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Ghulam A. Shabir[a]
[a] Abbott Laboratories, Witney, Oxfordshire, UK

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Systematic Strategies in High-Performance Liquid Chromatography Method Development and Validation

Ghulam A. Shabir
Abbott Laboratories, Witney, Oxfordshire, UK

This paper concerns the systematic strategies in high-performance liquid chromatography method development and validation. Currently, there is no single source and/or a lack of detailed recommendations on method validation that helps analytical scientists to perform validation in a systematic manner in pharmaceutical industry. Therefore industry depends on the scientist's knowledge and experience to develop simple and efficient methods of analysis. This is why much effort in this paper is focused on the development of validated methods in the pharmaceutical industry. Therefore, guidelines were given for analytical development and validation in this field; the methyl *p*-hydroxybenzoate and propyl *p*-hydroxybenzoate was used as model. In addition, a rapid method using HPLC coupled with photdiode array detection was developed and validated for the simultaneous determination of methyl *p*-hydroxybenzoate and propyl *p*-hydroxybenzoate in a liquid pharmaceutical sample.

Keywords good method validation practice; HPLC; method development; methyl p-hydroxybenzoate; pharmaceutical industry; propyl p-hydroxybenzoate; step-by-step approach to establishing a method validation

INTRODUCTION

Method validation issues are especially important in the analytical field when quantification is made. When a test method has been developed it is important to validate it to confirm that it is suitable for its intended use. The method validation is today an essential concern in the activity of analytical chemistry laboratories. Validation has received considerable attention in the literature. The U.S. FDA (1) describes in Section 211.165 (e) under CGMP, industries should include validation parameters such as accuracy, sensitivity, specificity, and reproducibility in their test method validation and documented. Validation and documentation may be accomplished in accordance with Section 211.194(a). These requirements include a statement of each method used in testing the sample to meet proper standards of accuracy and reliability, as applied to the tested product. The U.S. FDA has also proposed industry guidance for Analytical Procedures and Methods Validation (2). International Organization for Standardization/International Electrotechnical Commission (ISO/IEC) 17025 includes a chapter on the validation of methods (3) with a list of nine validation parameters. The ICH has introduced a guideline (4) on the validation of analytical procedures. The document includes definitions for eight validation characteristics. The U.S. Pharmacopoeia (USP) has published specific guidelines for compendial method validation (5) and more recently has introduced a new chapter on verification of compendial procedures (6).

Method validation is a critical step for any product release for marketing authorization. The literature contains diverse approaches to performing method validation (7–22). Many analytical methods appearing in the literature have not been through a thorough validation exercise and thus should be treated with caution until full validation has been carried out. Also there is no method validation reported with GMP/GLP considerations. Validation of a new method is a costly and very time-consuming exercise. However, the result of not carrying out method validation could result in litigation, failure to get product approval, costly repeat analysis, and loss of business and market share (23).

Currently, there is no completely worldwide single source or final guideline on method validation that helps analysts to perform validation in a systematic manner and most importantly under GMP/GLP considerations. Therefore industry depends on the analyst's knowledge and experience to develop simple and efficient methods of analysis. The ICH achieved a great deal in harmonizing the definitions of the required validation characteristics and their basic requirements. However, they provide only a basis for a general discussion of the validation parameters, their calculation and interpretation. Also ICH has not explained step-by-step approaches and most importantly GMP/GLP considerations that required during method validation to meet regulatory requirements. This impacts regulatory submissions.

The first section of this paper deals with systematic development and validation of analytical methods for pharmaceutical analysis. The second section reviews and

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Address correspondence to Ghulam A. Shabir, Oxford Brookes University, School of Life Sciences, Oxford, OX3 0BP, UK. E-mail: gshabir.oxford@yahoo.com

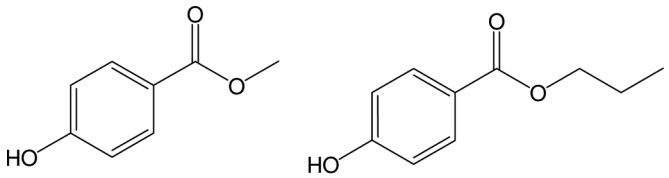

FIG. 1. Structures of: (a) methyl *p*-hydroxybenzoate and (b) propyl *p*-hydroxybenzoate.

demonstrates practical approaches to method validation with reference to simultaneous determination of methyl *p*-hydroxybenzoate and propyl *p*-hydroxybenzoate in a commercially available liquid pharmaceutical sample using high-performance liquid chromatography (HPLC).

Methyl *p*-hydroxybenzoate (MHB) and propyl *p*-hydroxybenzoate (PHB) (0.05 to 0.25%) (Fig. 1), alone or in combination with other esters of *p*-hydroxybenzoic acid or with other antimicrobial agents are used as a preservatives in cosmetic, food and pharmaceutical formulations (24). The formulator must be fully aware of the procedure for preservative systems in a product need to be analysed to establish their effectiveness throughout the shelf life of the products; hence, the simultaneous determination of these preservatives in commercial pharmaceutical products is particularly important both for quality assurance and consumer safety.

