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ORIGINAL RESEARCH PAPER

A new simple liquid chromatographic assay for gentamicin in presence of methylparaben and propylparaben

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ABSTRACT

Gentamicin sulfate is a potent broad spectrum aminoglycoside antibiotic which is used against Gram-positive and Gram-negative bacteria. A simple, isocratic HPLC method for separation, identification and determination of gentamicin and parabens (methylparaben and propylparaben) was developed and validated. To our knowledge there is no report about simultaneous determination of those three analytes in pharmaceutical products. The optimum chromatographic conditions were achieved on CN column with a mobile phase consisting of 0.15% triethylamine in 10 mM KH_2PO_4 aqueous solution (final pH 3.0 adjusted with H_3PO_4) and methanol in the ratio 70:30 (v/v), providing selective quantification of analytes within 5 min. The method was successfully validated according to ICH guidelines acceptance criteria in terms of selectivity, linearity, accuracy, precision and robustness. The linearity of the method was proved in defined concentrations ranges for gentamicin (0.32–1.04 mg mL^{-1}), methylparaben (0.0072–0.0234 mg mL^{-1}) and propylparaben (0.0008–0.0026 mg mL^{-1}). Relative standard deviations calculated for all analytes in precision testing were <2% (analysis repeatability) and <3% (intermediate precision). Recovery values were between 98.87% and 101.67%. Chromatographic parameters are not significantly influenced by small variations of column temperature, pH and molarity of KH_2PO_4. Finally, the method was successfully applied for quantitative determination of gentamicin and parabens in commercially available solution for injection. Proposed HPLC method is found to be promising in terms of simplicity, analysis times and non-use of derivatization and ion-pair agents.

KEYWORDS

gentamicin, liquid chromatography, parabens, validation

INTRODUCTION

The aminoglycosides are one of antibiotics classes widely used in human and veterinary medicine for the treatment of bacterial infections. This class is characterized as glycoside substituted 1,3-diaminoinositols. Gentamicin is produced by fermentation of *Micromonospora purpurea* and other related soil microorganisms. Such fermentation produces a mixture of major components designated gentamicins A, B, and C. Gentamicin C is a broad-spectrum antibiotic, a mixture that contains five congeners C1, C1a, C2, C2a, and minor C2b which differ from each other in their methylation on the purpurosamine ring (Fig. 1). The pharmaceutically active substance used therapeutically is gentamicin sulfate. Aminoglycosides inhibit protein synthesis by virtue of their ability to bind to the 16S unit within the 30S ribosomal subunit of bacteria. Irreversible binding leads to formation of nonsense proteins killing the bacteria [1]. Gentamicin is commonly reserved for aerobic Gram-negative infections, with less potency against Gram-positive bacteria [2].

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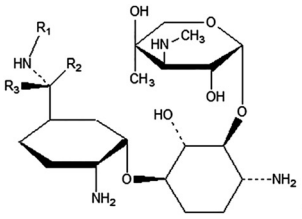
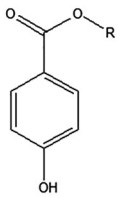


Gentamicin	R_1	R_2	R_3
C1	CH_3	CH_3	H
C1a	H	H	H
C2	H	CH_3	H
C2a	H	H	CH_3
C2b	CH_3	H	H



Paraben	R
Methyl	$-CH_3$
Propyl	$-C_3H_7$

Fig. 1. Chemical structure of major gentamicin components and parabens

Most of the literature data concerns analysis of gentamicin using liquid chromatography methods. The absence of a strong UV-absorbing chromophore in gentamicin makes conventional UV detectors not sensitive enough. Pre- and post-column derivatizations are the ways to improve gentamicin retention during HPLC analysis while also enabling UV, fluorescence or electrochemical detection. The indirect methods involve determination after derivatization with *o*-phthalaldehyde [3], 9-fluorenylmethyl chloroformate [4], phenyl isocyanate [5], benzenesulfonyl chloride [6], 1-fluoro-2,4-dinitrobenzene [7], fluorescamine [8]. However, derivatization could be time-consuming and derivatives are frequently unstable.

Ion-pair chromatography (IP) is well-suited to gentamicin because of its polar, charged and basic characteristics under IP conditions. The counterions could be the negatively charged nonvolatile alkylsulfonates [9, 10], but more suitable counterions in HPLC/MS applications are volatile fluorinated carboxylic acids [11–13]. Trifluoroacetic acid (TFA) is especially selective to gentamicin because other aminoglycosides are not retained on reversed-phase columns with TFA [12–14]. However, the use of ion-pairing relatively volatile agents reduces the life cycle of columns.

