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DEVELOPMENT STUDIES ON DETERMINATION OF PRESERVATIVES DECOMPOSITION PRODUCTS

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Abstract: Preservatives are chemical substances whose role is to protect medicinal products against harmful changes caused by microorganisms. They are added to sterile medicinal products, such as eye drops and multi-dose solutions for injections, as well as to non-sterile products, such as water oral solutions, creams, gels, suppositories and capsules with liquid content. The most commonly used preservatives include: benzyl alcohol, butyl, ethyl, methyl and propyl p-hydroxybenzoates and their sodium salts. In medicinal products benzyl alcohol slowly oxidizes to benzaldehyde and benzoic acid while esters of p-hydroxybenzoic acid hydrolyze to p-hydroxybenzoic acid. HPLC methods were elaborated for identification and quantitative determination of the parabens, benzyl alcohol, active substances as well as their impurities in pharmaceuticals: oral solutions Amertil and Efforil (contain cetirizine hydrochloride or etilefrine hydrochloride and parabens), eye drops Difadol (contains diclofenac sodium and benzyl alcohol) and cream Tenasil (contains terbinafine hydrochloride and benzyl alcohol). The HPLC systems consisted of columns: Supelcosil LC-DP, Inertsil ODS-3 or Discovery HS F5 and three mobile phases – mixtures of acetonitrile with buffers of various pH (3, 5 and 7) in proportions 45 : 55 (v/v). These systems have been characterized with appropriate selectivity (all the R_S values > 2) and sensitivity (LOD approx. 0.01 µg/mL). They also demonstrated satisfactory precision and a linear dependence between the analyte content and the peak area.

Keywords: benzyl alcohol, esters of p-hydroxybenzoic acid, HPLC

Preservatives are chemical substances whose role is to protect food products, stimulants, medicinal products and cosmetics against harmful changes caused by microorganisms (1). When added in proper concentrations, preservatives inhibit the growth of microorganisms during manufacturing and use of medicinal products (2). They should be tasteless, odorless and colorless. In concentrations used, they should be soluble and non-toxic as well as physiologically and chemically compatible. At present, it is recommended – as more effective – to use mixtures of preservatives.

Preservatives are added to sterile medicinal products, such as eye drops and multi-dose solutions for injections, as well as to non-sterile products, such as water oral solutions, creams, gels, suppositories and capsules with liquid content. It is allowed in Poland to use as preservatives the substances listed in Appendix no. 1 to the Regulation of Health Minister dated 16 January 2003, on preservatives, sweeteners, colors and antioxidants that may be included in medicinal products (Journal of Laws No. 19, 2003, item 169). These compounds comprise phenols, alcohols, organic acids, organic mercuric compounds and quaternary ammonium bases. The most commonly used preservatives include: benzyl alcohol, butyl, ethyl, methyl and propyl p-hydroxybenzoates and their sodium salts. According to the aforementioned Regulation, all preservatives should meet the requirements of the European Pharmacopoeia and their content in medicinal products should be determined quantitatively.

Benzyl alcohol, as a preservative in medicinal products (oral solutions and solutions for injections), is used in concentration up to 2%. Benzyl alcohol reveals incompatibilities with oxidizing agents and strong acids. Its antimicrobial activity is reduced in the presence of non-ionic surfactants, such as, for example, polysorbate 80 (1, 3). In medicinal products it oxidizes slowly to benzaldehyde and benzoic acid when exposed to air (4). Three papers have been found on determination of benzaldehyde contents in injections with diclofenac with the use of polarographic method (5) and in

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Table 1. Times of retention (t_R), resolution between the neighboring peaks (R_S) and peak asymmetry (A10%) of tested preservatives and their decomposition products in chromatographic system acc. to Ph. Eur. monographs for methylparaben. Mobile phase: 0.05 M solution of KH_2PO_4 and methanol 35 : 65 (v/v). Columns used: Zorbax Rx C18 (in accordance with the monograph) and columns selected for tests ODS-3, DP, HS F5.

Substance*	Column Rx C18			Column ODS-3			Column DP			Column HS F5		
	t_R	R_S	A10%	t_R	R_S	A10%	t_R	R_S	A10%	t_R	R_S	A10%
p-HBAc	1.5	–	1.4	2.8–3.2	–	triple peak	3.2–3.3	–	double peak	3.6-4.1	–	triple peak
MP	2.2	2.6	1.9	5.1	7.5	1.2	3.9	1.3	1.3	6.3	7.2	1.1
EP	2.7	2.3	1.7	6.6	5.1	1.2	4.0	0.6	1.2	7.7	5.3	1.2
PP	3.7	3.9	1.5	10.0	7.5	1.1	4.3	0.9	1.1	10.0	7.0	1.1
BP	5.7	5.6	1.3	14.8	14.7	1.0	4.6	1.2	1.1	13.7	6.8	1.1
BA-ol	2.1	1.1	1.1	4.5	2.8	1.2	3.7	1.8	1.1	4.7	–	1.4
BAc	1.9	–	0.8	3.8	–	1.8	3.4	–	1.4	5.6	3.8	1.8
BAld	2.49	2.0	1.5	5.7	4.2	0.9	4.0	1.3	1.8	6.0	1.8	1,1

* abbreviations see text.

injections with diclofenac, piroxicam and vitamin B complex with the use of GC method (6, 7). Benzyl alcohol and diclofenac were determined in various medicinal products with spectrophotometric method (8).