Strategy for the Method Development and Validation

Everyday many analytical scientists face the need and challenge to develop and validate new analytical methods. Whereas individual approaches exhibit considerable diversity, following the systematic approach given in Fig. 2 can simplify the process. In the feasibility phase, the analyst will determine whether the assigned task can be successfully accomplished by using available resources. Research is defined as the activity aimed at discovering new knowledge on the compound in hopes that such information will be useful in developing a new method. The development phase is the translation of research findings into a new analytical method and the systematic use of knowledge or understanding gained from research, directed towards the analytical method, including the design and development of prototypes and processes. The development phase must include studies of robustness, system suitability, and the stability of analytical solutions as well as the mobile phase. In the optimization study, the developed method can be further improved to gain greater confidence on the generation of analytical data. The current developed approach emphasis the allocation of greater resources during the development and optimization phases. This allows the analyst to have more confidence on the quality of data generated and therefore considerably reduces the resources that are required for the process of validation. The purpose of the characterization study is to determine reliable method performance limits from the analytical performance characteristics and set acceptance criteria for the method validation. As a best practice, the characterization protocol needs to be written and approved before execution. Prior to execution of the protocol, it is necessary that the analytical system itself is adequately designed, maintained, calibrated, and validated. In all cases proper documentation should be archived to support the validation process. All

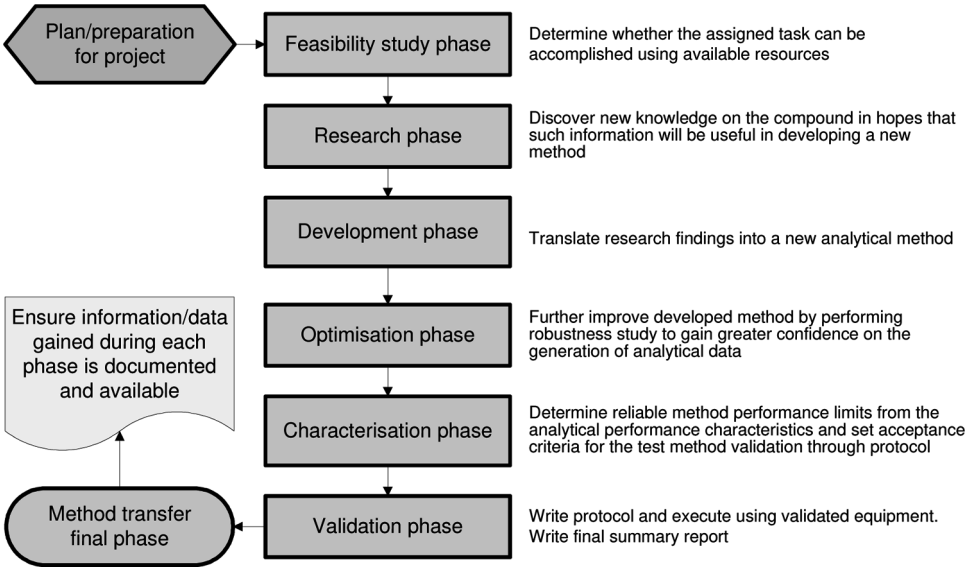

FIG. 2. Systematic approach in method development and validation.

personnel involved in the characterization protocol activities must be trained prior to performing their function. On completion of the characterization study, the results/data should be critically assessed from a statistical point of view.

Validation is the last and critical step for the success of the whole method development project. If the validation fails, it can be seen as a wasted resource and inevitably can delay the product release date. Here, validation protocol needs to be written and approved by an appropriate cross-functional team. Upon successfully completing the validation the data should be statistically analyzed and its acceptance criteria by appropriate experts in order to test its validity. Method transfer plays an important role in expediting drug candidates through development stages. Method transfer is not a trivial task and requires careful planning and constant communication between the laboratory personnel involved in the transfer process. Method transfer could occur within the same organization or between pharmaceutical companies and analytical service providers. To have a successful transfer, the analytical method itself must be robust and the equipment differences between the delivering and receiving parties should be carefully evaluated.

EXPERIMENTAL

Chemicals and Reagents

Methyl *p*-hydroxybenzoate (purity >99%) and propyl *p*-hydroxybenzoate (purity >99%) used were purchased from Sigma (St. Louis, MO). Potassium dihydrogen phosphate, sodium hydroxide (analytical reagent grade), and methanol (HPLC grade) were obtained from Merck (Darmstadt, Germany). Distilled water was de-ionized by using a Milli-Q system (Millipore, Bedford, MA).

HPLC Instrumentation and Conditions

The Knauer HPLC system (Berlin, Germany) equipped with a model 1000 LC pump, model 3950 autosampler, model 2600 photodiode-array (PDA) detector and a vacuum degasser was used. The data were acquired via Knauer ClarityChrom Workstation data acquisition software. The mobile phase was an aqueous solution of 50% (v/v) methanol containing 0.2 M potassium dihydrogen phosphate, adjusted to pH 6.05 ± 0.05 with 1 M NaOH. The flow rate was set to 1.0 mL/min. The injection volume was 20 μL and the detection wavelength was set at 258 nm. HPLC analysis was performed isocratically at ambient temperature using a Lichrosorb C_8 (150×4.6 mm, 5 μm particle size) column (Jones Chromatography, Hengoed, UK).

