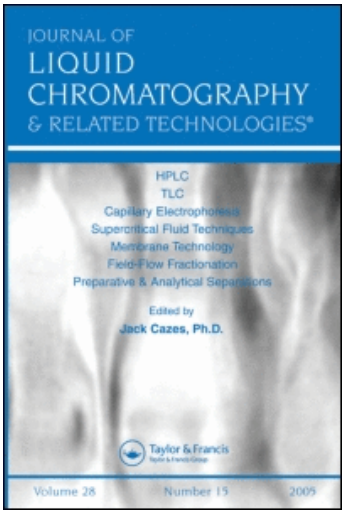

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Method Development and Validation of Preservatives Determination (Benzyl Alcohol, Ethylene Glycol Monophenyl Ether, Methyl Hydroxybenzoate, Ethyl Hydroxybenzoate, Propyl Hydroxybenzoate, and Butyl Hydroxybenzoate) using HPLC

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Abstract: The purpose of the research described herein was to develop a new high performance liquid chromatography (HPLC) method for the assay of benzyl alcohol, ethylene glycol monophenyl ether, methyl hydroxybenzoate, ethyl hydroxybenzoate, propyl hydroxybenzoate, and butyl hydroxybenzoate preservatives. The use of a reversed-phase C_{18} column (250 mm × 4.0 mm and 5 μm) using a gradient elution system enabled six compounds to be separated simultaneously in a single chromatographic run in less than 12 minutes. The method was successfully validated following ICH guidelines, and it has been demonstrated to be reliable for the assay of benzyl alcohol, ethylene glycol monophenyl ether, methyl hydroxybenzoate, ethyl hydroxybenzoate, propyl hydroxybenzoate, and butyl hydroxybenzoate preservatives used in the manufacturing of drug products.

Keywords: HPLC, Method validation, Benzyl alcohol, Ethylene glycol monophenyl ether, Methyl hydroxybenzoate, Ethyl hydroxybenzoate, Propyl hydroxybenzoate, Butyl hydroxybenzoate

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INTRODUCTION

Best practices in method development and validation is equally important in the analysis of both active components and preservatives/excipients (inactive ingredients) used in the manufacturing of drug products. Preservatives are an important class of chemicals used to inhibit the growth of microorganisms harmful to industrial products. Preservatives contribute to the safe and stable supply of drugs, food products, and raw materials. Three primary classes of these compounds are known: antimicrobials, antioxidants, and chelating agents. Preservatives, some of them naturally occurring, are used in a wide range of applications to maintain overall product quality. Some preservatives act as antimicrobial agents, some act as antioxidants, and some can perform both functions. The ability of a chemical to act as a preservative depends very much on the environment, so that factors such as type of the product, water content, pH, and storage conditions need to be considered when selecting preservatives. Antimicrobials and antioxidants are added to pharmaceutical products to prolong shelf life and maintain sterility.

The most commonly used preservatives in drug formulations are benzyl alcohol, ethylene glycol monophenyl ether (EGPE) also called 2-phenoxyethanol, methyl hydroxybenzoate (MH), ethyl hydroxybenzoate (EH), propyl hydroxybenzoate (PH), butyl hydroxybenzoate (BH) (Figure 1). These excipients are effective antimicrobial and antifungal agents, which are widely used alone or in combination with other esters of *p*-hydroxybenzoic acid as preservatives in foods, beverages, cosmetics, and pharmaceutical formulations.[1–3] Benzyl alcohol is prepared by the distillation of benzyl chloride with potassium or sodium carbonate. Benzyl alcohol is a clear, colorless, oily liquid with a faint aromatic odor and a sharp, burning taste. It can oxidize slowly in air to benzaldehyde and benzoic acid. It does not react with water and can be stored in metal or glass containers. Methyl hydroxybenzoate and propyl hydroxybenzoate are used together since they have a synergistic effect.[4] Methyl hydroxybenzoate is prepared by the esterification of *p*-hydroxybenzoic acid with methanol, ethyl hydroxybenzoate is prepared by the esterification of *p*-hydroxybenzoic acid with ethanol, propyl hydroxybenzoate is prepared by the esterification of *p*-hydroxybenzoic acid with *n*-propanol, and butyl hydroxybenzoate is prepared by the esterification of *p*-hydroxybenzoic acid with *n*-butanol. Many existing analytical procedures are available in literature for the determination of present excipients studied, either alone or in combination with other drugs by HPLC and other techniques.[5–18] However, I did not find any HPLC method dealing with simultaneous determination of all six compounds (benzyl alcohol, ethylene glycol monophenyl ether, methyl hydroxybenzoate, ethyl hydroxybenzoate, propyl hydroxybenzoate, and butyl hydroxybenzoate) in a single chromatographic run, either alone or in formulated products. Such a method is important, as there seems to be an increasing trend in using combinations of