Esters of p-hydroxybenzoic acid (parabens) show desirable preserving action within a quite wide range of pH, from weak-acid to weak-alkaline (pH 4 – 8), though in general, they are the most effective in acid environment. Above pH 8, the effectiveness of its action decreases due to rapid progress of hydrolysis. The parabens are more active against yeast and mould than against bacteria. They are also more active against Gram positive than against Gram negative bacteria. Antibacterial action of individual esters increases together with the increased chain length, but their solubility decreases. This problem may be omitted by the use of more soluble sodium salts of the esters, however, this raises the pH of poorly buffered formulations. Antibacterial activity may also be increased by the use of a mix of esters, for example of methyl and propyl p-hydroxybenzoates.

Antibacterial effect of parabens decreases in the presence of macromolecular substances, such as polysorbate 80, as the micelles that are formed close the esters inside. Addition of propylene glycol (10%) increases the adverse interactions in the presence of non-ionic surfactants. Esters are also incompatible with other substances, such as: bentonite, magnesium trisilicate, talc, sorbitol and atropine (1, 3).

Esters of p-hydroxybenzoic acid are used as preservatives mainly in oral solutions and preparations administered on skin. In the past, these were also used in solutions for injections and in the eye drops, but at now this application is being abandoned because of their irritating effect. Additionally, in case of medicinal products administered on eyes, a significant disadvantage of parabens is their poor impact on Gram-negative bacteria, in particular against *Pseudomonas aeruginosa*. Most commonly, a mixture of two esters methyl and propyl is used at concentrations up to 0.25%. A product of esters hydrolysis, p-hydroxybenzoic acid, shows no antibacterial effect (2).

In the available literature, several studies were found describing determination of various esters of p-hydroxybenzoic acid in medicinal products with micellar electrophoresis (9, 10) and HPLC methods (11, 12). Only one of these papers takes into consideration determining the content of parabens hydrolysis product – p-hydroxybenzoic acid (12). Stability of water solutions of p-hydroxybenzoic

Table 2. Times of retention (t_R). resolution between the neighboring peaks (R_S) and peak asymmetry (A10%) of tested preservatives and their decomposition products in elaborated chromatographic systems on the ODS-3 column (Inertsil ODS-3, 5 µm, 250 × 4.6 mm).

Substance*	t_R			R_S			A10%		
	pH ~3	pH ~5	pH ~7	pH ~3	pH ~5	pH ~7	pH ~3	pH ~5	pH ~7
p-HBAc	3.6	3.3	2.4	–	–	–	1.1	1.2	1.2
MP	5.9	5.9	5.9	11.1	12.1	14.5	1.0	1.1	1.1
EP	7.9	8.4	7.9	7.8	7.7	7.5	1.0	1.0	1.1
PP	11.5	11.3	11.4	10.7	10.3	10.1	1.1	1.0	1.0
BP	17.6	17.3	17.3	12.8	12.3	12.1	1.0	1.0	1.1
BAol	5.1	5.1	5.2	–	4.4	11.4	1.1	1.1	1.1
BAc	5.6	4.2	2.6	2.2	–	–	1.1	1.6	1.3
BAld	8.4	7.9	8.6	11.8	11.2	13.3	1.1	1.0	1.0

* abbreviations see text.

Table 3. Times of retention (t_R), resolution between the neighboring peaks (R_S) and peak asymmetry (A10%) of tested preservatives and their decomposition products in elaborated chromatographic systems on the DP column (Supelcosil LC- DP, 5 µm, 250 × 4.6 mm).

Substance*	t_R			R_S			A10%		
	pH ~3	pH ~5	pH ~7	pH ~3	pH ~5	pH ~7	pH ~3	pH ~5	pH ~7
p-HBAc	3.8	3.5	2.8	–	–	–	1.0	1.3	1.1
MP	4.6	4.5	4.6	1.7	4.3	16.9	1.0	1.2	1.1
EP	5.1	4.9	5.0	7.8	1.7	2.1	1.0	1.3	1.1
PP	5.8	5.4	5.6	3.1	2.2	2.7	1.0	1.3	1.1
BP	6.7	6.1	6.5	3.9	2.7	3.2	1.0	1.3	1.1
BAol	4.4	4.3	4.4	–	2.9	17.9	1.0	1.4	1.1
BAc	4.5	3.7	2.8	1.0	–	–	1.0	1.6	1.1
BAld	5.2	5.0	5.1	3.8	3.1	4.3	1.0	1.2	1.1

* abbreviations see text.