Standard Preparation

An accurately weighed amount (40 mg) of MHB and 20 mg of PHB standard were placed in a 100 mL volumetric flask and dissolved in methanol (stock solution 1). A 10 mL aliquot of stock solution 1 was transferred to a second 100 mL volumetric flask and diluted to volume with the mobile phase yielding a final concentration of 0.04 mg/mL and 0.02 mg/mL, respectively.

Sample Preparation

Approximately 2.5 g of liquid pharmaceutical sample was accurately weighed, added to a 50 mL volumetric flask, and then diluted in the mobile phase.

RESULTS AND DISCUSSION

Method Development

The chromatographic separation of MHB and PHB was carried out in the isocratic mode using a mixture of 50% methanol in potassium phosphate buffer pH 6.05 ± 0.05 (50:50, v/v) as mobile phase. The column was equilibrated with the mobile phase flowing at 1.0 mL/min for about 20 min prior to injection. The column temperature was ambient. The PDA UV detector was set at 200–400 nm and 258 nm was chosen as the optimal wavelength for maximum detection sensitivity for both preservatives. Additionally, preliminary system suitability, precision, linearity, stability of solutions, and robustness 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 (standard and sample) gave very good peak shapes and resolution (Figs. 3 and 4). In Table 1, the retention times are reported.

Robustness

Robustness measures the capacity of an analytical method to remain unaffected by small but deliberate variations in method parameters during normal usage. Parameters that should be investigated are percent organic content in the mobile phase (±2%); pH of buffer in mobile phase (up to ±0.5 pH units); column temperature (±1 to 5°C); flow rate (±0.2 mL/min) and different HPLC columns (lots and/or suppliers). These parameters may be evaluated one factor at a time or simultaneously as part of a factorial experiment.

In the present study, robustness of the method was evaluated by the analysis of MHB and PHB under different experimental conditions such as changes in the composition of the mobile phase, flow rate, buffer pH, and columns from different batches. The percentage of methanol in the mobile phase was varied ±2%, buffer pH (±0.5 pH units) and the flow rate was varied ±0.2 mL/min. Their effects on the retention time asymmetry factor, recovery, and repeatability were studied. Deliberate variation of the method conditions had no significant effect on assay data

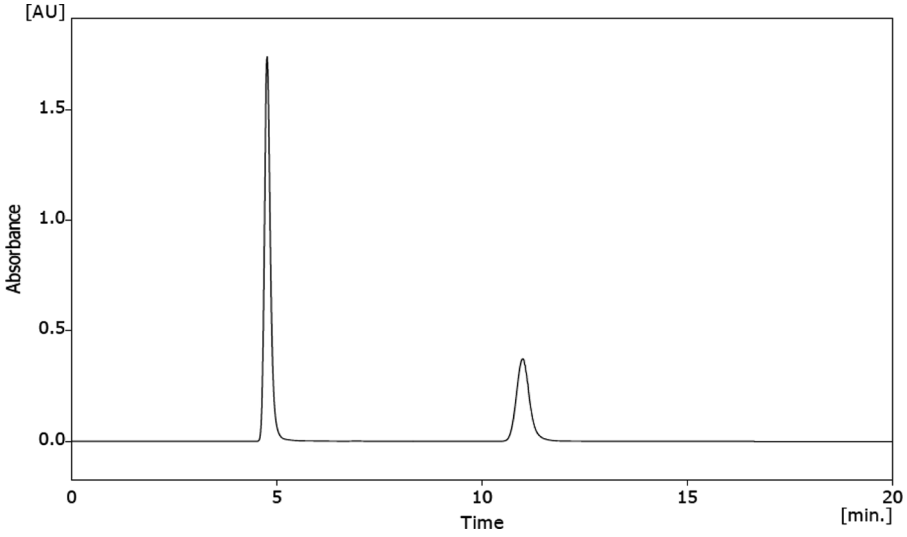

FIG. 3. HPLC chromatogram of reference standard MHB ($t_R = 4.98$ min) and PHB ($t_R = 11.85$ min).