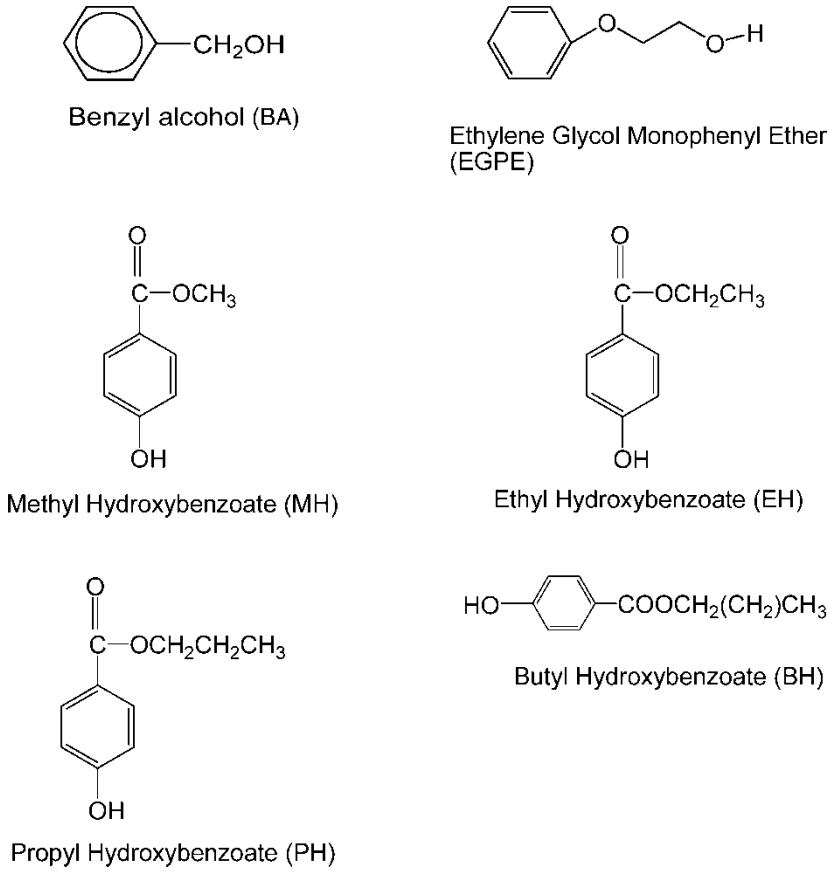

Figure 1. Chemical structure of the analytes, in order of elution.

excipients, not only in the food industry but also in pharmaceutical formulations and cosmetic products. Therefore, the objective of this present study was to develop and extensively validate a new, simple, accurate, and robust reversed-phase HPLC method for the assay of benzyl alcohol, ethylene glycol monophenyl ether, methyl hydroxybenzoate, ethyl hydroxybenzoate, propyl hydroxybenzoate, and butyl hydroxybenzoate preservatives in a single chromatographic run.

Validating the analytical method is a crucial part of successful product development, testing, and quality. The determination of preservatives both alone or in formulated products is important and provides a difficult analytical challenge. As a best practice,[19–21] in the subsequent investigation, the new and simple reversed-phase HPLC assay method was validated[22,23] for linearity, precision (repeatability and intermediate precision), accuracy, specificity, limit of detection, and limit of quantitation.