Table 4. Times of retention (t_R), resolution between the neighbouring peaks (R_S) and peak asymmetry (A10%) of tested preservatives and their decomposition products in elaborated chromatographic systems on the HS F5 column (Discovery HS F5, 5 µm, 250 × 4.6 mm).

Substance*	t_R			R_S			A10%		
	pH ~3	pH ~5	pH ~7	pH ~3	pH ~5	pH ~7	pH ~3	pH ~5	pH ~7
p-HBAc	4.0	3.9	2.4-2.5	–	–	–	1.2	1.3	double peak
MP	6.0	5.9	6.1	10.6	9.6	32.3	1.0	1.1	1.2
EP	7.3	7.2	7.4	5.9	5.6	5.6	1.0	1.1	1.3
PP	9.3	9.0	9.4	7.8	7.2	7.4	1.0	1.1	1.3
BP	12.2	11.7	12.3	9.4	8.6	8.6	1.0	1.0	1.4
BAol	5.2	5.1	5.1	–	–	26.2	1.0	1.1	1.1
BAc	5.6	6.8	2.9	2.3	4.8	–	1.1	2.7	1.1
BAld	7.8	7.7	7.5	10.6	2.2	9.8	1.0	1.0	1.3

* abbreviations see text.

Table 5. The linear relationship between analyte concentration (c) and peak area (x) for parabens and benzyl alcohol in elaborated chromatographic systems. The coefficients of correlation **r** given in brackets.

Substance*	c = ax + b					
	Column ODS-3		Column DP		Column HS F5	
	pH ~3	pH ~5	pH ~3	pH ~5	pH~3 p	H~5
MP	1.05E–05x–2.82 (r = 0.9989)	8.63E–06x–2.67 (r = 0.9990)	1.15E–05x–4.16 (r = 0.9970)	8.78E–06x–2.73 (r = 0.9990)	9.61E–06x–2.15 (r = 0.9991)	8.55E–06x–2.45 (r = 0.9992)
EP	1.06E–05x–1.77 (r = 0.9996)	8.73E–06x–1.63 (r = 0.9997)	1.19E–05x–3.54 (r = 0.9980)	9.09E–06x–2.27 (r = 0.9994)	1.10E–05x–3.34 (r = 0.9991)	8.77E–06x–1.74 (r = 0.9996)
PP	1.09E–05x–0.85 (r = 0.9999)	9.11E–06x–0.82 (r = 0.9999)	1.13E–05x–2.27 (r = 0.9990)	9.45E–06x–1.44 (r = 0.9996)	1.13E–05x–2.17 (r = 0.9995)	9.19E–06x–0.99 (r = 0. 9998)
BP	1.14E–05x–0.48 (r = 0.9999)	9.60E–06x–0.58 (r = 0.9999)	1.17E–05x–1.89 (r = 0.9993)	9.93E–06x–1.16 (r = 0.9999)	1.17E–05x–1.59 (r = 0.9998)	1.01E–05x–2.93 (r = 0.9997)
BAol	6.90E–04x–35.7 (r = 0.9994)	6.13E–04x–10.72 (r = 0.9998)	**	5.95E–04x–6.88 (r = 0.9999)	5.77E–04–3.40 (r = 0.9999)	5.93E–04x–8.63 (r = 0.9999)

* abbreviations see text. **- not calibrated due to non-complete separation of benzyl alcohol with benzoic acid (R = 1.0).

Table 6. The linear relationship between analyte concentration (c) and peak area (x) for p-hydroxybenzoic acid, benzoic acid and benzaldehyde in elaborated chromatographic systems. The coefficients of correlation **r** given in brackets.

Substance*	Wavelenght	c = ax + b					
		Column ODS-3		Column DP		Column HS F5	
		pH ~3	pH ~5	pH ~3	pH ~5	pH ~3	pH ~5
p-HBAc	254 nm	9.13E–06x–0.24 (r = 0.9999)	8.82E–06x–0.11 (r = 0.9999)	8.58E–06x–0.17 (r = 0.9999)	7.83E–06x–0.08 (r = 0.9999)	8.97E–06x–0.26 (r = 0.9999)	7.71E–06x–0.09 (r = 0.9999)
BAc	220 nm	1.29E–05x–0.14 (r = 0.9999)	1.06E–05x–0.06 (r = 0.9999)	**	1.07E–05x–0.13 (r = 0.9999)	1.36E–05x–0.24 (r = 0.9999)	9.87E–06x–0.05 (r = 0.9999)
BAld	254 nm	9.59E–06x–0.24 (r = 0.9999)	1.07E–05x–0.06 (r = 0.9999)	**	9.31E–06x–0.29 (r = 0.9999)	1.06E–05x–0.40 (r = 0.9998)	8.71E–06x–0.11 (r = 0.9999)

* abbreviations see text.** not calibrated due to non-complete separation of benzyl alcohol with benzoic acid (R = 1.0).