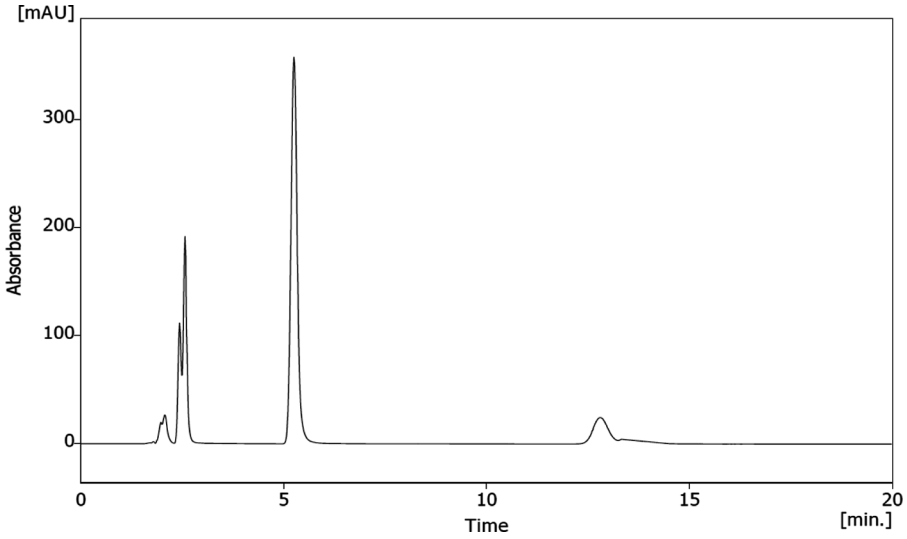

FIG. 4. HPLC chromatogram of the sample.

TABLE 1
System suitability parameters and results of MHB and PHB

Parameter	Acceptance criteria	Results	
		MHB	PHB
Retention time (min)	–	4.98	11.85
Injection repeatability[a]	$N = 5$ for RSD $\leq 2\%$	0.10	0.13
Peak asymmetry	≤ 2.0	1.12	1.25
Capacity factor	>2.0	2.82	6.32
Resolution	>2.0	–	13.89
Theoretical plates	>2000	4093	4862

[a]Six replicate injections, peak area, RSD (%).

or on chromatographic performance, indicating the robustness of the method and its suitability for routine use and transfer to other laboratories.

System Suitability Tests

System suitability testing is essential for the assurance of the quality performance of the chromatographic system. The amount of testing required will depend on the purpose of the test method. For dissolution or release profile test methods using an external standard method, capacity factor, tailing factor, and RSD are minimum recommended system suitability tests. For acceptance, release, stability, or impurities/degradation methods using external or internal standards, capacity factor, tailing factor, resolution and

RSD are recommended as minimum system suitability testing parameters. In this study, system suitability testing was performed to determine the accuracy and precision of the system from six replicate injections of solutions containing 0.04 and 0.02 mg/mL of MHB and PHB, respectively. The percent relative standard deviations (RSD) of the peak area were found to be less than 0.14% for both preservatives. The retention factor (also called capacity factor, k) was calculated using the equation $k=(t_r/t_0)-1$, where t_r is the retention time of the analyte and t_0 is the retention time of an unretained compound; in this study, t_0 was calculated from the first disturbance of the baseline after injection and capacity factor value was obtained 2.82 for MHB peak and 6.32 for PHB peak. The plate number (also known as column efficiency, N) was calculated as $N=5.54\ (t_r/w_{0.5})^2$ where $w_{0.5}$ is the peak width at half peak height. In this study, the theoretical plate number was 4093 and 4862 for MHB and PHB, respectively. Resolution is calculated from the equation $R_s=2(t_2-t_1)\ /\ (t_{w1}+t_{w2})$. Where t_1 and t_2 are retention times of the first and second eluted peas, respectively, and t_{w1} and t_{w2} are the peak widths. The resolution for PHB peak was 13.89. The asymmetry factor was calculated using the USP method. The peak asymmetry value for each MHB and PHB peaks were 1.12 and 1.25, respectively. The proposed method met these requirements within the USP and U.S. FDA accepted limits (Table 1) (25,26).

Method Validation

Before validation activities begun, required validation parameters and associated acceptance criteria must be established, documented, and approved. The level and extent of method validation requirements must take into account the stage of product development, source (new or established test methods), type (physical property, potency, impurity), and intended use (investigational, in-process/release, process or cleaning validation, stability) of the test method. Required validation parameters by ICH, USP, and FDA are listed in Table 2 and are described in detail in the following paragraphs. Also before undertaking the task of method validation, it is necessary that the analytical system itself is adequately designed, maintained, calibrated and validated. The staff carrying out the validation experiments must be properly trained on equipment and validation protocol. In all cases proper validation documentation should be archived to support the validation process.

Stability of Analytical Solutions

The stability of the samples and standards under various conditions should also be studied in method validation. Conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling and analysis. Samples and standards should be tested over at least a 24 h period and quantitation of components should be determined by comparison to freshly prepared standards. Acceptable stability is 2.0% changes in standard or sample response, relative to freshly prepared standards. The mobile phase is considered to have acceptable stability if the aged mobile phase produces equivalent chromatography (capacity factors, resolution, or tailing factor) and assay results are within 2% of the value obtained with fresh mobile phase. For impurity methods, the sample, standard solutions, and mobile phase will be stable for 24 h under defined storage conditions. Acceptable stability is 20% change in standard or sample response at the limit of quantitation, relative to freshly prepared standards. If a solution is not stable at room temperature,

TABLE 2
The ICH, USP and FDA validation parameters requirements

ICH/USP validation parameter	Additional FDA validation requirement	FDA CGMP (legal) requirement	Other type of method[d]
Specificity[a]	Sensitivity	Accuracy	Cleaning
Accuracy[a]	Recovery	Sensitivity	Specific tests
Precision: Repeatability[a]	Reproducibility	Specificity	Concentration
Intermediate precision[a]	Robustness	Reproducibility	Response
Reproducibility[c]	Stability		Physical method
Detection and Quantitation limits[a]	System suitability		
Linearity and Range[a]			
Ruggedness[a] and Robustness[a,c]			

[a]ICH and USP requirement.
[b]Included in the USP.
[c]Included in ICH publication but not part of required parameter.
[d]Not included yet in ICH publication.