EXPERIMENTAL

Chemicals and Reagents

Tetrahydrofuran (THF), acetonitrile and methanol (HPLC-grade) were obtained from Merck (Darmstadt, Germany). Benzyl alcohol, ethylene glycol monophenyl ether, methyl hydroxybenzoate, ethyl hydroxybenzoate, propyl hydroxybenzoate, and butyl hydroxybenzoate were purchased from Sigma chemicals (St. Louis, MO, USA). De-ionized distilled water was used throughout the experiment.

HPLC System and Analytical Conditions

A PerkinElmer (Norwalk, CT) HPLC system equipped with a module LC 235C diode array detector (DAD), series 200 LC pump, series 200 autosampler, and series 200 peltier LC column oven were used in this work. The data were acquired via PE TurboChrom Workstation data acquisition software using PE Nelson series 600 LINK interfaces. The mobile phase consisted of water as solvent A and acetonitrile as solvent B. The solvents were pumped as a gradient at 1 mL/min, starting at 70% A and 30% B and were maintained for 5 min. Over a 5 min period, the solvent was changed to 40% A and 60% B, which was maintained for 2 min. Over a 2 min period, the solvent was changed to 30% A and 70% B, which was maintained for 6 min, and then changed to 70% A and 30% B, which was maintained for 15 min. The chromatographic separation was achieved using a 100 RP 18 (250 mm $\times$ 4.0 mm, i.d., 5 μm particle size) Lichrospher column, filtered with a RP 18, 5 μm guard column obtained from Phenomenex (Macclesfield, UK). The column temperature was held at 30 $\pm$ 0.5°C. The injection volume was 10 μL and the detection wavelength was set at 258 nm.

Preparation of the Standard and Sample Solutions

Accurately weighed amounts (250, 60, 30, and 90 mg) of methyl hydroxybenzoate, ethyl hydroxybenzoate, propyl hydroxybenzoate, and butyl hydroxybenzoate standards were placed in a 100 mL volumetric flask and dissolved in methanol (stock 1). Accurately weighed amounts (250, and 120 mg) of benzyl alcohol, and ethylene glycol monophenyl ether standards were placed in a 100 mL volumetric flask and dissolved in methanol (stock 2). Pipette 1.0 mL aliquot of (stock 1) solution and 10 mL aliquot of (stock 2) solution to a second 100 mL volumetric flask, add 30 mL of tetrahydrofuran and make up to volume with methanol. The final working concentrations were 0.025 mg/mL for methyl hydroxybenzoate, 0.006 mg/mL for ethyl hydroxybenzoate, 0.003 mg/mL for propyl hydroxybenzoate, 0.009 mg/mL for butyl

hydroxybenzoate, 0.25 mg/mL for benzyl alcohol; and 0.12 mg/mL for ethylene glycol monophenyl ether.

Linearity experiments were performed by preparing each preservative of benzyl alcohol, ethylene glycol monophenyl ether, methyl hydroxybenzoate, ethyl hydroxybenzoate, propyl hydroxybenzoate, and butyl hydroxybenzoate standard solution in the range of 75–450, 30–320, 5–45, 3–9, 0.5–6, and 6–12 μg/mL, respectively.

RESULTS AND DISCUSSION

Method Development

The main criteria for developing a successful and robust HPLC method determination of six preservative components in a pharmaceutical liquid product were as follows: the method should be stability indicating, free of interference from other materials in the formulation, robust, and simple enough for routine use in a quality control laboratory. The first step was to find the appropriate wavelength, which is usually a compromise for different compounds with different absorption maximum. The main factor is the sensitivity of determination of any degradation products presented in a very low concentration, especially in the beginning of the stability tests. Therefore, 258 nm was chosen. The optimization of mobile phase was first done with a binary mixture of water and acetonitrile or methanol under isocratic mode. It was found that it is not possible to separate all six components with acceptable resolution in a single run. Therefore, gradient mode was chosen to obtain a good separation and achieve an adequate resolution. The optimal composition was tested using different speeds of mobile phase.

Finally, for sufficient separation of benzyl alcohol, ethylene glycol monophenyl ether, methyl hydroxybenzoate, ethyl hydroxybenzoate, propyl hydroxybenzoate, and butyl hydroxybenzoate, the mobile phase was composed of water as solvent A and acetonitrile as solvent B. The solvents were pumped as a gradient at 1.0 mL/min, starting at 70% A and 30% B and were maintained for 5 min. Over a 5 min period, the solvent was changed to 40% A and 60% B, which was maintained for 2 min. Over a 2 min period, the solvent was changed to 30% A and 70% B, which was maintained for 6 min, and then changed to 70% A and 30% B, which was maintained for 15 min.