Gentamicin and its related substances are highly water soluble, so hydrophilic interaction liquid chromatography (HILIC) can be considered as elective choice for their analysis [15, 16].

The amino and hydroxyl groups in gentamicin structure are apparently the source of their electrochemical activity. Direct detection methods for analysis of gentamicin include evaporate light scattering [14, 17] and pulsed electrochemical detection [12]. The official method for determination of the composition and related substances of gentamicin prescribed by the British and U.S. Pharmacopeias is liquid chromatography combined with pulsed electrochemical detection (LC-PED) [18, 19].

Mass spectrometry is the method of choice and offers the required sensitivity and selectivity for confirmation of gentamicin. Several methods based on liquid chromatography coupled to mass spectrometry (LC-MS/MS) are available in literature [20–23].

Parabens (Fig. 1) are antifungal and antibacterial agents, widely used as preservatives in pharmaceuticals. Most methods based on liquid chromatography for simultaneous determination of parabens and pharmaceutically active compounds are available in literature [24, 25]. To our knowledge, there is no report about simultaneous determination of gentamicin and parabens in pharmaceutical products. This paper describes development, validation and application of a selective and easy-to-use RP-HPLC method with UV detection for control of gentamicin and parabens (methylparaben and propylparaben) in solution for injection.

EXPERIMENTAL

Chemicals and reagents

Analytical grade potassium dihydrogen phosphate and edetate sodium were purchased from Merck (Darmstadt, Germany) while sodium metabisulfite, triethylamine and orthophosphoric acid were provided from Sigma-Aldrich (Steinheim, Germany). HPLC grade methanol (JT Baker, Phillipsburg, NJ, USA) was used as organic modifier. CRS standards of gentamicin sulfate, methyl 4-hydroxybenzoate (methylparaben) and propyl 4-hydroxybenzoate (propylparaben) were used. Gentavet 80 mg mL^{-1} solution for injection was produced by Evrolek-Pharmacija d.o.o. (Šabac, Serbia).

HPLC instruments and analytical conditions

Chromatographic analysis was performed on chromatograph Dionex UltiMate 3000 system (Thermo Fisher Scientific, Germering, Germany) equipped with quaternary pump, autosampler, thermostatted column compartment and PDA detector. Mobile phase was degassed using Sineks Laboratory Mod UCI-75 ultrasonic bath. The column used for separation was Phenomenex Luna® 5 µm CN 100 Å (150 x 4.6 mm), thermostated at 40 °C. A binary mobile phase consisting of 0.15% triethylamine in 10 mM KH_2PO_4 aqueous solution (final pH 3.0 adjusted with H_3PO_4) and methanol in the ratio 70:30 (v/v) was used. The sample injection volume was 10 µL. The flow rate was 1 mL min^{-1}, and detection was performed at 200 nm.

Preparation of solutions

Standard solutions. As a solvent, 10 mM KH_2PO4 was used for the preparation of all solutions during testing.

Placebo Gentavet 80 mg mL^{-1} solution for injection (containing edetate sodium and sodium metabisulfite) was used for selectivity testing. Stock standard solutions of gentamicin (8 mg mL^{-1}, calculated on gentamicin base),

methylparaben (0.18 mg mL^{-1}) and propylparaben (0.02 mg mL^{-1}) were prepared in solvent with addition of methanol. Working standard solution was obtained by diluting stock standard solutions with solvent to obtain following concentrations: 0.8 mg mL^{-1} (gentamicin base), 0.018 mg mL^{-1} (methylparaben) and 0.002 mg mL^{-1} (propylparaben).

Real sample testing. One milliliter of Gentavet 80 mg mL^{-1} solution for injection was diluted with the solvent to 100 mL.

Method validation

Selectivity. Selectivity testing of the method was performed by injecting three solutions under optimal chromatographic conditions: placebo solution, working standard solution and a real sample solution. Selectivity was tested by examining the chromatogram of placebo solution at retention times corresponding to gentamicin sulfate, methylparaben and propylparaben.

Linearity. For linearity assessment, five solutions of mixture of analytes were prepared by diluting working standard mixture with the solvent. Linearity of all compounds was tested in ranges of 0.32–1.04 mg mL^{-1} for gentamicin sulfate, 0.72–2.34 $\times$ 10^{-2} mg mL^{-1} for methylparaben and 0.8–2.60 $\times$ 10^{-3} mg mL^{-1} for propylparaben. Each point of the calibration graph corresponded to the mean value obtained from three independent measurements.