TABLE 3
Stability of MHB and PHB in solution ($n = 5$)

Time (h)	Area (RSD, %)	Height (RSD, %)	Recovery (%)	Percent of initial
MHB				
0	0.44	0.08	99.98	
24	0.38	0.10	99.88	99.89
PHB				
0	0.14	0.09	99.97	
24	0.21	0.11	99.87	99.90

then decreasing the temperature to 2–8°C can improve the stability of the samples and standards.

In this study, test solutions of three batches of MHB and PHB were prepared using the conditions cited in Section standard preparation. They were chromatographed at the beginning and after 24 h. The stability of MHB, PHB, and the mobile phase were calculated by comparing area response and area percent of two standards at 0.04 and 0.02 mg/mL over time. The solutions were stable during the investigated 24 h and the RSD was <1.0% for peak area and height. Standard solutions stored in a capped volumetric flask on a laboratory bench under normal lighting conditions for 24 h, were shown to be stable with no significant change in MHB and PHB concentration over this period (Table 3). This is indicated (0.4% changes in area between $T = 0$ h and $T = 24$ h).

Linearity/Range

The linearity of a test procedure is the ability (within a given range) to produce results that are directly proportional to the concentration of analyte in the sample. The range is the interval between the upper and lower levels of the analyte that have been determined with precision, accuracy, and linearity using the method as written. ICH guideline specifies a minimum of five concentration levels, along with certain minimum specified ranges. For assay, the minimum specified range is 80–120% of the target concentration. For an impurity test, the minimum range is from the reporting level of each impurity to 120% of the specification. For content uniformity testing, the minimum range is 70–130% of the target concentration and for dissolution testing, ±20% over the specified range of the test. In practice the linearity study should be designed to be appropriate for the intended analytical method. Acceptability of linearity data is often judged by examining the correlation coefficient and *y*-intercept of the linear regression line for the response versus concentration plot. The regression coefficient (r^2) is >0.998 is generally considered as evidence of acceptable fit of the data to the regression line.

In the present study, linearity was studied using seven solutions in the concentration range 0.010–0.070 mg MHB/mL and 0.005–0.035 mg PHB/mL, ($n = 3$). The regression equations were found by plotting the peak area (y) versus the MHB and PHB concentration (x) expressed in mg/mL. The correlation coefficient (r^2) obtained for the regression line demonstrates that there is a strong linear relationship between peak area and concentration of MHB and PHB (Table 4).

Accuracy/Recovery Studies

The accuracy of an analytical method is the degree of closeness between the true value of analytes in the sample and the value determined by the method and is some times called trueness. Accuracy is usually determined in one of four ways. First, accuracy can be assessed by analysing a sample of known concentration (reference materials) and comparing the measured value to the true value. The second approach is to compare test results from the new method with results from an existing alternate

TABLE 4
Linearity assessment of the HPLC method for the assay of MHB and PHB

Methyl *p*-hydroxybenzoate			Propyl *p*-hydroxybenzoate		
Concentration (mg/mL)	Concentration as % of 0.04 mg/mL	Area (mAU s) ($n = 3$)	Concentration (mg/mL)	Concentration as % of 0.02 mg/mL	Area (mAU s) ($n = 3$)
0.010	25	402	0.005	25	187
0.020	50	1146	0.010	50	646
0.030	75	1902	0.015	75	1089
0.040	100	2635	0.020	100	1511
0.050	125	3328	0.025	125	1987
0.060	150	4088	0.030	150	2428
0.070	175	4826	0.035	175	2907
$y_{MP} = 73.507x - 322.14$ ($r^2 = 0.9999$)			$y_{PP} = 90.157x - 266.71$ ($r^2 = 0.9998$)		

well-characterized procedure that is known to be accurate. The third approach is based on the recovery of known amounts of analyte is performed by spiking analyte in blank matrices. For assay methods, spiked samples are prepared in triplicate at three levels over a range of 50–150% of the target concentration. For impurity methods, spiked samples are prepared in triplicate at three levels over a range that covers the expected impurity content of the sample, such as 0.1 to 2.5% (w/w). The percent recovery should then be calculated. The fourth approach is the technique of standard additions, which can also be used to determine recovery of the spiked analyte. This approach is used if it is not possible to prepare a blank sample matrix without the presence of the analyte. Accuracy criteria for an assay method (FDA) is that the mean recovery will be $100 \pm 2\%$ at each concentration over the range of 80–120% of the target concentration. For an impurity method, the mean recovery will be within 0.1% absolute of the theoretical concentration or 10% relative, whichever is greater, for impurities in the range of 0.1 to 2.5% (w/w). In this study, accuracy of the method was evaluated by fortifying a MHB and PHB sample solutions with three known concentrations of reference standard 0.02, 0.04, 0.06 mg/mL and 0.01, 0.02, 0.03 mg/mL (50–150%), respectively. Percent recoveries were calculated from differences between the peak areas obtained for fortified and unfortified solutions. Mean recoveries ($n = 3$) for sample MHB and PHB were found to be 99.72% and 99.61%, respectively, as shown in Table 5.