Several analytical columns were tried in order to reach acceptable specificity and selectivity. We first tried RP-8 columns, but the analytes were retained on these columns and gave poor separation and resolution as well. A shift to RP-18 columns, among which was the Lichrospher column, proved to be superior to others and exhibited excellent separation with shorter retention time (Figure 2). The column was equilibrated with the mobile phase flowing at 1.0 mL/min for 20 min prior to injection. Standard and sample solutions of 10 μL were injected automatically into the column.

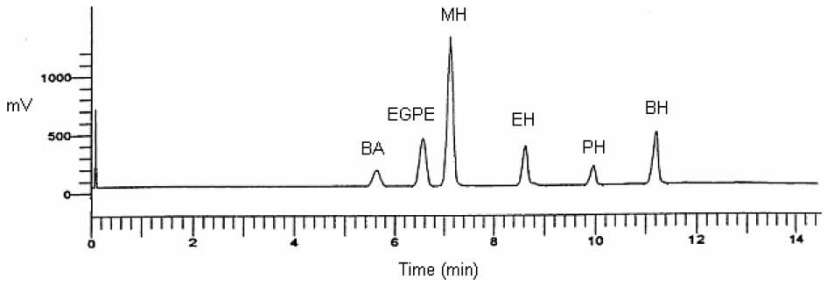

Figure 2. Representative HPLC chromatogram obtained for a benzyl alcohol (BA), Ethylene glycol monophenyl ether (EGPE), methyl hydroxybenzoate (MH), ethyl hydroxybenzoate (EH), propyl hydroxybenzoate (PH) and butyl hydroxybenzoate (BH) preservatives.

The column temperature was optimized and held at $30 \pm 0.5^{\circ}C$. Robustness verification studies were also performed in the method development phase. The robustness of an analytical method is defined as the measure of its capacity to remain unaffected by small but deliberate variations in the method parameters and provides an indication of its reliability during normal usage. One way to gauge robustness is to examine some relevant factors, which might influence the reliability of the developed method. Selected factors, namely the mobile phase composition, flow rate, temperature, and column from different lots were investigated. In all cases, good separations of all six preservative were always achieved, indicating that the analytical method remained selective and robust for benzyl alcohol, ethylene glycol monophenyl ether, methyl hydroxybenzoate, ethyl hydroxybenzoate, propyl hydroxybenzoate, and butyl hydroxybenzoate components under the optimized conditions.

Method Validation

Linearity

Linearity was studied using five solutions in the concentration range 0.5 to 450 $\mu g/mL$ and each one injected in duplicate. The regression equation was found by plotting the peak area (y) versus the preservative concentration (x) expressed in $\mu g/mL$. The correlation coefficients $\geq (r^2 = 0.9997)$ obtained in each case for the regression line demonstrates that there is a strong linear relationship between peak area and concentration of preservatives (Table 1).

Accuracy

The accuracy of an analytical method is determined by how close the test results obtained by that method come to the true value. It can be determined

Table 1. Linearity assessment of the HPLC method for the assay of preservatives

Preservatives	Concentration (μg/mL)	Equation for regression line	Correlation coefficient
Benzyl alcohol	75–450	y = 13521x − 5214.6	0.9998
Ethylene glycol monophenyl ether	30–320	y = 35477x + 150480	0.9997
Methyl hydroxybenzoate	5–45	y = 51493x + 5E + 06	0.9999
Ethyl hydroxybenzoate	3–9	y = 23502x + 92488	0.9999
Propyl hydroxybenzoate	0.5–6	y = 9809.7x + 15374	1.000
Butyl hydroxybenzoate	6–12	y = 31589x + 80305	0.9999

by application of the analytical procedure to an analyte of known purity (for the drug substance) or by recovery studies, where a known amount of standard is spiked in the placebo (for drug product). In the present study, a number of different solutions were prepared with a known added amount of each preservative and injected in triplicate. Percent recoveries of response factor (area and concentration) were calculated as can be seen in Table 2, and it is evident that the method is accurate within the desired range.

