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Short communication

HPLC determination of chlorhexidine gluconate and *p*-chloroaniline in topical ointment

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Abstract

A novel fast isocratic reversed-phase HPLC method for simultaneous determination of chlorhexidine and its degradation product *p*-chloroaniline was developed. Zorbax SB Phenyl column (75 mm × 4.6 mm, 3.5 μm) was used for the separation. Mobile phase composed of acetonitrile and buffer solution of 0.08 M sodium phosphate monobasic containing 5 ml of triethylamine (0.5%) and adjust with 85% phosphoric acid to pH 3.0 in ratio 35:65 (v/v) pumped isocratically at flow rate 0.6 ml min^{-1} was used. UV detection was performed at 239 nm, the total analysis time was about 10 min.

The method is suitable for practical routine analysis of topical ointment in the quality control laboratory.

Keywords: HPLC; Chlorhexidine; Ointment

1. Introduction

Chlorhexidine gluconate, is an aqueous solution of chlorhexidine gluconate, chemically 2,4,11,13-tetraazatetradecanediimidamide, *N*,*N*″-bis(4-chlorophenyl)-3,12-diimino-, di-D-gluconate [1].

Chlorhexidine is the most popular antiseptic of biguanides. It has potent antimicrobial activity against most Gram-positive and some Gram-negative bacteria but not against spores. In human use is chlorhexidine applied to prevent and treat the redness, swelling, and bleeding gums associated with gingivitis [2]. In veterinary medicine, chlorhexidine is used as a general purpose disinfectant for cleansing wounds, skin, instrument, and equipment [3]. Because of its antiseptic properties and low potential for systemic or dermal toxicity, chlorhexidine has been incorporated into shampoos, ointments, skin and wound cleansers, teat dips, surgical scrubs, etc. [4].

High performance liquid chromatography has been widely used for the determination of chlorhexidine in formulations. The basic HPLC method for determination of chlorhexidine and its degradation product *p*-chloroaniline is described in USP 29. Gradient of acetonitrile and buffer solution (contains monobasic sodium phosphate, triethylamine, pH adjusted with phosphoric acid to pH 3.0) was used for the chromatography. Detection was at 239 nm and the flow-rate was about 1.5 ml min^{-1} [1].

Recently, there has been found a number of reports dealing with determination of chlorhexidine by liquid chromatography using ion-pair reversed-phase HPLC [5–12]. An isocratic reversed-phase liquid chromatographic method was developed for determination of five active substances, including chlorhexidine, in an ointment using Zorbax RX-C 8 (4.6 mm × 150 mm, 5 μm) column and a mobile phase containing sodium dodecyl sulfate and 2-propanol [13]. The hydrolytic pathway of chlorhexidine was investigated. HPLC methods with gradient mode were used for the assay of chlorhexidine and its known degradation products [14,15]. HPLC methods for assay of chlorhexidine and *p*-chloroaniline using gradient elution and acetate buffer [16] or ammonia solution [17] as a part of the mobile phase were developed. HPLC methods for determination of chlorhexidine in urine [18], in human serum [19,20], in saliva [8,9,21], in ophthalmic solution [22], in ointment [23] or in suspension [24] were published. Chlorhexidine was determined together with other antimicrobial agents used in cosmetics using ion interaction RP-HPLC [25]. Other methods reported in the literature used LC-ESI-MS for the determination of chlorhexidine [26,27]. An isocratic HPLC method for the simultaneous

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determination of chlorhexidine and p-chloroaniline using Zorbax C8 column and mobile phase composed of methanol and 0.2 mol l^{-1} phosphate at flow-rate 1.0 ml min^{-1} was developed. The analysis time was about 17 min [28].

None of these methods were developed and validated for the determination of chlorhexidine and its degradation product in topical ointment using HPLC isocratic mode without adding ion-pair reagent. The isocratic mode is favourable for routine quality control laboratory. From stability testing point of view this approach supplied more reproducible results and it is less time consuming because no reequilibration is needed in comparison with the utilization of gradient elution.

The aim of this work is to develop a novel fast isocratic high-performance liquid chromatography method for the separation of chlorhexidine gluconas and its degradation product *p*-chloroaniline in ointment for veterinary use. It is favourable, for the routine analysis, to develop fast methods with analysis time less than 10 min.