Precision Studies

The precision of an analytical method is the closeness of a series of individual measurements of an analyte when the analytical procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of pharmaceutical matrix. The precision is calculated as coefficient of variation (CV), i.e., relative standard deviation (RSD). The measured RSD can be subdivided into three categories: repeatability (intra-day precision), intermediate precision (inter-day precision), and reproducibility (between laboratories precision (4). Repeatability is obtained when the analysis is performed in one laboratory by one analyst using the same equipment on the same day. It should be determined from a minimum of nine determinations covering the specified range of the procedure (for example, three levels, three repetitions each) or from a minimum of six determinations at 100% of the test or target concentration. According to the FDA (26) for instrument precision or injection repeatability study, should be a minimum of 10 injections of one sample solution is made to test the performance of the chromatographic instrument. Intermediate precision is the results from within lab variations due to random events such as different days, analysts, equipment, etc. Precision criteria for an assay method are that the instrument precision and the intra-assay precision (RSD) will be $\leq 2.0\%$. For impurity assay, at the limit of quantitation, the instrument precision will be $\leq 5.0\%$ and the intra-assay precision will be $\leq 10.0\%$.

The reproducibility is determined by testing homogeneous samples in multiple laboratories often as part of inter-laboratory crossover studies. An example of reproducibility criteria for an assay method could be that the assay results obtained in multiple laboratories will be statistically equivalent or the mean results will be within 2.0% of the value obtained by the primary testing laboratory. Reproducibility is not normally expected if intermediate precision is performed.

In this study, the precision of the method was investigated with respect to repeatability and intermediate precision. The repeatability (intra-day precision) of the method was evaluated by assaying six replicate injections of the MHB and PHB at 100% of test concentration 0.04 and 0.02 mg/mL, respectively on the same day. The (RSD, %) of the retention time (min) and peak area were calculated as shown in Table 6. Intermediate precision (inter-day precision) was demonstrated by two analysts using two instruments and evaluating the relative peak area percent data across the two instrument systems at three different concentration levels (50, 100, and 150%) that cover the assay method range of MHB (0.01–0.07 mg/mL) and (0.005–0.035 mg/mL) of PHB. The RSD across the systems and analysts were calculated from the individual relative peak areas mean values at the 50, 100, and 150% of the test concentration. The inter-day RSD ($n = 3$) are given in Table 7. All the data are within the acceptance criteria of 2.0%.

TABLE 5
Recovery of MHB and PHB from samples with known concentrations

Sample #	Percent of nominal	Amount of analyte (mg/mL) Added	Found	Recovery (%) and confidence limits
Methyl *p*-hydroxybenzoate				
1	50	0.025	0.0249	99.60 ± 0.10
2	100	0.045	0.0449	99.77 ± 0.13
3	150	0.055	0.0549	99.80 ± 0.11
Propyl *p*-hydroxybenzoate				
1	50	0.010	0.0100	100.0 ± 0.11
2	100	0.020	0.0199	99.50 ± 0.09
3	150	0.030	0.0298	99.33 ± 0.12

TABLE 6
Demonstration of the repeatability of the HPLC assay

	Methyl *p*-hydroxybenzoate		Propyl *p*-hydroxybenzoate	
Injection #	t_R (min)	Area (mAU s)	t_R (min)	Area (mAU s)
1	4.983	2226	11.850	941
2	4.983	2235	11.850	943
3	4.982	2236	11.851	947
4	4.983	2221	11.851	939
5	4.983	2226	11.850	942
6	4.983	2231	11.850	943
Mean (6)	4.983	2229	11.850	943
RSD (%)	0.008	0.262	0.004	0.283

TABLE 7
Demonstration of the intermediate precision of the HPLC assay

	Instrument 1			Instrument 2		
Percent of nominal	50	100	150	50	100	150
Methyl *p*-hydroxybenzoate						
Analyst 1	1023[a]	2634	4987	1019	2636	4986
Analyst 2	1027[a]	2630	4982	1026	2629	4971
Mean instruments	1024	2633	4982			
Mean analysts	1025	2632	4984	1026	2632	4978
Instruments (%RSD)[b]	0.20	0.13	0.15			
Analysts (RSD, %)	0.28	0.11	0.07			
Propyl *p*-hydroxybenzoate						
Analyst 1	522	999	1502	530	992	1516
Analyst 2	518	996	1510	524	997	1511
Mean instruments	523	996	1509			
Mean analysts	520	998	1506	527	994	1513
Instruments (%RSD)	0.96	0.29	0.38			
Analysts (RSD, %)	0.54	0.21	0.37			

[a]Peak area (mAU s), $n = 3$.
[b]Acceptance criteria (x) RSD, % < 2.0.

