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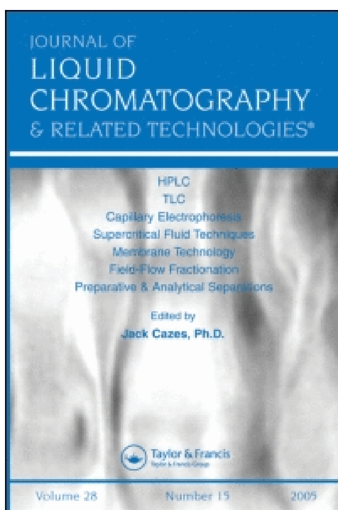

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Evaluation and Application of Best Practice in Analytical Method Validation

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Evaluation and Application of Best Practice in Analytical Method Validation

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Abstract: Method validation is an important part of analytical chemistry to confirm that the method employed for a specific test is suitable for its intended use. As such, it is an essential requirement for any package of information submitted to regulatory agencies in support of new product marketing or clinical trial applications. Currently, there is no single source or final guideline on analytical method validation that helps analysts to perform validation in a systematic manner. Therefore, industry depends on the analyst's knowledge and experience to develop simple and efficient methods of analysis. The intention of this paper is to review regulatory requirements and role of the pharmacopeias and to study how analytical method development and validation are typically carried out at present, and to formulate this into a simple step by step approach. Such a template was not only used as the foundation of this research programme, but could also serve as a simple systematic guide for other practitioners and those new to the field. Furthermore, it was recognized that this protocol should satisfy the requirements of the most strategically important regulatory bodies.

Keywords: Method validation, Pharmacopoeias role, Best practice, HPLC analysis, Regulatory agencies, 4-Hydroxybenzoic acid ester

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INTRODUCTION

Analytical method development and validation play a major role in the discovery, development, and manufacture of pharmaceuticals. The official test method that results from these processes are used by quality control laboratories to ensure the identity, purity, potency, and performance of drug product 'quality', essential for drug safety and efficacy. In the pharmaceutical and biotechnology industries, a current major issue is the high cost of research in introduction of new drugs. In essence, it takes several hundred million dollars to discover, develop, and gain regulatory approval. One of the reasons research and development (R&D) is so costly in pharmaceuticals is that most new drug candidates fail to reach the market. Failure can result from toxicity, carcinogenicity, manufacturing difficulties, inadequate efficacy, and analytical problems. Therefore, there is a need for high throughput in order to maximize patent lifetime and, consequently, generate the profits to support the research and to increase the speed with which the product can be delivered to the market. All the different stages of pharmaceutical R&D are underpinned by analysis so that high throughput is actually dependent on effective and efficient analysis within which simple effective method development and comprehensive analytical method validation is of fundamental importance. A wide variety of materials are used in the pharmaceutical and diagnostic industries. All of these materials must be analysed in some way or other and, just as importantly, the method of analysis must be validated, i.e., it must be shown that the method is fit for its intended purpose.

In the pharmaceutical industry, analytical method validation is very much a major issue as analysis is used primarily to control drug quality. This is important in its own right and, also, in that drug safety and efficacy are dependent on it. Different chemical entities with varying chemical and physical properties are used. These may include starting materials, intermediates, final drug substances, and the final formulated pharmaceutical products. The pharmaceutical analyst will be concerned with applying analytical methods to the determination of stability/shelf life, purity, side-product identity, dissolution, etc. Here, the analyst is required to develop new methods of analysis appropriate to the information required. In many cases, the analyte may be known but is present in a new sample matrix, such that a new sample preparation method is needed. The knowledge gained in the method development phase is important when it comes to validating the method efficiently. Frequently, high performance liquid chromatography (HPLC) is the analytical method of choice in pharmaceutical analysis because of its specificity (i.e. all the components of a sample are separated from one another before the measurement is made, so that its results arise from the analyte and from nothing else). Although HPLC is a relatively mature technique, the analyst is continually required to innovate by adapting current methodology, or indeed,

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developing completely new protocols. For example, the coupling of HPLC with another technique such as mass spectrometry (MS) can be especially powerful.

In the diagnostic industry, the variety of materials is further expanded due to the complexity of medical devices and their corresponding reagents. Such materials may include polymers, surfactants, enzymes, cofactors, stabilizers, etc. The diagnostic analyst is, therefore, required to apply other techniques apart from HPLC in the analysis of key materials. An in depth knowledge of the materials and their critical properties as applied to their use in the diagnostic device is necessary. Innovation is again needed if there is no directly applicable methodology reported in the literature. Once an analytical method is developed, validation is conducted in order to prove its use for the intended application.

Validation is a critical step for any product release for marketing authorization. The literature contains diverse approaches to performing method validation.[1–8] 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.[9] Validation of a new method is a costly and 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.[9] Validation is the proof needed to ensure that an analytical method can produce results that are reliable, reproducible, and are fit for the purpose intended. Choosing the validation criteria depends on the method type. In general, method validation parameters that should be studied are linearity, range, accuracy, precision (repeatability and intermediate precision), specificity, limit of detection, and limit of quantitation.

The International Conference on Harmonization (ICH) guidelines[10,11] 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. It is the responsibility of the analyst to identify parameters that are relevant to the performance of the given analytical procedure, as well as to design proper validation protocols including acceptance criteria and to perform an appropriate evaluation.