Table 7. Times of retention of selected medicinal substances and their impurities in the elaborated chromatographic systems.

Substance*	Retention time [min]					
	Column ODS-3		Column DP		Column HS F5	
	pH ~3	pH ~5	pH ~3	pH ~5	pH ~3	pH ~5
C	2.7	6.4	40.2	6.2	26.3	7.0
pCB-ol	23.4	25.2	5.9	7.2	10.6	15.4
pCBP-on	49.8	53.17	7.2	9.0	17.7	27.3
Imp. A	2.0	4.9	50.6	9.6	30.1	21.2
D	28.1	18.4	na	5.3	11.1	37.7
Imp. A	23.9	23.8	na	8.0	10.1	14.5
Imp. B	66.1	63.2	na	10.8	19.1	30.0
Imp. C	30.1	29.5	na	8.4	11.7	17.2
Imp. E	3.9	4.3	na	4.1	3.8	4.5
T	2.7	71.1	na	15.8	60.1	62.9
Imp. A	1.7	24.0	na	5.9	11.7	8.2
Imp. B	2.6	68.2	na	15.5	64.0	61.0
Imp. E	1.9	>120	na	>80	>120	>120
E	1.7	2.3	15.2	4.3	6.3	4.9
Imp. A	1.7	2.3	14.5	4.5	7.3	5.3

* abbreviations see text. na – not analyzed (due to non-complete separation of benzyl alcohol with benzoic acid).

acid n-butyl ester exposed to light (254 nm) was also investigated (13).

The European Pharmacopoeia specifies the permissible amount of impurities in parabens and recommends the HPLC method for testing of parabens purity. However, there are no guidelines on their levels in medicinal products. They may not be considered as unknown impurities, as they often exceed the level of 0.2% adopted in line with ICH guidelines.

For determination of benzyl alcohol and testing of its purity the European Pharmacopoeia recommends a GC method. However, this method may not be sufficient, therefore a different method for testing of purity and determination of active substance should be applied. Simultaneous analysis with use of the two methods increases the costs.

The purpose of this paper was to elaborate HPLC methods for identification and quantitative determination of the parabens and benzyl alcohol in pharmaceuticals. It was assumed that the content of medicinal substances and preservatives as well as their purity, taking into consideration the impurities coming from the active substance as well as from the preservatives, would be determined in one chromatographic system.

For the testing the following were selected: expired preparations (Amertil, Effortil, Difadol) – their content of preservatives and their impurities had been determined earlier, during their period of validity – and a multi-dose preparation in its period of validity (Tenasil cream) – the amount of benzyl alcohol oxidation products may increase after opening of this preparation. This compilation allows estimation of the products of preservatives decomposition that are formed when storing under normal conditions as well as their rate of formation, and may facilitate determination of limits for these impurities.

EXPERIMENTAL

Test materials

LGC standards: methyl (**MP**), ethyl (**EP**), propyl (**PP**) and butyl (**BP**) p-hydroxybenzoates (parabens); p-hydroxybenzoic acid (**p-HBAc**); benzyl alcohol (**BAol**); benzaldehyde (**BAld**); benzoic acid (**BAc**); certizine dihydrochloride (**C**), p-chlorobenzophenone (**p-CBPon**), p-chlorobenzhydrol (**p-CBHol**), A impurity acc. to Eu. Ph.; diclofenac sodium (**D**) and A, B, C and E impurities acc. to Eu. Ph.; etilefrine hydrochloride (**E**) and A

Table 8. Results of preservatives decomposition products determination (content expressed as percentage in relation to the parabens or benzyl alcohol content) in selected medicinal products with the use of elaborated chromatographic systems.

Assay [%]	Effortil						Amertil					
	Column ODS-3		Column DP		Column HS F5		Column ODS-3		Column DP		Column HS F5	
	pH ~3	pH ~5	pH ~3	pH ~5	pH ~3	pH ~5	pH ~3	pH ~5	pH ~3	pH ~5	pH ~3	pH ~5
p-HBAc*	9.82	10.67	9.81	10.32	9.99	10.02	0.41	0.45	–**	0.43	–**	0.42
	Difadol						**Tenasil**					
Assay [%]	Column ODS-3		Column DP		Column HS F5		Column ODS-3		Column DP		Column HS F5	
	pH ~3	pH ~5	pH ~3	pH ~5	pH ~3	pH ~5	pH ~3	pH ~5	pH ~3	pH ~5	pH ~3	pH ~5
BAc*	< LOQ	< LOQ	no	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	no	< LOQ	< LOQ	< LOQ
BAld*	0.03	0.04	no	0.03	0.03	0.03	0.26	0.26	no	0.25	0.24	0.28

* abbreviations see text. no – not determined. LOQ – limit of quantitation. **note: p-HBAc peak did not separate completely from the placebo peak.

impurity acc. to Eu. Ph.; terbinafine hydrochloride (**T**) and A, B and E impurities acc. to Eu. Ph.