Precision

The precision of the analytical method, reported as %RSD, was estimated by measuring repeatability (intra-day precision) on ten replicate injections at 100% test concentration.

Intermediate precision (inter-day variation) was demonstrated by two analysts using two HPLC systems, and evaluating the relative peak area percent data across the two HPLC systems at three concentration levels (50, 100, and 150%). The %RSD values presented in Table 3 were less than 2% in all cases, and illustrated the good precision of the chromatographic method.

Table 2. Recovery studies of the HPLC method for the assay of preservatives

	Applied concentration (% of target)		
Preservatives	50	100	150
Benzyl alcohol	98.9 (±0.92)[a]	99.9 (±0.15)	99.2 (±0.46)
Ethylene glycol monophenyl ether	98.2 (±0.52)	98.6 (±0.71)	97.8 (±0.13)
Methyl hydroxybenzoate	99.6 (±0.55)	99.9 (±0.15)	99.2 (±0.45)
Ethyl hydroxybenzoate	99.3 (±0.57)	99.8 (±0.13)	99.2 (±0.45)
Propyl hydroxybenzoate	99.3 (±0.48)	99.8 (±0.12)	99.2 (±0.49)
Butyl hydroxybenzoate	99.0 (±0.99)	99.6 (±0.53)	99.6 (±0.53)

[a]The coefficient of variation.

Limit of Detection and Quantification

The limit of detection (LOD) and quantitation (LOQ) tests for the procedure were performed on samples containing very low concentrations of analyte. LOD is defined as the lowest concentration of analyte that can be detected above baseline noise. Typically, this is three times the noise level. LOQ is defined at the lowest concentration of analyte that can be reproducibly quantitated above the baseline noise with a signal to noise of 10. In this study, the LOD for benzyl alcohol, ethylene glycol monophenyl ether, methyl hydroxybenzoate, ethyl hydroxybenzoate, propyl hydroxybenzoate, and butyl hydroxybenzoate was 20, 8, 2, 1, 0.024, 2 μg/mL and LOQ was 75, 30, 5, 3, 0.5, and 6 μg/mL, respectively (Table 3).

Specificity

Injections of the blank were performed to demonstrate the absence of interference with the elution of the benzyl alcohol, ethylene glycol monophenyl ether, methyl hydroxybenzoate, ethyl hydroxybenzoate, propyl hydroxybenzoate, and butyl hydroxybenzoate preservatives. These results demonstrate (Figure 3) that there was no interference from the other compounds and, therefore, confirm the specificity of the method. Forced degradation studies were performed to evaluate the specificity of each preservative under four stress conditions (heat, UV light, acid, base). Solutions of each preservative were exposed to 50°C for 1 h, UV light using a Mineralight UVGL-58 light for 24 h, acid (1M hydrochloric acid, HCl) for 24 h, and base (1M sodium hydroxide, NaOH) for 4 h. A summary data of the stress results is shown in Table 4, which showed no changes in retention times of each preservative

Table 3. Method validation results

	Preservatives					
Parameter	BA	EGPE	MH	EH	PH	BH
Repeatability (Peak area %RSD, $n = 10$)	0.67	1.02	1.42	0.36	0.79	0.57
Intermediate precision ($n = 3$)	0.56	0.69	0.40	0.29	0.61	0.36
Instrument %RSD						
Analyst %RSD	0.52	0.59	0.38	0.43	0.51	0.27
LOD (μg/mL)	20	8	2	1	0.024	2
LOQ (μg/mL)	75	30	5	3	0.5	6
System suitability (Peak area %RSD, $n = 6$)	0.81	0.57	0.83	0.16	0.53	0.41

