg/mL
Linearity range	0.59 – 2.20 µg per band	$4.48 \cdot 10^{-3}$ – $2.46 \cdot 10^{-2}$ mg/mL
Regression coefficients P = a c + b ± S_e	a = 534.28 b = 69.667 ± 55.29	a = 23.673 b = –0.0357 ± 0.01679
Standard deviation of the regression coefficients	S_a = 37.52 S_b = 51.51	S_a = 0.709 S_b = 0.0106
Correlation coefficient, r	r = 0.9879	r = 0.9969
Determination coefficient r^2	r^2 = 0.9759	r^2 = 0.9938
Precision n = 5 level 50%: level 100%: level 150%:	 x_m = 263.68 RSD = 1.70% x_m = 637.84 RSD = 1.44% x_m = 1026.26 RSD = 1.17%	 x_m = 95.94% RSD = 2.92% x_m = 96.75% RSD = 2.23% x_m = 103.26% RSD = 1.89%
Intermediate precision n = 5 level 50%: level 100%: level 150%:	 x_m =228.10 RSD = 2.05% x_m =743.42 RSD = 2.01% x_{in} =1062.44 RSD = 1.27%	 x_m = 100.22% RSD = 3.38% x_m = 99.98% RSD = 3.30% x_m = 99.60% RSD = 2.04%
Recovery, (%) n = 3 level 80%: level 100%: level 120%:	 101.48% RSD = 2.65% 101.26% RSD = 2.07% 100.97% RSD= 2.40%	 105.31% RSD = 0.89% 97.29% RSD = 2.11% 97.75% RSD = 1.74%

P – peak area; c – concentration [µg per band]; a and b – regression coefficients, S_e – standard error of the estimate, S_a – standard deviation of the slope a, S_b – standard deviation of the intercept, x_{in} – arithmetic mean; RSD – relative standard deviation.

Table 2. The results of neomycin determination in pharmaceutical preparation with statistical evaluation.

Preparation	Declared concentration	Determined concentration	
		TLC method	Spectrophotometric method
Neomycinum (tablets)	250 mg	x_m = 253.15 SD = 5.2908 RSD = 2.09%	x_m = 254.70 SD = 2.5145 RSD = 2.47%
		Fisher-Snedecor test: F_{obs} = 4.43; $F_{1-\alpha}$ = 5.05 for f = 5, α = 0.05 $F_{obs} < F_{1-\alpha}$ statistically insignificant	

f – number of degrees of freedom, α – significance level, SD – standard deviation, RSD – relative standard deviation, F_{obs} – calculated experimental value; $F_{1-\alpha}$ – critical value from the Snedecor law table.

might be present in the analysis. Resolution of the peaks appearing in the chromatograms was 3.94.

A plot of concentration against mean peak area of NDC was linear over the range from 0.5918 µg per band to 2.1960 µg per band. The correlation coefficient (r) and determination coefficient (r^2) obtained for significance level 0.05 and n = 7 were close to 0.99 that proved a highly significant linear correlation (Fig. 3). The y-intercept of the linear equation for NDC was statistically insignificant. The regression equation of the calibration plot, values of standard deviation of the slope (S_a), standard deviation of the intercept (S_b) and standard error of the estimate (S_e) are presented in Table 1.

Based on parameters of the curve (P = –25.48 + 650.27C, r = 0.9846 and S_e = 38.48), in low range of concentrations, the LOD and LOQ (µg per band) values were 0.1953 and 0.5918, respectively. These low values indicated satisfactory sensitivity of the method.

Accuracy of the method expressed as % recovery at three concentration levels was from 100.97 to 101.48%. Good precision and intermediate precision with % RSD less than 2.10% was observed. Detailed results are presented in Table 1. In all the deliberately varied chromatographic conditions (composition of the mobile phase, change of stationary phase), the retention parameters of NDC remained unchanged.

The usefulness of the method was examined by determination of neomycin in the tablets. There was no influence of additional components present in tested preparation such as macrogol, sodium starch glycolate type A, talc, magnesium stearate, sucrose and the components of the substrate on the determination results. Satisfactory results of the quantitative determination were obtained, which were characterized by good repeatability of measurements (RSD = 2.09%). The concentration determined by the developed method was comparable to the results obtained by spectrophotometric method (Table 2).

