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Short communication

Determination of combined *p*-hydroxy benzoic acid preservatives in a liquid pharmaceutical formulation by HPLC

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Abstract

This paper describes a reversed-phase high performance liquid chromatographic (RP-HPLC) assay method for the determination of combined *p*-hydroxy benzoic acid (ethylparaben (EP), methylparaben (MP) and propylparaben (PP)) preservatives in a liquid pharmaceutical formulation. The chromatographic separation was achieved with potassium phosphate buffer (pH 7.05)-methanol (47.5:52.5, v/v) as mobile phase, a Spherisorb C_{18} column (250 mm × 4.6 mm) and UV detection at 254 nm. The analysis time was <8 min. The method was validated with respect to linearity, precision, accuracy, selectivity, specificity and ruggedness. The calibration curves showed good linearity over the concentration range of 2–140 μg/ml. The correlation coefficient were >0.9999 in each case. The relative standard deviation (R.S.D.) values for intra- and inter-day precision studies were <1%. The procedure describe here is simple, selective and is suitable for routine quality control analysis and stability tests.
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Keywords: Combined *p*-hydroxy benzoic acid; Preservatives; Quality control of pharmaceuticals; Reversed-phase HPLC; Stability

1. Introduction

Some organic acids and their esters are commonly used single preservatives, but more often combinations of preservatives as antimicrobial agents in cosmetic, food and pharmaceutical products [1] to prevent alteration and degradation of the product formulation. However, these preservatives may be harmful to consumers due to their tendency to induce allergic contact dermatitis. Methyl hydroxybenzoate (methylparaben, MP), ethyl hydroxybenzoate (ethylparaben, EP) and propyl hydroxybenzoate (propylparaben, PP) (Fig. 1) have been widely used as antimicrobial and anti-fungal agents in food, beverages, cosmetics and pharmaceuticals [2] because of their broad antimicrobial spectrum with good stability and non-volatility [3]. Hence, the simultaneous determination of these preservatives in commercial pharmaceutical products is particularly important both for quality assurance and consumer safety.

Combined *p*-hydroxy benzoic acid is a combination of three esters including MP, EP and PP and its theoretical composition is given in the Table 1. Each of these preservatives are commonly used preservatives, and are used in medicinal products. Many HPLC

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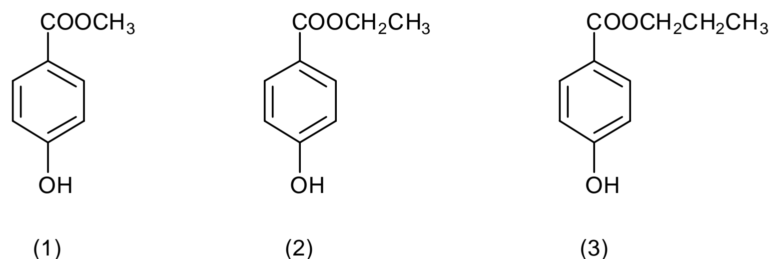

Fig. 1. Structures of the analytes, in order of elution: (1) MP; (2) EP; (3) PP.

methods have been reported for the determination of acidic preservatives in food, cosmetics and pharmaceuticals [4–16]. However, there have been no reports concerning stability tests for, and the determination of, combined *p*-hydroxy benzoic acid preservatives by reversed-phase HPLC. The purpose of the present study was to develop and validate a new HPLC method for the determination of combined *p*-hydroxy benzoic acid preservative compounds in a liquid pharmaceutical formulation suitable for bulk, final product release and stability testing. Thereafter, this method was successfully applied for the separation, quantification and a stability study of all the compounds in the liquid pharmaceutical formulation.

2. Experimental

2.1. Materials

Methyl 4-hydroxybenzoate (purity > 99%), ethyl 4-hydroxybenzoate (purity > 99%) and propyl 4-hydroxybenzoate (purity > 99%) used were purchased from Sigma (St. Louis, MO). Potassium dihydrogen phosphate and sodium hydroxide, of analytical reagent grade, and methanol, of HPLC grade, were obtained from Merck (Darmstadt, Germany). De-ionized distilled water was used throughout the experiment.