Precision. Two levels of method precision were tested: repeatability and intermediate precision. Six solution of the mixture of analytes at 100% of the test concentration (0.8 mg mL^{-1} for gentamicin sulfate, 0.018 mg mL^{-1} for methylparaben and 0.002 mg mL^{-1} for propylparaben) were prepared and injected. Intermediate precision was tested in the same manner by another analyst, another day, and with different set of reagents.

Accuracy. Accuracy of the method was tested by spiking real sample (Gentavet 80 mg mL^{-1} solution for injection with working standard mixture at concentration levels 80, 100 and 120%). Triplicate determinations at all concentration levels were used.

Robustness. Robustness was tested by small variations of selected parameters: column temperature and mobile phase composition (methanol content, triethylamine concentration, pH and molarity of KH_2PO_4). Working standard solution and real sample solution were injected under each of tested chromatographic conditions, and peak areas of tested analyst were monitored.

System suitability testing. To ensure the validity of the analytical procedure, a system suitability test was established and parameters were determined by injecting 5 replicates of standard solution. Parameters such as theoretical plates, peak symmetry and relative standard deviation were calculated.

RESULTS AND DISCUSSION

Method development and optimization

The main objective was to develop a simple and sensitive HPLC method with UV detection for determination of gentamicin in presence of methylparaben and propylparaben. Optimum conditions, which are necessary for the quantitative analysis of compounds, were established by selection of column type and temperature, varying mobile phase composition and observing their effects on separation appearance and symmetry of the peak.

Gentamicin is a polar compound with high water solubility and low solubility in most organic solvents, so usually is not partitioned in reversed-phase system. Normal-phase chromatographic analysis is also inadequate considering long retention of the compound. Gentamicin lacks UV-absorbing chromophores and has to be derivatized to gain UV/VIS-detectability. These physicochemical properties create a challenge to develop HPLC method suitable for routine use in quality control laboratories.

Gentamicin has several primary and secondary amino groups and, therefore, is easily derivatized. The reactive hydroxyl groups further increase the possibility to form different derivatives [26]. Method development activities were initiated using *o*-phthalaldehyde (OPA), the widely used reagent with best solubility characteristics among derivatization agents. OPA reacts in the presence of 2-mercaptoethanol as reducing agent in basic conditions with primary amines producing yellow UV-absorbing derivatives. Derivatization increases lipophilicity, but analyzed compounds retain base characteristics, so ion-pair chromatography was applied using sodium pentanesulfonate as a counterion. Separation and retention are assumed to result from ion-pair formation between protonated gentamicin and anionic alkylsulfonate ions and the different interactions between the ion pair and the hydrophobic column phase. The protonated primary and secondary amino groups of gentamicin responsible for ion-pair formation are the same ones that undergo OPA derivatization. As organic modifier in mobile phase acetonitrile was used. Proposed conditions on typical C18 column resulted in broad and unreproducible chromatographic peak shapes, not even by change of alkylsulfonates as ion-pairing reagents (hexane-, heptane- and octanesulfonate). In further method development, composition of the mobile phase was changed in terms of organic modifier. The results achieved using methanol, instead of acetonitrile, were very similar. Gradient separation resulted in better separation and retention, but still poor reproducibility.

The next phase of method development includes modification of stationary phase. According to gentamicin multiple basic sites, cyano column was selected with abilities to attract strong dipole and separates polar molecules. To accomplish dominant dipole-dipole interactions between an analyte and stationary phase, gentamicin should be present in ionic form and dissolved in phosphate buffer (10 mM KH_2PO_4). The pH 3.0 of the mobile phase has profound

effects on the elution of gentamicin. The chromatographic pattern is improved by triethylamine (TEA), useful when column silica backbone contains free silanol group. When this silanol is ionized, it might produce ionic retention mechanism where the analyte in positive ionic form will retain longer on the column. In case of gentamicin, which contains several amino groups, those interactions are strong and produce a hideous tailing chromatogram peak. TEA protects or deactivates the silanol from interacting with gentamicin. The amount of TEA was optimized to 10 mM (enough to prevent peak tailing).