2. Experimental

2.1. Chemicals and reagents

Working standards of chlorhexidine gluconas 20% solution, *p*-chloroaniline, and ethylparaben were used for the purpose of this study. Chlorhexidine gluconas 20% solution active substance was provided by Herbacos (Pardubice, Czech Republic). *P*-Chloroaniline was obtained from Merck (Darmstadt, Germany). The reference standard of ethylparaben (internal standard) was bought from Sigma–Aldrich (Prague, Czech Republic).

Acetonitrile Chromasolv for HPLC gradient grade, sodium dihydrogen phosphate dihydrate, triethylamine, and formic acid were provided by Sigma–Aldrich (Prague, Czech Republic). Phosphoric acid 85% p.a. was obtained from Merck (Darmstadt, Germany). HPLC grade water was prepared by Milli-Q reverse osmosis Millipore (Bedford, MA, USA) and it met all European Pharmacopoeia requirements.

2.2. Chromatographic system

A Shimadzu LC-2010 C system (Shimadzu, Kyoto, Japan) with built-in UV–vis detector was used to perform all the analyses. Chromatographic software Class VP 6.12 was used for data collection and processing.

Supelco Discovery C18 column (250 mm × 4.0 mm, 5 μm) was bought from Sigma–Aldrich (Prague, Czech Republic). LiChroCART PUROSPHER RP 18 column (125 mm × 4 mm, 5 μm) was purchased from Merck [Darmstadt, Germany]. Zorbax SB Phenyl column (75 mm × 4.6 mm, 3.5 μm) was obtained from Agilent Technologies (Prague, Czech Republic).

2.3. Reference standard preparation

Reference standard solution for chlorhexidine analysis was prepared in 100.0 ml volumetric flask by dissolving of 63.75 mg of chlorhexidine gluconas 20% solution and 0.5 mg of degradation product *p*-chloroaniline in a mixture of acetonitrile and formic acid 1% 20:80 (v/v). Thereafter, 4.0 ml of internal standard ethylparaben stock solution (10.0 mg/10.0 ml of ethylparaben working standard) were added and the flask was topped up to the volume with a mixture of acetonitrile and formic acid 1% 20:80 (v/v).

2.4. HPLC

Zorbax SB Phenyl column (75 mm × 4.6 mm, 3.5 μm) was used for the separation. Mobile phase composed of acetonitrile and buffer solution in ratio 35:65 (v/v) pumped isocratically at flow rate 0.6 ml min^{-1} was used. Buffer solution was prepared using of 0.08 M sodium phosphate monobasic containing 5 ml of triethylamine (0.5%) and adjusted with 85% phosphoric acid to pH 3.0. Detection wavelength 239 nm was chosen for the chromatography according to absorption spectra of separated components.

3. Results and discussion

3.1. Chromatography

The method development was carried out according to the pharmacopoeial method USP 29. The isocratic elution and mobile phase composed of acetonitrile and buffer solution pH 3.0 (70:30 v/v) was tested for the analysis. Supelco DISCOVERY C18 column (250 mm × 4.6 mm, 5 μm) was used for the analysis. The method gave peak of chlorhexidine in 2 min, the it was not convenient because of high peak tailing. Mobile phase composed of acetonitrile and buffer solution pH 3.0 in different volume ratios was tested. LiChroCART PUROSPHER RP 18 (125 mm × 4 mm, 5 μm) was tested for the analysis. *P*-Chloroaniline was not separated from chlorhexidine. The second problem was peak tailing of chlorhexidine.

A column with different packing material, Zorbax SB Phenyl column and mobile phases consisting of acetonitrile and phosphate buffer pH value from 2.5 to 5.0 in different volume ratios were also tested. To suppress the tailing of chlorhexidine peak, triethylamine was added into the mobile phase. The column produced the best chromatography when the pH 3.0 buffer solution (buffer solution prepared as mention above) was used in the mobile phase.

Finally, chromatography with a standard reference solution (Fig. 1) as well as with a real sample (after isolation procedure) was performed using mobile phase of acetonitrile and buffer solution 35:65 (v/v) as mobile phase at flow rate 0.6 ml min^{-1}. The analysis time of standard solution at those conditions was about 6 min. It was necessary to prolong the analysis time of a real sample to 10 min because of placebo elution peak in minute 9.