Specificity/Selectivity

Sometimes there is some confusion over which term that should be used in characterising a method, specificity or selectivity. The differences between the two terms is that selectivity refers to a method that gives responses for a number of substances and can distinguish the analyte(s) response from all other responses. Specificity refers to a method that gives response for only one single analyte. In chromatography with UV detectors it is unusual that a method responds only to one analyte and therefore the term selectivity is appropriate. The selectivity of the method should be evaluated by processing blank samples with and without the addition of analytes and inject them to test for interferences. The selectivity of the method is very important to enable accurate analyte quantification

Other potential sample components are generated by exposing the analyte to stress conditions sufficient to degrade it to 80–90% purity. For bulk pharmaceuticals, stress conditions such as heat (50 to 80°C), light (600 FC of UV), acid (0.1 M HCl), base (0.1 M NaOH), and oxidant (3% H_2O_2) are typical. For formulated products, heat, light, and relative humidity (70 to 80% RH) are often used. The resulting mixtures are then analyzed, and the analyte peak is evaluated for peak purity and resolution from the nearest eluting peak. An example of specificity criterion for an assay method is that the analyte peak will have baseline chromatographic resolution of at least 2 from all other sample components. In this study, the HPLC-PDA/UV isoplot chromatogram (Fig. 5) demonstrates a good separation of the MHB and PHB. The isoplot chromatogram data consist of UV absorption spectra from 200 to 400 nm for each point along the chromatogram. Also injections of the extracted placebo were performed to demonstrate the absence of interference with the elution of the MHB and PHB. These results demonstrate (Fig. 6) that there was no interference from the other materials in the liquid formulation and, therefore, confirm the specificity of the method.

Forced degradation studies were also applied to MHB and PHB reference standards at a concentration of 0.04 mg/mL and 0.02 mg/mL, respectively, to verify that none of the degradation products interfered with quantitation of the drug. Hydrolytic degradation was studied by heating the drug under reflux at 80°C in 0.1 M HCl and 0.1 M NaOH for 4 h. The samples were then cooled to room temperature and neutralized. Oxidative degradation was studied by treating the drug with 3% H_2O_2 at room temperature (24 ± 1°C) for 4 h.

Solutions containing 0.04 and 0.02 mg/mL of each degraded sample were prepared and injected in triplicate. A summary of the stress results for retention time, peak area and resolution are shown in Table 8. Under acidic and alkaline degradation hydrolysis conditions, the MHB content decreased and additional unknown polar peaks were observed near the solvent front (Fig. 7). No degradation was observed under oxidative condition. This was further confirmed by peak purity analysis on a PDA UV detector. The MHB and PHB analytes obtained by acidic hydrolysis were well resolved from the additional peak, indicating the specificity of the method.

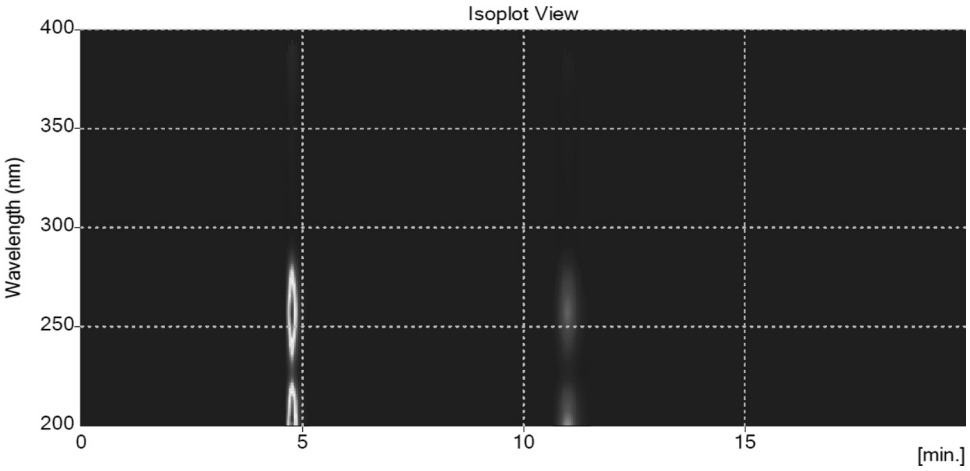

FIG. 5. HPLC-PDA/UV isoplot chromatogram of MHB and PHB.