Preparations containing: cetirizine dihydrochloride and methyl and ethyl p-hydroxybenzoates – Amertil syrup 1 mg/mL s. CP8001, expiry date: 12. 2009 (Biofarm Sp. z o.o.); diclofenac sodium and benzyl alcohol – Difadol, solution for intramuscular injections 25 mg/g, s. 01AK1104, expiry date: 11. 2007 (WZF Polfa); etilefrine hydrochloride and methyl and propyl p-hydroxybenzoates – Effortil oral drops 7.5 mg/g, s. 433452D, expiry date: 09. 2009 (Boehringer Ingelheim); terbinafine hydrochloride and benzyl alcohol – Tenasil cream s. 010709, expiry date: 07. 2012 (Pharmaceutical Laboratory HOMEOFARM sp. z o.o.).

The preparations were previously tested in November and December 2010.

HPLC reagents of high purity: acetonitrile (Rathburn, Scotland); deionized water (Millipore); 85% phosphoric acid; KH_2PO_4 and K_2HPO_4 (AppliChem).

HPLC columns: used for reconstruction of pharmacopoeia monograph for parabens: Zorbax Rx C18, 5 mm, 150 × 4.6 mm, Agilent; used for elaboration of new methods: Supelcosil LC-DP, 5 mm, 250 × 4.6 mm, Supelco; Inertsil ODS-3, 5 mm, 250 × 4.6 mm, MZ-Analysentechnik GmbH; Discovery HS F5, 5 mm, 250 × 4.6 mm, Supelco.

Apparatus: PC controlled liquid chromatograph with SPD-10AVvp UV detector, LC-10ATvp pumps and DGU-14A mobile phase degasser, SCL-10AVvp controller, automatic sample injector SIL-10AVvp – manufactured by Shimadzu, Japan.

***Solutions used for HPLC*:** solvent for solutions preparation: acetonitrile and water mixture 45 : 55 (v/v); standard solutions for determination of calibration curves: paraben solutions in a range of concentrations from 10 to 200 µg/mL; solutions of p-hydroxybenzoic acid in a range of concentrations 0.03–50 µg/mL; solutions of benzyl alcohol in a range of concentrations 10–1700 µg/mL; solutions of benzaldehyde in a range of concentrations 0.05–75 µg/mL; solutions of benzoic acid in a range of concentrations 0.03–50 µg/mL; placebo solutions: weigh about 50 mg of every excipient and dissolved in solvent; tested solutions obtained from pharmaceutical preparations: Amertil – concentration of medicinal substance ca. 0.1 mg/mL, concentration of MP ca. 0.135 mg/mL, concentration of PP ca. 0.015 mg/mL; Difadol – concentration of medicinal substance ca 1 mg/mL, concentration of BAol ca. 1.4 mg/mL; Effortil – concentration of medicinal substance ca. 0.75 mg/mL, concentration of MP ca. 0.07 mg/mL, concentration of PP ca. 0.03 mg/mL;

Table 9. Comparison of retention times of active substance, preservatives and products of their decomposition in selected preparations obtained by elaborated chromatographic systems.

Substance*	Effortil					
	Column ODS-3		Column DP		Column HS F5	
	pH ~3	pH ~5	pH ~3	pH ~5	pH ~3	pH ~5
E	1.7	2.3	15.2	4.3	6.3	4.9
Imp. A	1.7	2.3	14.5	4.5	7.3	5.3
p-HBAc	3.6	3.5	3.8	3.5	4.0	3.9
MP*	5.9	5.9	4.6	4.5	6.0	5.9
PP*	11.5	11.3	5.8	5.4	9.3	9.0
Substance*	**Amertil**					
	Column ODS-3		Column DP		Column HS F5	
	pH ~3	pH ~5	pH ~3	pH ~5	pH ~3	pH ~5
C*	2.7	6.4	40.2	6.2	26.3	7.0
Imp. A	2.0	4.9	50.6	9.6	30.1	21.2
p-CBol	23.4	25.2	5.9	7.2	10.6	15.4
p-CBPon	49.8	53.17	7.2	9.0	17.7	27.3
p-HBAc	3.6	3.5	3.8	3.5	4.0	3.9
MP	5.9	5.9	4.6	4.5	6.0	5.9
PP	11.5	11.3	5.8	5.4	9.3	9.0
Substance*	**Didafol**					
	Column ODS-3		Column DP		Column HS F5	
	pH ~3	pH ~5	pH ~3	pH ~5	pH ~3	pH ~5
D	28.1	18.4	na	5.3	11.1	37.7
Imp. A	23.9	23.8	na	8.0	10.1	14.5
Imp. B	66.1	63.2	na	10.8	19.1	30.0
Imp. C	30.1	29.5	na	8.4	11.7	17.2
Imp. E	3.9	4.3	na	4.1	3.8	4.5
BAolc	5.1	5.1	4.4	4.3	5.	5.1
BAc	5.6	4.2	4.5	3.7	5.6	6.8
BAld	8.4	7.9	5.2	5.0	7.8	7.7
Substance*	**Tenasil**					
	Column ODS-3		Column DP		Column HS F5	
	pH ~3	pH ~5	pH ~3	pH ~5	pH ~3	pH ~5
T	2.7	71.1	na	15.8	60.1	62.9
Imp. A	1.7	24.0	na	5.9	11.7	8.2
Imp. B	2.6	68.2	na	15.5	64.0	61.0
Imp. E	1.9	> 120	na	> 80	> 120	> 120
BAolc	5.1	5.1	4.4	4.3	5.	5.1
BAc	5.6	4.2	4.5	3.7	5.6	6.8
BAld	8.4	7.9	5.2	5.0	7.8	7.7