The method using internal standard was used for the purposes of this study. Methyl-, ethyl-, and propylparaben were tested as internal standards. Ethylparaben was successfully separated from other compounds in solution and has been used as internal standard.

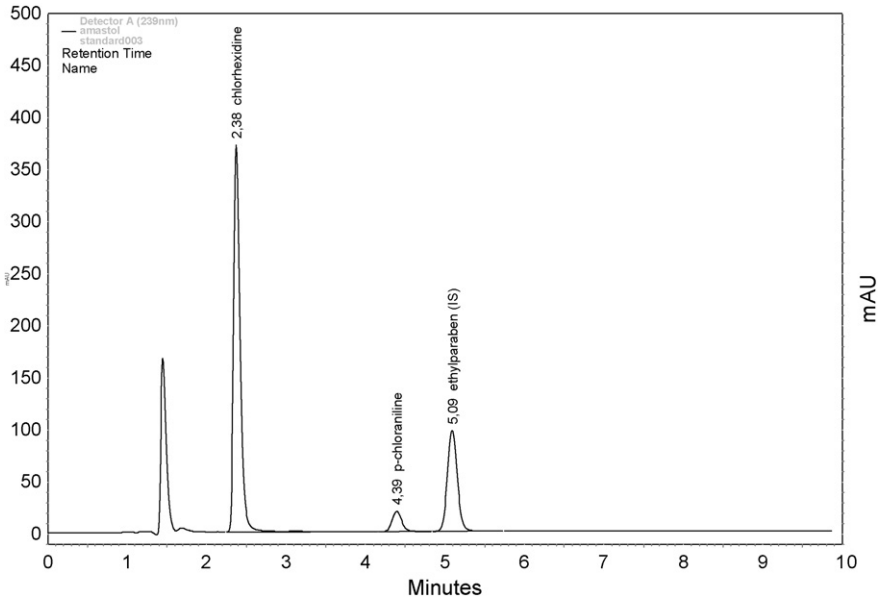

Fig. 1. Chromatogram: chromatogram at 239 nm, analysis of compounds in standard solution—chlorhexidine gluconas (active substance), *p*-chloroaniline (degradation product) and ethylparaben (IS).

3.2. Determination of chlorhexidine in topical ointment

The novel method developed in our study was used for Amastol neo ointment stability testing analysis. The concentration of chlorhexidine in reference standard solutions 127.5 mg l^{-1} corresponded to concentrations in a real sample of topical preparation.

The isolation procedure was based on extraction with organic solvent. Methanol and acetonitrile were tested as extraction media. No satisfactory results related to the recovery were achieved. Mixtures of acetonitrile with phosphate buffer (in pH range from 2.5 to 6.0) or with phosphoric acid in various volume ratios were tested. Lower pH values gave better results. The influence of elevated (water bath 40–80 °C) or reduced temperature (−20 °C) and optimal time of sonication and centrifugation (10–30 min) were examined. The phosphoric acid (strong inorganic acid) was not suitable for the analysis, and therefore a 1% solution of formic acid was tested. Finally, a mixture of acetonitrile and formic acid 1% 20:80 (v/v) was used as extraction medium.

A 0.5 g of the ointment was accurately weighted and transferred into a centrifuge flask. Twenty milliliters of working solutions of internal standard ethylparaben were added. Isolation procedure included 20 min in hot water bath at 80 °C,