Table 1
Theoretical composition of combined *p*-hydroxy benzoic acid

Component	Theoretical composition (%)
Methyl hydroxy benzoate	73.21
Ethyl hydroxy benzoate	16.07
Propyl hydroxy benzoate	10.71
Total	99.99

2.2. Equipment

A Perkin-Elmer HPLC system, equipped with a model series 200 UV-Vis detector, series 200 LC pump, series 200 autosampler and series 200 peltier LC column oven, was used to chromatograph the solutions. Separation was achieved using a Waters μ-Bondapak C_{18} column (300 mm × 3.9 mm). The data were acquired via Perkin-Elmer TurboChrom Workstation data acquisition software, Version 6.1.0 using PE Nelson series 600 LINK interfaces. An Oxford A2205D analytical balance was used for weighing standards and samples. In addition, a Millipore filtration assembly, and a Corning PC-351 stirrer were used in this study. The mobile phase was an aqueous solution of 52.5% (v/v) methanol containing 0.2 M potassium dihydrogen phosphate, adjusted to pH 7.05 ± 0.05 with 1 M sodium hydroxide. The mobile phase was filtered through 0.45 μm membrane filter and degassed before use. The flow rate was 2.0 ml/min. UV detection was performed at 254 nm. The injection volume was 20 μl. All Samples and reference standards were diluted with mobile phase.

2.3. Standard preparation

Twenty milligram of each of the standards was accurately weighed, added to separate 100 ml volumetric flasks and then dissolved in methanol. Five millilitre aliquots of MP, 1 ml of EP and 1 ml of PP of stock solution were added to the same 100 ml volumetric flask and diluted in mobile phase, yielding a final concentration of 10, 2 and 2 μg/ml, respectively.

2.4. Sample preparation

Approximately 1 g of liquid pharmaceutical sample was accurately weighed, added to a 100 ml volumetric flask and then diluted in mobile phase. The sample was filtered through a 0.45 μm membrane filter and injected into the chromatograph.

Table 2
Retention times (min) of compound MP, EP and PP

Peak no.	Compound	Approximately RT (min)
1	Methyl *p*-hydroxy benzoate	2.90
2	Ethyl *p*-hydroxy benzoate	4.20
3	Propyl *p*-hydroxy benzoate	6.81

3. Results and discussion

3.1. Method development

The chromatographic separation of combined *p*-hydroxy benzoic acid (pK_a ∼ 8.4) was carried out in the isocratic mode using a mixture of 52.5% methanol in potassium phosphate buffer pH 7.05 ± 0.05 (52.5:47.5, v/v) as mobile phase. The column was equilibrated with the mobile phase flowing at 2.0 ml/min for about 1 h prior to injection. The column temperature was ambient. Twenty microlitre of standard and sample solutions were injected automatically into the column. Subsequently, the liquid chromatographic behaviours of both drugs were monitored with a UV detector at 254 nm. Additionally, preliminary system suitability, precision, linearity and ruggedness studies performed during the development of the method showed that the 20 μl injection volume was reproducible and the peak response was significant at the analytical concentration chosen. Chromatograms of the resulting solutions gave good separation and resolution (Fig. 2) and co-elution of excipients was not observed (Fig. 3) at the same retention time as MP, EP and PP. In Table 2, the retention times are reported. The analysis time for standards and samples for all compounds was ca. 8 min (Fig. 2).

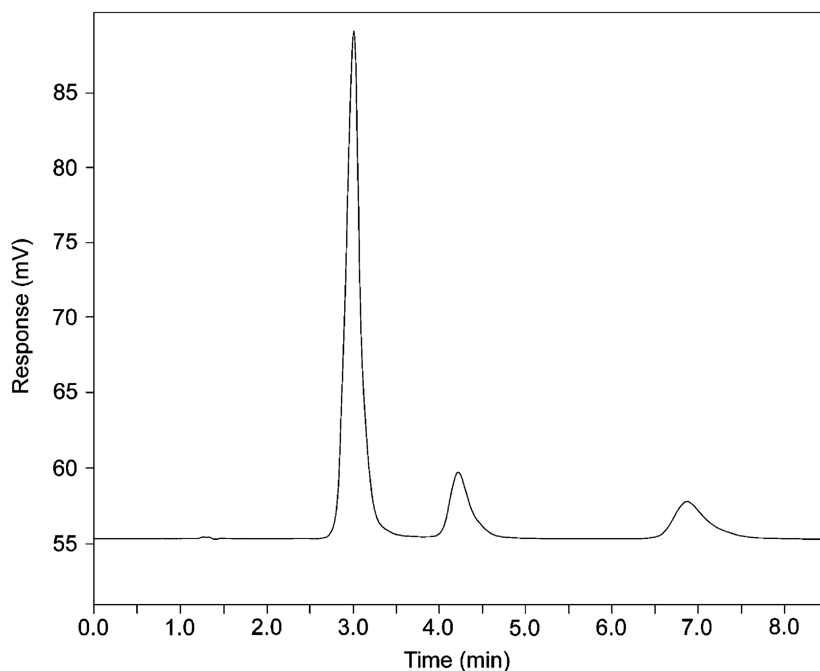

Fig. 2. HPLC chromatogram of the sample with 0.3% of combined *p*-hydroxy benzoic acid.