* abbreviations see text. na – not analyzed (due to non-complete separation of benzyl alcohol with benzoic acid). Imp. A, B, C, E – impurity for each substance by Ph. Eur.

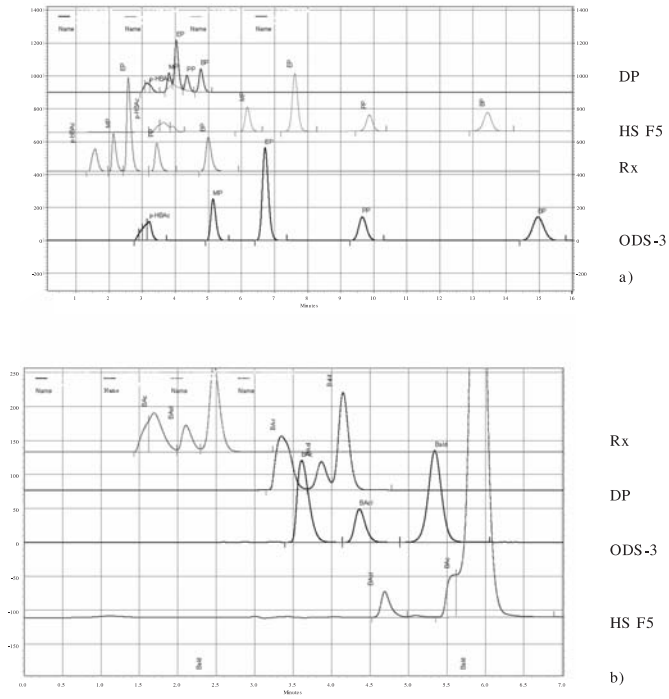

Figure 1. Chromatograms in chromatographic system acc. to Eur. Ph. monographs for parabens on the columns: Zorbax Rx C18 (recommended by Eur. Ph.) and ODS -3, HS F5, DP
a) parabens (MP, EP, PP, BP) and p-hydroxybenzoic acid (p-BAc)
b) benzyl alcohol (BAol), benzaldehyde (Bald) and benzoic acid (BAc)

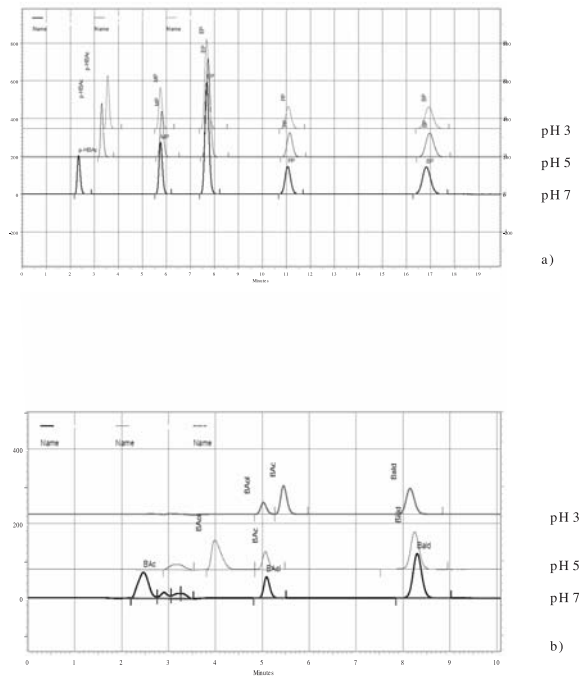

Figure 2. Chromatograms in elaborated chromatographic systems on the column ODS-3
a) parabens (MP, EP, PP, BP) and p-hydroxybenzoic acid (p-HBAc)
b) benzyl alcohol (BAol), benzaldehyde (Bald) and benzoic acid (BAc)