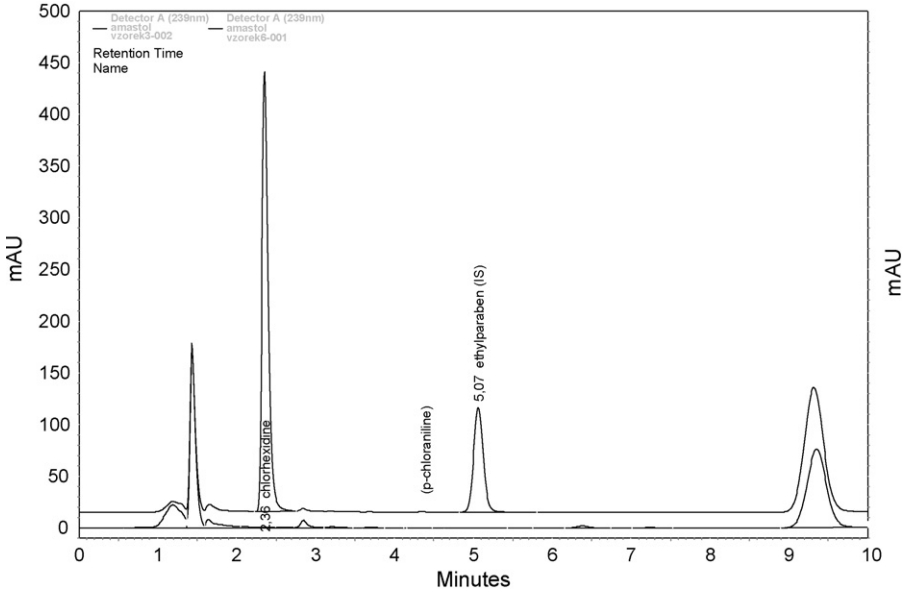

Fig. 2. Chromatogram: chromatogram at 239 nm, analysis of topical pharmaceutical preparation containing chlorhexidine gluconas, and placebo chromatogram to demonstrate method selectivity.

Table 1
Method validation results

Parameter		Clorhexidine	*p*-Chloroaniline	Critetia
SST				
Repeatibility—retention time[a]	R.S.D (%)	0.37	0.07	$X<1\%$
Repeatibility—area[a]	R.S.D (%)	0.07	0.33	$X<1\%$
Theoretical plates[b]		4002	7109	$N>2000$
Resolution[b]		7.16	9.99	$R_{ij}>1.5$
Asymmetry[b]		1.81	1.18	$T<2$
Validation				
Precision[c]	R.S.D.(%)	1.72	1.87	$X<5\%$
Linearity[d]	Correlation coefficient	0.99964	0.99901	$R>0{,}9990$
	Intercept	-0.0180 ± 0.024	0.0066 ± 0.003	
	Slope	0.1749 ± 0.002	0.4855 ± 0.002	
Accuracy[c]	Recovery (%)	99.72	100.38	$X=100 \pm 5\%$
	R.S.D (%)	2.72	1.53	$X<5\%$
LOD (mg ml^{-1})		–	$6{,}65.10^{-5}$	
LOQ (mg ml^{-1})		–	$2{,}22.10^{-4}$	
Sample stability 4 °C[e]	%changes in response factor	0.17	0.52	$X<1\%$
20 °C		0.64	0.53	$X<1\%$
Selectivity		No interference	No interference	

[a] Made in six replicates.
[b] Made in six replicates.
[c] Six samples injected three times each.
[d] Linearity range 5.12×10^{-2} to 17.85×10^{-2} mg ml^{-1} chlorhexidine, three replicates. Linearity range 5×10^{-4} to 6×10^{-3} mg ml^{-1} *p*-chloroaniline, three replicates.
[e] One-day stability.

followed by 20 min of sonication and centrifugation for 15 min at 6000 rpm. The supernatant was filtered through a flute filter and injected into the chromatographic system.

The above-described isolation procedure met the requirements of recovery in range of 95–105% for tested compounds. The chromatogram shown in Fig. 2 illustrates the separation of compounds tested after isolation from a pharmaceutical preparation. In placebo background there were not detected a coeluting peaks. This was verified during the method validation.

3.3. Method validation

The method was validated according to ICH guideline recommendation Q2 (R1) [29], and guidelines valid in our laboratory. Method validation covers System Suitability Test (repeatability, number of theoretical plates, resolution, and asymmetry) and the determination of validation parameters including accuracy, precision, selectivity, linearity and short-term stability. The method validation results are summarized in Table 1. All tested parameters met the requirements of regulative authorities.

4. Conclusions

The aim of this work was to develop a fast novel stability indicating HPLC method for determination of chlorhexidine and its degradation product *p*-chloroaniline in veterinary ointment. The significant advantages of the method are the fast (components are separated in 5.50 min) and simultaneous determination of substances by using the isocratic reversed phase HPLC without ion-pair reagent as a part of the mobile phase.