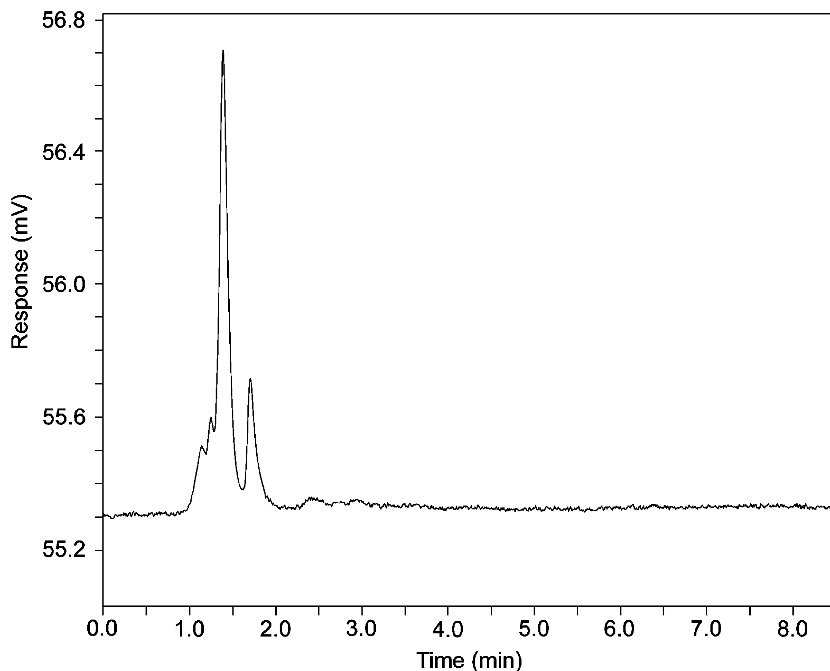

Fig. 3. HPLC chromatogram of placebo.

For the determination of method ruggedness within a laboratory during method development a number of chromatographic parameters were evaluated, such as flow rate, column temperature, different room temperature and humidity, mobile phase composition and pH, columns from different batches, different equipment and the quantitative influence of the variables were determined. For each parameter studied two injections of both standard and sample solutions were chromatographed. In all cases the influence of the parameters were found within a previously specified tolerance range. This shows that the method for determination of EP, MP and PP in liquid pharmaceutical formulation was reproducible and robust.

3.2. Method validation

3.2.1. Linearity/range

The linearity test was performed using seven different amounts of MP, EP and PP in the range of 20–140% around the theoretical values (MP 10 μg/ml, EP and PP 2 μg/ml).

Solutions corresponding to each concentration level were injected and linear regression analysis of the MP, EP and PP peak areas (y) versus MP, EP and PP concentration (X) were calculated (Table 3), MP (r^2 = 0.9999), EP (r^2 = 1.0000) and PP (r^2 = 0.9999).

3.2.2. System precision

The system precision was examined by analysing six determinations of the same batch of product at 100% of the test concentration. The samples were stored at 25 °C for six months. The relative standard deviation (R.S.D.) of the areas of each paraben peak were found to be less than 0.9% (Table 3), which confirms that the method is sufficiently precise.

3.2.3. Method/intermediate precision

The precision of the method for MP, EP and PP was assessed by the assay of five samples containing the nominal amount of MP, EP and PP. Intermediate precision was studied by assaying five samples prepared by different analysts, using a different HPLC column, on a different day. The R.S.D. values across the system and analysts were calculated and found to be less than 0.6% (Table 3) for each of the multiple sample preparations, which demonstrates excellent precision for the method.

Table 3
Method validation results

Validation step	Parameter	MP	EP	PP	Criteria
System precision	R.S.D. (%)[a]	0.037	0.582	0.827	$X < 2$
Method precision					
Analyst 1	R.S.D. (%)[b]	0.163	0.421	0.356	$X < 2$
Analyst 2	R.S.D. (%)	0.365	0.529	0.561	$X < 2$
Analyst 1 and 2	R.S.D. (%)	0.300	0.480	0.444	$X < 2$
Linearity ($n = 7$)[c]	Correlation coefficient	0.9999	1.0000	0.9999	$X > 0.9990$
Standard stability[d]	Change in response factors (%)	0.140	0.140	0.140	$X < 2$
Sample stability		0.275	0.276	0.275	$X < 2$
System suitability	R.S.D. (%, $n = 6$)	0.09	0.19	0.24	$X < 2$

[a] Six injections.
[b] Five preparations each, two injections of each preparation.
[c] At 20, 40, 60, 80, 100, 120 and 140% levels.
[d] Two-day stability data.