The results of determination of neomycin obtained by TLC and spectrophotometric methods were analyzed statistically using Fisher-Snedecor test (Table 2). The calculated experimental value F_{obs} for TLC and spectrophotometric methods were compared with the critical value: $F_{1-\alpha}$ (for f = 5, α = 0.05), extracted from the Snedecor law table. Based on the results of statistical analysis ($F_{obs} > F_{1-\alpha}$) it was found that the compared methods were not different considering precision in statistically significant way.

CONCLUSIONS

In reaction of neomycin with DBS a yellow complex of neomycin derivative was formed which can be used in direct TLC–densitometric analysis at λ = 460 nm.

The method meets the acceptance criteria for validation and may be useful for the determination of neomycin in the form of derivative with DBS in pharmaceutical products.

REFERENCES

1. Zejc A., Gorczyca M. Chemistry of Drugs (Polish), PZWL, Warszawa 2002.
2. Bonomo R.A., Van Zile P.S., Li Q., Shermock K.M., McCormik W.G., Kohut B.: Expert Rev. Anti Infect. Ther. 5, 773 (2007).
3. Sheth V.M., Weitzul S.: Dermatitis 19, 181 (2008).
4. Gennaro A.R. Ed.: Remington`s Pharmaceutical Sciences, 17th edn., p. 1181, Mack Publ., Easton, PA 1985.
5. Amin A.S., Issa Y.M.: Spectrochim. Acta A 59, 663 (2003).
6. El-Shabrawy V.: Spectrosc. Lett. 35, 99 (2002).
7. Cruz Sarraguça M., Oliveira Soares S., Almeida Lopes J.: Vib. Spectrosc. 56, 184 (2011).
8. Confinop M., Bontchev V, P.: Microchim. Acta 102, 305 (1990).
9. Marques M.R.C., Hackmann E.R.M., Saito T.: Anal. Lett. 23, 1005 (1990).
10. Pendela M., Adams E., Hoogmartens J.: J. Pharm. Biomed. Anal. 36, 751 (2004).
11. Adams E., Schepers R., Gathu L.W., Kibaya R., Roets E., Hoogmartens J.: J. Pharm. Biomed. Anal. 15, 505 (1997).
12. Megoulas N.C., Koupparis M.A.: J. Chromatogr. A 1057, 125 (2004).
13. Hanko V.P., Rohrer J.S.: J. Pharm. Biomed. Anal. 43, 131 (2007).
14. Krzek J., Starek M., Kwiecień A., Rzeszutko W.: J. Pharm. Biomed. Anal. 24, 629 (2001).
15. Hubicka U., Krzek J., Woltyńska H., Stachacz B.: J. AOAC Int. 92, 1068 (2009).
16. Kurzawa M., Jastrzębska A., Szłyk E.: Chem. Pap. 63, 255 (2009).
17. Kurzawa M., Jastrzębska A., Szłyk E.: Acta Pol. Pharm. Drug Res. 62, 163 (2005).
18. Huidobro A.L., García A., Barbas C.: J. Pharm. Biomed. Anal. 49, 1303 (2009).
19. Srisom P., Liawruangrath B., Liawruangrath S., Slater J.M., Wangkarn S.: J. Pharm. Biomed. Anal. 43, 1013 (2007).
20. Ackermans M.T., Everaerts F.M., Beckers J.L.: J. Chromatogr. 606, 229 (1992).
21. Lin J.-K., Chang J.-Y.: Anal. Chem. 47, 1634 (1975).
22. Romero R., Bagur M.G.; Sánchez-Viñas M., Gázquez D.: Chromatographia 51, 404 (2000).
23. Romero R., Sánchez-Viñas M., Gázquez D., Bagur M.G., Cuadros-Rodriguez L.: Chromatographia 53, 481 (2001).
24. Castillo M.A., Castells R.C.: Chromatographia 54, 711 (2001).
25. Witek A., Hopkala H., Matysik G.: Chromatographia 50, 41 (1999).
26. Krzek J., Piotrowska J.: Acta Pol. Pharm. Drug Res. 68, 853 (2011).
27. ICH-Q2 (R1) Validation and Analytical Procedures: Text and Methodology, International Conference on Harmonization, Geneva, November 2005: http://www.ich.org.

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