The isocratic mode is favourable for routine quality control laboratory. This approach can give more reproducible results and moreover it is less time consuming because no reequilibration of the HPLC system is needed in comparison with the use of more complicated gradient elution.

The novel method was successfully applied for long-term stability tests of topical ointment Amastol neo HBF in the quality control laboratory.

Acknowledgement

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References

[1] USP 29–NF 24 United States Pharmacopoeial Convention Inc., 2006.
[2] http://www.uhe.com/chlorhex.htm, April 2006.
[3] http://www.emea.eu.int/pdfs/vet/mrls/010796en.pdf, April 2006.
[4] http://www.merckvetmanual.com/mvm/index.jsp?cfile=htm/bc/191804.htm, April 2006.
[5] A. Richard, M. Elbaz, G. Andermann, J. Chromatogr. 298 (1984) 356–359.
[6] C. Yue, J. Poff, M.E. Cortés, R.D. Sinisterra, C.B. Faris, P. Hildgen, R. Langer, V.P. Shastri, Biomaterials 25 (2004) 3743–3750.
[7] N.J. Medlicott, I.G. Tucker, M.J. Rathbone, D.W. Holborow, D.S. Jones, Int. J. Pharm. 143 (1996) 25–35.
[8] T. Pesonen, J. Holmalahti, J. Pohjola, J. Chromatogr. B: Biomed. Appl. 665 (1995) 222–225.
[9] Y.W.F. Lam, D.C.N. Chan, S.Y. Rodriguez, J.H. Lintakoon, T.H. Lam, J. Chromatogr.: Biomed. Appl. 612 (1993) 166–171.
[10] M. Bauer, C. Degude, L. Mailhe, J. Chromatogr. 315 (1984) 457–464.
[11] B. Wyhowski-Bukanski, M.O. Masse, Int. J. Cosmet. Sci. 6 (1984) 283–291.

[12] L. Gagliari, et al., J. Chromatogr. 348 (1985) 321–326.
[13] S. Izumoto, Y. Machida, N. Hiroyuki, K. Nakamura, H. Nakai, T. Sato, J. Pharm. Biomed. Anal. 15 (1997) 1457–1466.
[14] Y. Ha, A.P. Cheung, J. Pharm. Biomed. Anal. 14 (1996) 1327–1334.
[15] W.H. Doub, et al., J. AOAC Int. 79 (1996) 636–639.
[16] W.K. Gavlick, J. Chromatogr. A 623 (1992) 375–380.
[17] H. Below, N. Lehan, A. Kamer, Microchim. Acta 146 (2004) 129–135.
[18] L.R. Brougham, H. Cheng, K.A. Pitman, J. Chromatogr. B. 383 (1986) 365–437.
[19] P. Wainwright, M. Cooke, Analyst 111 (1986) 1343–1344.
[20] K. Kudo, N. Ikeda, A. Kiyoshima, Y. Hino, N. Nishida, N. Inoue, J. Anal. Toxicol. 26 (2002) 119–122.
[21] N.J. Medlicott, D.G. Ferry, I.G. Tucker, M.J. Rathborne, D.W. Holborow, D.S. Jones, J. Liq. Chromatogr. 17 (1994) 1605–1620.
[22] A.P. Cheung, R. Mavar, C. Carlson, W.K. Chiang, J. Pharm. Biomed. Anal. 9 (1991) 41–45.
[23] X.F. Ji, K.Y. Yu, W. Zhang, Yaowu-Fenxi-Zazhi 17 (1996) 363–365.
[24] Y.M. Xu, G.Y. Wong, J. Liq. Chromatogr. Relat. Technol. 22 (1999) 2071–2091.
[25] E. Marengo, V. Gianotti, S. Angioi, M.C. Gennaro, J. Chromatogr. A 1029 (2004) 57–65.
[26] F.M. Musteata, J. Pawliszyn, J. Pharm. Biomed. Anal. 37 (2005) 1015–1024.
[27] K. Usui, T. Hishinuma, H. Yamaguchi, N. Tachiiri, J. Goto, J. Chromatogr. B 831 (2006) 105–109.
[28] D. Zhang, H. Liang, J. Zeng, G. Rao, J. West China Univer. Med. Sci. 26 (1995) 447–451.
[29] http://www.ich.org/LOB/media/MEDIA417.pdf, April 2006.