Finally, the optimized HPLC parameters were as follows: stationary phase, Phenomenex Luna® 5 µm CN 100Å; flow rate, 1 mL min^{-1}; column temperature, 40 °C; detection wavelength, 200 nm; isocratic program with a binary mobile phase consisting of 0.15% triethylamine in 10 mM KH_2PO_4 aqueous solution (final pH 3.0 adjusted with H_3PO_4) and methanol in the ratio 70:30 (v/v). Under optimal chromatographic conditions, gentamicin is eluted as a single peak. It could be important, because most analysts use a gentamicin standard that contains an unknown mixture of the components, so identification of components is not based on individual standards. Additionally, under optimal chromatographic conditions methylparaben and propylparaben were successfully separated, so the proposed method provides selective quantification of those three analytes within 5 min. The big advantage of this method is non-use of derivatization which could be time-consuming and derivatives are frequently unstable. Ion-pairing relatively volatile agents which can reduce the life cycle of columns are also avoided. According to literature data, there is no report about use of cyano columns in determination of gentamicin. This column is very compatible with LC/MS and enables UV detection of gentamicin in proposed reversed-phase mode.

Method validation

The performance and validation of the method were evaluated considering the guidelines of the ICH [27] by determining selectivity, linearity, accuracy, precision and robustness.

Selectivity. Selectivity testing of the method was performed by injecting placebo solutions, working standard solutions and a real sample solution under optimal chromatographic conditions. Fig. 2 clearly demonstrates that all analytes are well separated and no interfering peaks were observed. Thus, the selectivity of the method is confirmed.

Linearity. The ICH guidelines specified a minimum of five concentration levels for linearity testing, along with certain minimum specified ranges. For an assay, the minimum specified range is from 80 to 120% of the target concentrations. In the present study, linearity was tested in the concentration range 0.32–1.04 mg mL^{-1} for gentamicin sulfate, 0.0072–0.0234 mg mL^{-1} for methylparaben and 0.0008–0.0026 mg mL^{-1} for propylparaben. The specified range for all three analytes is from 40 to 130% of nominal concentration.

The correlation coefficients (r) obtained for the regression line demonstrates the excellent relationship between peak area

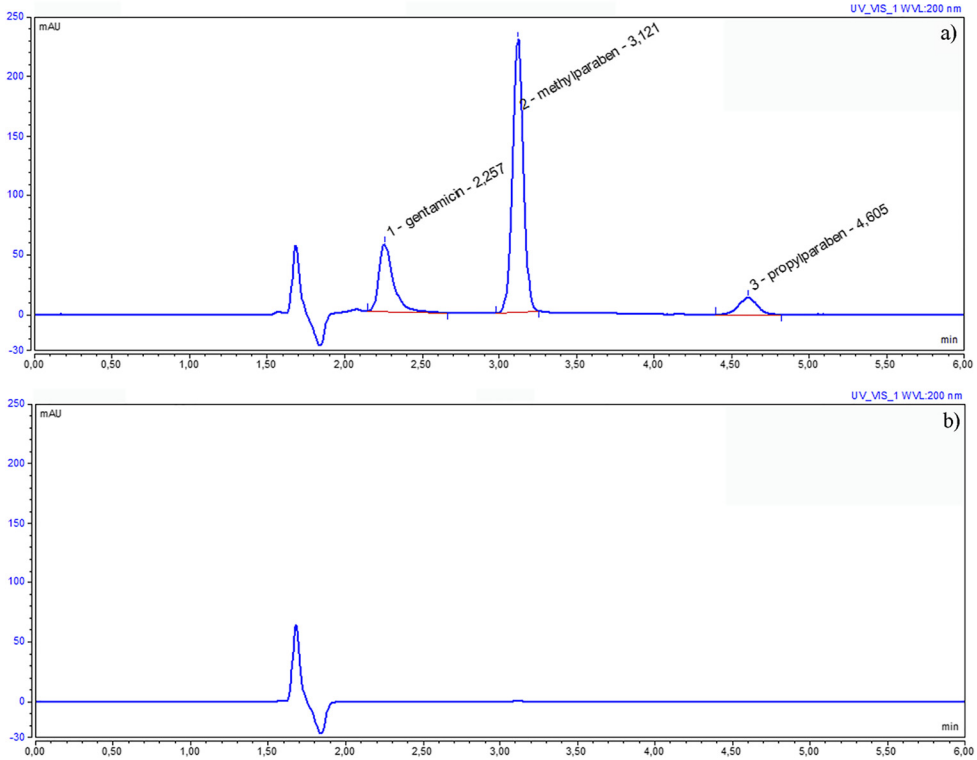


Fig. 2. Chromatograms: a) Gentavet solution (gentamicin sulfate 0.8 mg mL^{-1}; methylparaben 0.018 mg mL^{-1}; propylparaben 0.002 mg mL^{-1}) and b) Gentavet placebo solution