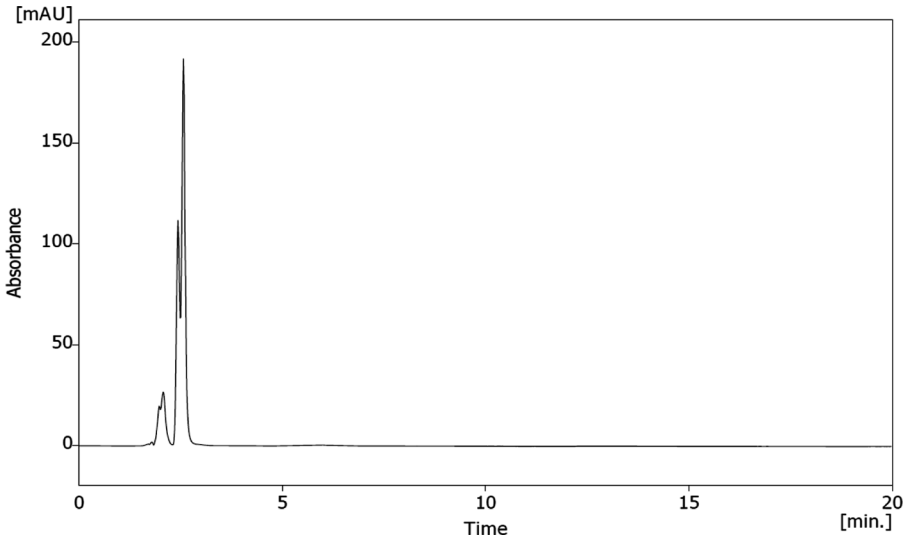

FIG. 6. HPLC chromatogram of placebo.

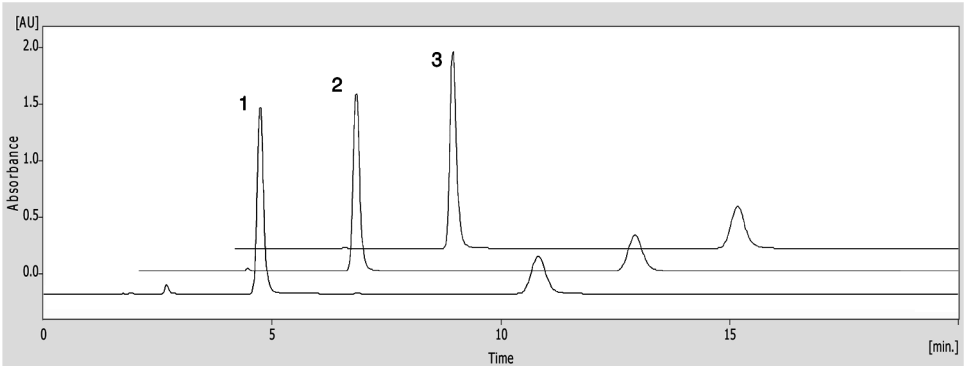

FIG. 7. HPLC chromatograms of hydrolysed MHB and PHB under stress conditions (1) acid at 80°C for 4 h; (2) base at 80°C for 4 h; (3) oxidative, 3% H_2O_2 at room temperature for 24 h.

TABLE 8
Results of the stress conditions experiments

		MHB		PHB		
Stress conditions	Sample treatment	t_R (min)	Area (%)	t_R (min)	Area (%)	R
Reference	Fresh solution	4.983	70.2	11.850	29.9	13.8
Acid	1 M HCl at 80°C for 4 h	4.733	66.7	10.817	30.7	7.4
Base	1 M NaOH at 80°C for 4 h	4.750	68.4	10.867	30.8	7.6
Oxidative	3% H_2O_2 at room temperature for 24 h	4.982	69.9	11.841	30.01	13.7

Limits of Detection and Quantitation

The limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected, not quantitated. It is expressed as a concentration at a specified signal-to-noise ratio, usually 3:1 (4). The limt of quantitation (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method with a signal-to-noise ratio of 10:1. LOD and LOQ may also be calculated based on the standard deviation (σ) and the slope of the calibration curve(s) at levels approximating the LOD according to the formulae:

$$\text{LOD} = 3.3\,\sigma/\text{s}$$

$$\text{LOQ} = 10\,\sigma/\text{s}$$

The standard deviation of the response can be determined based on the standard deviation of the blank, on the residual standard deviation of the regression line, or the standard deviation of y-intercepts of regression lines. In this study, the LOD values for MHB and PHB were found to be 0.022 µg/mL (s/n = 3.05) and 0.009 µg/mL (s/n = 3.07), respectively. The LOQ values for MHB and PHB were found to be 10 µg/mL (s/n = 10.25) and s/n = 10.30 (5 µg/mL), respectively. The RSD for six injections of the LOQ solutions for MHB and PHB was <2.0%.

CONCLUSION

In the present paper the detailed recommendation and rules given by the FDA, USP, and ICH for analytical methods have been applied and modified to the field for analysis of pharmaceutical samples. This paper also contains guidelines for systematic method development and validation demonstrating proof of principle with a great potential to achieve excellent accuracy and precision of the quantification of results. HPLC coupled t PDA, for identification and/or quantification is today an established method in the pharmaceutical industry. In this paper I also have developed and validated HPLC-PDA method that can be reliably used in simultaneously determination of liquid pharmaceutical samples containing methyl *p*-hydroxybenzoate and propyl *p*-hydroxybenzoate preservatives. I hope that I have provided a complete guide to help analysts to understand how to perform an analytical method validation under GMP and GLP environment that meets FDA, USP, and ICH requirements.

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