3.2.4. Accuracy/recovery studies

A known quantity of pure combined *p*-hydroxy benzoic acid was added to the sample to give a concentration range of 75–125% ($n = 3$) of that in a test preparation. These solutions were chromatographed and the amount of combined *p*-hydroxy benzoic acid recovered calculated. Good recovery of combined *p*-hydroxy benzoic acid was observed as shown in the Table 4.

3.2.5. Specificity and selectivity

Injections of the extracted placebo were performed to demonstrate the absence of interference with the elution of the combined *p*-hydroxy benzoic acid. These results demonstrate (Fig. 3) that there was no interference from the other materials in the liquid pharmaceutical formulation, and therefore confirm the specificity of the method.

Table 4
Accuracy/recovery of combined *p*-hydroxy benzoic acid from samples with known concentration

Sample	Percent of nominal	Amount of standard (mg)		Recovery (%)[a]	R.S.D. (%)[a]
		Spiked	Found		
1	75	4.5	3.7	82.0	0.4
2	100	9.0	8.4	94.0	0.7
3	125	135	122	91.0	0.6
Mean				89.0	

[a] $n = 3$.

3.2.6. Stability of analytical solutions

Sample and standard solutions were chromatographed immediately after preparation and then re-assayed after storage at room temperature for 24 h. The results given in Table 3 showed that there was no significant change (<0.14% response factor) in combined *p*-hydroxy benzoic acid concentration over this period.

3.2.7. Measurement of ruggedness

Analytical methods developed for use in quality control (QC) laboratories ideally are rugged. Retention times for the analytes of interest will not change significantly from day-to-day or from laboratory-to-laboratory if the method is considered rugged. To determine the ruggedness of the chromatographic methodology developed for ME, EP and PP, experimental conditions were purposely altered and chromatographic characteristics were evaluated. In particular the pH of the mobile phase was adjusted to 6.5 and 7.5. Thus, the normal pH for the method is 7.05. The effected temperature was also studied. Standard and sample solutions were prepared and injected at early 20 °C and again at 27 °C. Also a standard solution and mobile phase were stored in the refrigerator and then injected. Also the same solutions were injected at 27 °C. In all cases studied, the retention times of these preservatives (MP, EP and PP) were 2.9, 4.2 and 6.8 min, respectively. The coefficient of variation for retention time was less than 1%. Good separation was always achieved, indicating that the

Table 5
Stability results

Components	Specifications (total area %)	Interval time in months					
		0	3	6	9	12	18
Batch 1							
MP	65.0–0.5	73.4	73.3	73.2	74.2	73.4	73.3
EP	14.9–8.2	16.8	15.9	15.8	16.1	15.8	16.0
PP	8.6–2.8	10.2	10.4	10.98	9.5	10.9	10.7
Batch 2							
MP	65.0–0.5	73.2	72.9	73.6	73.6	73.5	72.5
EP	14.9–8.2	16.3	15.8	15.9	16.2	15.6	15.6
PP	8.6–2.8	10.3	11.5	10.5	9.7	10.9	10.3

HPLC method remained selective for all components under the measured conditions.

3.2.8. System suitability test

A system suitability test was performed to determine the accuracy and precision of the system by injecting six replicate injections of combined *p*-hydroxy benzoic acid standard solutions. The relative standard deviation (R.S.D.) of the peak areas responses was measured. The R.S.D. for MP (0.09%), EP (0.19%) and PP (0.24%) as can be seen in Table 3.

3.2.9. Method application and stability studies

The purpose of stability testing is to provide evidence on how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors such as temperature, humidity and light, and enables storage conditions to be recommended and re-test and shelf lives to be established. For this purpose, samples containing MP, EP and PP preservatives were packed and stored in International Conference on Harmonisation (ICH) certified stability chambers maintained at 25 °C and 60% RH. The samples were withdrawn periodically (0, 3, 6, 9, 12 and 18 months) and analysed (Table 5).

4. Conclusion

An RP-HPLC assay method with UV spectrophotometric detection on a C_{18} column was developed successfully for the determination of combined *p*-hydroxy benzoic acid preservatives. The method was validated and the results obtained were accurate and precise with R.S.D. $< 1\%$, and no significant interfering peaks were detected. The method can be used for the routine quality control analysis (batch analysis and stability tests) of compounds in pharmaceutical products containing 0.3% of MP, EP and PP preservatives and the degradation products of the active compound. This method was successfully applied for the identification, quantitative analysis and stability tests of all compounds in the liquid pharmaceutical formulation.

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