and concentration of gentamicin sulfate (r = 0.9991), methylparaben (r = 0.9996) and propylparaben (r = 0.9998). Correlation coefficient as well as statistical insignificance of calibration curve intercepts ($P > 0.05$) proved linearity of the method in defined concentration ranges. Linearity parameters for all three analytes are presented in Table 1.

Precision. Precision was performed at two levels: repeatability and intermediate precision. Repeatability (intra-day assay precision) was determined from six determinations at 100% of the test concentration (0.8 mg mL^{-1} for gentamicin sulfate, 0.018 mg mL^{-1} for methylparaben and 0.002 mg mL^{-1} for propylparaben). Mean value, standard deviation and relative standard deviations were calculated. Precision criterion for an assay method is that intra-assay precision will be ≤2% [28].

In this study, intermediate precision (within-laboratory variation) was demonstrated by two operators, in two days, using different set of reagents. Precision criterion for an assay method is that intermediate precision will be ≤3% [28].

Results of precision testing are presented in Table 2. Relative standard deviations were within specified limits, which proved the suggested method is precise for the simultaneous determination of gentamicin, methylparaben and propylparaben.

Accuracy. Accuracy in this assay method was performed using spiked samples prepared in triplicate at three levels (80%, 100% and 120%) of nominal concentration. Percent recoveries of response factor (area/concentration) were calculated. Results of accuracy testing are presented in Table 2. Recovery values were 98.87%–99.78% (gentamicin), 99.03–101.67% (methylparaben) and 99.02–101.40% (propylparaben) which are within the required range (98–102%) [28]. The obtained results proved the method's accuracy for the simultaneous determination of all three analytes.

Robustness. According to ICH Q2 (R1), the evaluation of robustness of an analytical procedure should be considered during the development. The robustness should reveal the reliability of an analysis with respect to small, but deliberate variations in method parameters. Robustness was tested by small variations of selected parameters: column temperature

Table 1. Linearity parameters of the proposed method

	Gentamicin sulfate	Methylparaben	Propylparaben
Range (mg mL^{-1})	0.32–1.04	0.0072–0.0234	0.0008–0.0026
a	7.9307	1113.8	991.99
b	−0.4259	0.2341	0.0511
r	0.9991	0.9996	0.9998
p	0.0737	0.7273	0.1080
SE intercept	0.1576	0.3219	0.0225
SE slope	0.1984	18.0076	11.3298

a, slope; *b*, intercept; *r*, correlation coefficient; *p*, statistical significance of calibration curve intercept ($P > 0.05$); SE intercept, standard error of intercept; and SE slope, standard error of slope.

Table 2. Results of precision and accuracy testing

	Gentamicin sulfate	Methylparaben	Propylparaben
Precision			
Repeatability*			
Concentration of analyte (mg mL^{-1})	0.8	0.018	0.002
Mean value (mg mL^{-1})	0.83	0.0182	0.0021
Standard deviation (mg mL^{-1})	6×10^{-3}	2×10^{-4}	4.17×10^{-5}
RSD (%)	0.76	1.10	1.97
Intermediate precision*			
Concentration of analyte (mg mL^{-1})	0.8	0.018	0.002
Mean value (mg mL^{-1})	0.82	0.0179	0.0020
Standard deviation (mg mL^{-1})	1.01×10^{-2}	2.46×10^{-4}	3.82×10^{-5}
RSD (%)	1.30	1.37	1.89
Accuracy**			
Concentration 1 (mg mL^{-1})	0.64	0.0146	0.0016
Recovery (%)	99.63	101.67	101.40
Concentration 2 (mg mL^{-1})	0.80	0.0182	0.0020
Recovery (%)	98.87	101.15	99.02
Concentration 3 (mg mL^{-1})	0.96	0.0216	0.0024
Recovery (%)	99.78	99.03	99.51

* $n = 6$.
** $n = 3$.
Concentration 1–80%; concentration 2–100%, concentration 3–120%.