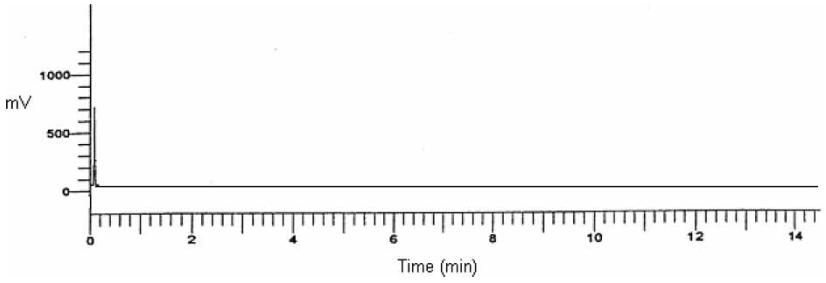

Figure 3. Representative HPLC chromatogram of the blank run.

by peak purity analysis on a DAD UV detector and, therefore, confirms the specificity of the method.

Measurement of Robustness and System Suitability

During robustness testing, a method must prove to be able to remain unaffected by small, but deliberate variations in method parameters, thus showing its own reliability during normal usage. It is advisable to simultaneously study the possible variations of method parameters in an interval chosen symmetrically around the optimised conditions. This interval represents the variations expected during method transfer and routine use in quality control testing. In this case, the seven selected parameters were the same considered in the optimisation step. Their experimental domain is reported in Table 5. This showed that the method for determination of benzyl alcohol, ethylene glycol monophenyl ether, methyl hydroxybenzoate, ethyl hydroxybenzoate, propyl hydroxybenzoate, and butyl hydroxybenzoate was reproducible and robust.

Table 4. Assay (%) of preservatives under stress conditions

Stress conditions	Sample treatment	t_R (min) (BA, EGPE, MH, EH, PH, BH)	Assay (%) (BA, EGPE, MH, EH, PH, BH)
Reference	Fresh solution	5.63, 6.57, 7.13, 8.63, 9.96, 11.21	99.84, 97.96, 98.99, 99.96, 98.87, 99.80
Acid	1M HCl for 24 h	5.64, 6.56, 7.12, 8.64, 9.95, 11.22	98.77, 97.92, 98.97, 99.90, 98.82, 99.74
Base	1M NaOH for 4 h	5.65, 6.54, 7.12, 8.62, 9.95, 11.20	98.65, 98.78, 98.88, 99.97, 98.93, 99.88
Heat	50°C for 1 h	5.62, 6.55, 7.14, 8.64, 9.93, 11.23	98.38, 96.89, 98.92, 99.94, 98.80, 99.67
Light	UV Light for 24 h	5.61, 6.54, 7.10, 8.61, 9.94, 11.22	98.18, 97.96, 98.99, 99.96, 98.87, 99.80

Table 5. Experimental domain of the factors during robustness testing

Factor	Experimental domain	Optimised conditions
Sample solvent	Mobile phase, methanol, THF	Methanol, THF
Analytical column (different lots)	C_{18}-C_{18}	C_{18}
Percent organic solvent (gradient system)	0–5 min: 28–32; 5–7 min: 58–62; 7–10 min: 68–72; 10–13 min: 28–32	30, 60, 70, 30
Flow rate (mL/min)	0.8–1.2	1.0
Injection volume (μL)	8–12	10
T (°C)	28–32	30
Wavelength (nm)	248–268	258

A system suitability test was performed to determine the accuracy and precision of the system by making six replicate injections of preservative standard solutions. The RSD of the peak areas responses was measured. The %RSD of peak areas averaged was ≤ 0.84 ($n = 6$) for each preservative (Table 3).

CONCLUSION

A simple, specific, and sensitive HPLC method for assaying benzyl alcohol, ethylene glycol monophenyl ether, methyl hydroxybenzoate, ethyl hydroxybenzoate, propyl hydroxybenzoate, and butyl hydroxybenzoate has been developed. This method is capable of separating six compounds simultaneously in a single analytical run in less than 12 minutes. The method has been extensively validated and it has been shown that it is very reliable, being linear, accurate, and precise, both in upper and lower concentration range. Therefore, it can be applied reliably for quantification of all six preservative compounds. Finally, validity of the method has been proven by applying it to samples of incoming raw material of benzyl alcohol, ethylene glycol monophenyl ether, methyl hydroxybenzoate, ethyl hydroxybenzoate, propyl hydroxybenzoate, and butyl hydroxybenzoate preservatives used in manufacturing of drug products.

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