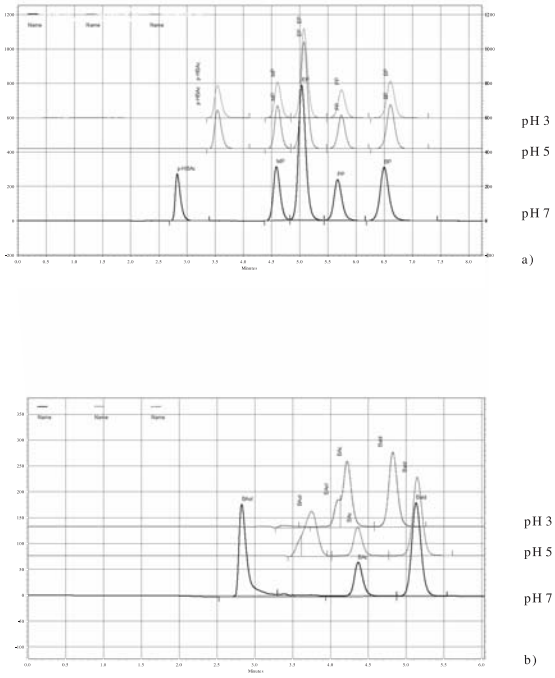

Figure 3. Chromatograms in elaborated chromatographic systems on the column DP
a) parabens (MP, EP, PP, BP) and p-hydroxybenzoic acid (p-HBAc)
b) benzyl alcohol (BAol), benzaldehyde (Bald) and benzoic acid (BAc)

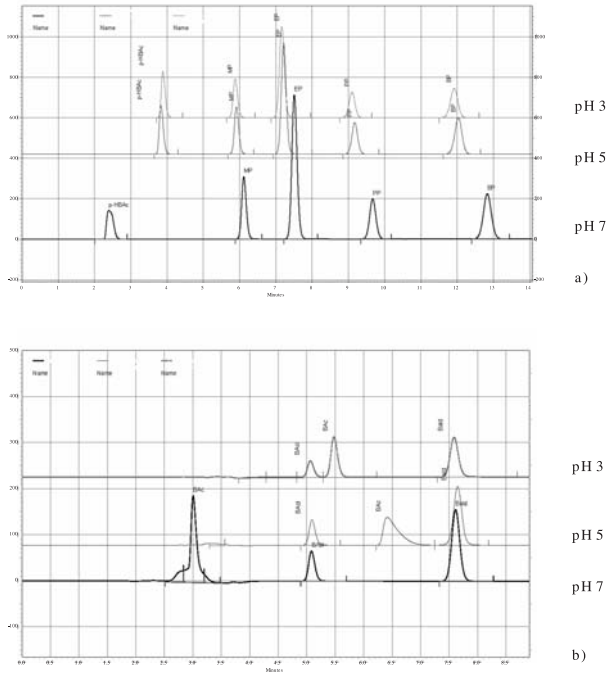

Figure 4. Chromatograms in elaborated chromatographic systems on the column HS F5
a) parabens (MP, EP, PP, BP) and p-hydroxybenzoic acid (p-HBAc)
b) benzyl alcohol (BAol), benzaldehyde (Bald) and benzoic acid (BAc)

Tenasil – concentration of medicinal substance ca. 1 mg/mL, concentration of BAol ca. 1 mg/mL; solutions for identity confirmation of: certizine dihydrochloride and impurities; diclofenac sodium and impurities, etilefrine hydrochloride and impurities, terbinafine hydrochloride and impurities.

RESULTS AND DISCUSSION

A solution was prepared of standard substances: methyl, ethyl, propyl and butyl parabens and p-hydroxybenzoic acid in the acetonitrile : water mixture (45 : 55, v/v) with the concentrations of approx.100 μg/mL (parabens) and 25 μg/mL (acid). This solution was used to reproduce the HPLC system acc. to Eu. Ph. for testing of the purity of parabens. Then, the solution of the following standard substances was prepared: benzyl alcohol, benzaldehyde and benzoic acid in the mixture of acetonitrile : water (45 : 55, v/v) in the concentrations of 800, 40 and 25 μg/mL, respectively, and it was verified whether the abovementioned HPLC system is suitable for determination of benzyl alcohol and its decomposition products (Tab. 1). The retention times for all standard substances are within the range from 1.7 to 5.7 min. Due to the presence of excipients in preparations, such retention times are being to short. Therefore, the system was modified by application of columns with a different filling material (DP, ODS-3, HS F5 columns) than the one recommended by Eur. Ph. (Zorbax Rx C18 column) with the same mobile phase, i.e., the mixture of 0.05 M KH_2PO_4 and methanol in the proportion of 35 : 65 (v/v). It turned out that the peak of p-hydroxybenzoic acid was divided and was not suitable for quantitative determinations. In the case of benzyl alcohol and the products of its decomposition on the ODS-3 and HS F5 columns the obtained separation was suitable, but the peaks were relatively wide and asymmetric (Tab. 1, Fig. 1).

For elaboration of new methods the columns with variable filling materials were selected: ODS-3 – alkyl groups bonded to spherical silica, with large specific surface (450 m^2/g); HS F5 – pentafluorophenyl propyl groups bonded to spherical silica, combining the features of reversed and normal phases, allowing for delaying of polar compounds retention with no delay in non-polar substances retention; DP – diphenyl groups bonded to spherical silica, ensuring higher selectivity for aromatic groups, with moderate polarity.