Table 3. Results of robustness testing

Optimal chromatographic conditions						
Mobile phase composition: 0.15% triethylamine in 10 mM KH_2PO_4 aqueous solution (final pH 3.0 adjusted with H_3PO_4)/methanol 70:30 (*v/v*)						
Flow rate: 1.0 mL min^{-1}						
Column temperature: 40°C						
C_s (mg mL^{-1}): 0.84 (gentamicin sulfate); 0.0186 (methylparaben); 0.002 (propylparaben)*						
	Gentamicin sulfate		Methylparaben		Propylparaben	
Variation	C_0 (mg mL^{-1})	Deviation (%)	C_0 (mg mL^{-1})	Deviation (%)	C_0 (mg mL^{-1})	Deviation (%)
Column temperature						
35 °C	0.791	−5.83	0.0182	−2.15	0,00195	0.39
45 °C	0.827	−1.55	0.0181	−2.69	0.00191	−4.50
Methanol content						
27%	0.810	−3.57	0.0177	−4.84	0.00178	−11.00
33%	0.771	−8,21	0.0193	3.76	0.00173	−13.50
pH						
2.6	0.853	1.55	0.0179	−3.76	0.00188	−6.00
3.4	0.900	7,14	0.0192	3.23	0.00197	−1.50
Triethylamine concentration						
0.135%	0.775	−7.74	0.0179	−3.76	0.00189	−5.50
0.165%			Not applicable (peak splitting)			
KH_2PO_4 molarity						
9 mM	0.799	−4.88	0.0178	−4.30	0.00197	−1.50
11 mM	0.824	−1.90	0.0177	−4.84	0.00184	−8.00

* $n = 3$.

and mobile phase composition (methanol content, triethylamine concentration, pH and molarity of KH_2PO_4). Variations of chromatographic parameters are presented in Table 3. The concentration of analyzed analyte was calculated according to Eq. (1).

$$C_0 = (A_i/A_s)\mathrm{x}\, C_s \quad (1)$$

A_i – peak area of analyzed compound in real sample solution
A_s – peak area of analyzed compound in standard working solution
C_s – concentration of analyzed compound in standard working solution (mg mL^{-1})
C_0 – concentration of analyzed compound in real sample solution (mg mL^{-1})

The results of robustness testing are presented in Table 3. The concentration of trimethylamine as well as methanol content are found to be critical parameters. Increasing of content of triethylamine up to 10% of the optimal chromatographic conditions causes peak splitting in chromatogram. According to these results, chromatographic parameters are not significantly influenced by small variations of column temperature, pH and molarity of KH_2PO_4.

System suitability testing. The system suitability parameters were as follows: number of theoretical plates (N).

2589 (gentamicin sulfate), 8439 (methylparaben) and 7184 (propylparaben); peak symmetry 1.10 (gentamicin sulfate), 1.02 (methylparaben) and 0.97 (propylparaben). Relative standard deviations calculated for peak area of all analytes were below the limits defined (≤5%). Those results suggest efficient performance of the system.

During research, the stability of working standard solution and real sample solution was studied. The assessment of stability was carried after 3, 6, 12, 24 and 36 h of storage at room temperature and in refrigerator (2–8 °C). Working standard solution and real sample solution were stable for 6 h when stored at room temperature and for 24 h when stored at 2–8 °C.

Analysis of pharmaceutical dosage form

Applicability in routine of proposed HPLC method was tested by analysis of three batches of Gentavet 80 mg mL^{-1} solution of injection, a commercially available dosage form for veterinary use. The contents of three analytes were 98.63% (78.90 mg mL^{-1}), 101.11% (1.82 mg mL^{-1}) and 97.00% (0.194 mg mL^{-1}) for gentamicin sulfate, methylparaben and propylparaben, respectively. All results are corresponded to the mean value from the three independent measurements. The contents of analytes are in accordance with Gentavet 80 mg mL^{-1} solution of injection specification.

CONCLUSION

A new, selective and easy-to-use HPLC method was found to be promising for the simultaneous determination of

gentamicin sulfate, methylparaben and propylparaben in pharmaceuticals. The method provides selective quantification of those three analytes within 5 min without interference from placebo. Obtained validation parameters met ICH guidelines and confirmed selectivity, linearity, accuracy, precision and robustness. Compared with the reported methods for the determination of gentamicin, the conditions outlined herein represent a significant improvement in terms of simplicity, analysis times and non-use of derivatization and ion-pair agents. According to obtained results, the proposed HPLC method is suitable for regular use as a part of quality control and can simplify the quality control procedures provided in current use.

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