Next, three other mobile phases of various pH were prepared: pH~3 – a mixture of acetonitrile with water in proportions of 45 : 55 (v/v) with addition of 85% phosphoric acid in the amount of 0.2 mL per one litre of the phase; pH~5 – a mixture of acetonitrile with 0.025 M solution of KH_2PO_4 in a proportion of 45 : 55 (v/v); pH~7 – a mixture of acetonitrile with a solution containing 0.0125 M of KH_2PO_4 and 0.0125 M of K_2HPO_4 in a proportion of 45 : 55 (v/v).

The tests with the use of the mobile phases were carried out on each of the columns. The remaining conditions of analysis have always been the same: temperature of column operation: 40°C; flow rate of the mobile phase: 1 mL/min; UV detection – 220 nm and 254 nm; sample dose volume – 20 μL.

The results for parabens and benzyl alcohol and their degradation products recorded for all columns and mobile phases are given (Tabs. 2–4 and Figs. 2–4). The mobile phase of pH ~7 was eliminated on all the columns because p-HBAc and BAc acids were eluted too fast and their peaks are often divided and may overlap the solvent peaks or placebo peaks in case of preparations analysis. Moreover, no determinations of benzyl alcohol and its decomposition products on the DP column, pH ~3 phase could be made because of non-complete separation of BAol and BAc peaks (Tab. 3, Fig. 3).

For the rest of combinations column–mobile phase, the calibration curves, relationship between the analyte concentration expressed in μg/mL and the peak area, analyses were performed. The concentration ranges were selected as those that are usually applied for determination the compounds in pharmaceutical preparation: parabens (10–200 μg/mL), benzyl alcohol (10–1700 μg/mL), p-hydroxybenzoic acid (0.03–50 μg/mL) and benzaldehyde (0.05–75 μg/mL) and benzoic acid (0.03–50 μg/mL) (Tabs. 5, 6). Limits of detection were determined at the level of 0.01 μg/mL by determining the S/N ratio. Next, the samples, which were prepared by dissolving the medicinal products in the mixture acetonitrile and water (45 : 55, v/v), have been examined in the selected systems. Retention times of medicinal substances and their impurities are given in Table 7.

Selection of columns with other filling material (DP, ODS-3, HS F5) and adequate mobile phases ensured proper selectivity (all values $R_S > 2$), shape of the peaks (Tabs. 2–4, Figs. 2–4) as well as sensitivity (0.01 μg/mL). It was proven that pH of the mobile phase influences the time of retention of p-hydroxybenzoic and benzoic acids (Tabs. 2–4, Figs. 2–4) and in the case of products testing – the time of retention of medicinal substances (Tab. 7). The systems that are most suitable for quantitative analysis of decomposition products were selected.

The selected systems were used to determine decomposition products of preservatives in medicinal products with no impurities indicated in their specifications. The results obtained in different systems were generally comparable (Tab. 8). For Amertil preparation, tested in the phase with a pH ~3, the results obtained only with the use of ODS-3 column have been presented, because in the case of DP and HS-F5 columns the p-HBAc peak is not entirely separated from the placebo peaks. Tenasil and Difadol preparations that contain benzyl alcohol were not tested on DP column with the pH ~3 phase due to the non-complete separation of BAol and BAc peaks.

Table 9 includes the comparison of retention times of active substance, preservative and their decomposition products.

CONCLUSIONS

The abovementioned results show that it is not possible to simply transfer the pharmacopoeial method of parabens purity testing to the columns with different filling material (Tab. 1, Fig. 1) and to apply these methods in the testing of purity of preparations containing parabens or benzyl alcohol. This method is not applicable in medicinal products due to too short retention times and a possibility that the peaks from placebo interfere with the peaks of p-hydroxybenzoic acid.

Therefore, new HPLC systems characterized with appropriate selectivity (all the R_S values > 2) and sensitivity (LOD approx. 0.01 μg/mL) have been elaborated. They allow for testing of parabens purity in the presence of parabens hydrolysis product (p-hydroxybenzoic acid) and testing of benzyl alcohol purity in the presence of its oxidation products (benzaldehyde and benzoic acid). Assessment of purity of the preservatives may be performed with satisfactory precision. These systems may also be used for quantitative determination of preservatives, as it was demonstrated that there is a linear dependence between the analyte content and the peak area in terms of concentrations usually used for determination of preservatives content in medicinal products.

The tests performed on the expired preparations revealed that the amount of preservatives decomposition products exceeded 0.2% (limit value for unknown impurities according to ICH), and confirmed the need of their determination.

The use of columns with various filling materials and mobile phases with a pH ranging from 3 to 7 makes it possible to match the system to the composition of an investigated product.

The proposed HPLC systems shall be optimized for testing other medicinal products, containing the preservatives.

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