Currently, there is no single source or final guideline on method validation that helps analysts to perform validation in a systematic manner. Therefore, industry depends on the analyst's knowledge and experience to develop simple and efficient methods of analysis. The other major problem pharmaceutical industries are facing in today's world, is that different validation data requirements are required for regulatory submissions for product approval depending upon the location of the regulatory body. For example, the release of any pharmaceutical product in Europe, Japan, and USA would require the use of ICH method validation criteria. However, the

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release of the very same product, by the same industry, in any other part of the world would force the use of their local regulatory guidelines. This inevitably becomes a costly process due to issues of documentation and personnel training, etc. Therefore, efforts are underway to streamline the method validation process through an idea commonly referred to as Harmonization by ICH. As an example, Health Canada Drugs Directorate has already started to align their method validation guidelines (Acceptable Methods) according to ICH.

The outcome of ICH efforts has been accepted by most regulatory bodies and pharmacopoeia, such as FDA[12,13] (the largest of the world's drug regulatory agencies, FDA is responsible for the approval of all drug products used in the USA) and USP.[14,15] Consequently, they have updated their general chapters. The USP established in 1820, contains legally recognized standards of identity, strength, quality, purity, packaging and labeling for drug substances, dosage forms, and other therapeutic products, including nutritional and dietary supplements. USP also contains monographs, which are recognized worldwide and may be enforceable by the US FDA and also by state agencies in the US.

The ICH guidelines 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. However, this has not removed the confusion in industries because ICH, as yet, has not explained various other method types such as a response test (to detect a specific substance in a sample as indicated by test signal response), concentration test (for quantitation of a specific substance in a sample), physical test (for determination of the physical characteristics of a product or material), and cleaning test (for evaluating the cleanliness of equipment and areas used for manufacturing). This impacts regulatory submissions.

The purpose of this review is (i) to critically evaluate current practices in method development and analytical method validation, in order to identify best practices, (ii) to apply best practices with some improvements, in such a way as to ensure good quality and provide new knowledge on a wide range of pharmaceutical substances, products, and compounds used in pharmaceuticals, diagnostics, and, finally, (iii) to draw upon the outcomes of the programme to be able to recommend the way forward with respect to ensuring that the ever evolving approaches to analytical method development and validation were enhanced, simple, systematic, efficient, and effective, while still being compliant with the requirements of regulatory agencies. Also, it is intended in this paper, to review and demonstrate practical approaches to method validation in detail with reference to an HPLC assay of 4-hydroxybenzoic acid ester (HBAE). HBAE alone or in combination with other esters of p-hydroxybenzoic acid, or with other antimicrobial agents, is used as a preservative in cosmetic and pharmaceutical formulations.

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PHARMACOPOEIA ROLE AND GLOBAL HARMONISATION

Pharmacopoeial standards for medicinal products and auxiliary substances are widely used for regulatory purposes in the fields of public health protection and commerce. Small wonder, therefore, that, with the existence of some 37 pharmacopoeias worldwide, those engaged in the marketing of these substances on an international scale are often faced with the need to undertake additional testing of their products beyond that which they may consider to be necessary, in order to ensure compliance with the often divergent specifications of these different pharmacopoeias. Such additional testing is expensive and difficult to justify in terms of patient protection. This problem has been recognized by the three major pharmacopoeial authorities.

In June 1989, on the 25th anniversary of the European Pharmacopoeia convention in Strasbourg and at the congress on the perspectives of international harmonization in Tokyo, multinational pharmaceutical companies expressed their need for the harmonization of the pharmacopoeias of Japan, Europe, and the US. The heads of these pharmacopoeias immediately decided to organize regular contacts among themselves and a procedure for rapprochement. In this way, the Pharmacopoeial Discussion Group (PDG) was founded and meets twice a year. About 50 compendial monographs on excipients and general methods of analysis proposed by national associations of manufacturers of pharmaceutical products have been selected for convergence and harmonization among the three pharmacopoeias. Proposals for harmonized texts are regularly published in the forum of the three pharmacopoeias for public enquiry (Pharmeuropa, US Pharmacopoeial Forum and the Japanese Pharmacopoeial Forum).

As commented by Halperin,[16] harmonization at the world level rarely means identical standards (unlike the results obtained in Europe), but rather the elimination of elements of disharmony whenever possible and whenever useful to international trade. Indeed, many parameters are involved and there are many conflicts between monographs, methods of analysis, and reagents. Furthermore, attaining identical standards is complicated by expanding markets. Hence, the first stage of harmonization involves the elimination of standards that are not scientifically justified, the revision of a thorough evaluation of analytical test methods, obsolete specifications, and the search for compatibility between the chosen standards. To be effective, it requires much explanation and public relations between all the partners concerned so that the constraints and limits of each are known.

The pharmacopoeias also participate in the work on the rapprochement of licensing dossiers within the framework of ICH. ICH is a joint initiative involving both regulatory bodies and the pharmaceutical research based industry (Europe, Japan and the United States of America) as equal partners in the scientific and technical discussions of the testing procedures that are required to ensure and assess the safety, quality, and efficacy of medicines. The pharmacopoeias have observer status in the quality working party and

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biotech working party and, notably, have participated in the elaboration of guidelines on analytical validation, impurities, residual solvents, and specifications. Where appropriate, they integrate the general principles of these guidelines into their specifications.

Following the adoption of the last quality guidelines by ICH steering committee in October 1989 on specifications, test procedures, and acceptance criteria for new drug substances and new drug products, and chemical substances, it is intended that the regulators of the three regions (U.S. FDA, EU/Europe and MHW Japan) will recognize as interchangeable, procedure and acceptance criteria of any of the three pharmacopoeias where harmonization of this procedure and criteria have been successfully completed. To signify the harmonized status of these procedures, the three pharmacopoeias have agreed to include a statement in their respective texts that indicates that the procedures and acceptance criteria from all three pharmacopoeias are considered equivalent and are, therefore, interchangeable. This agreement takes effect as soon as the three pharmacopoeias publish the common text that was agreed on.

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CRITICAL EVALUATION OF CURRENT BEST PRACTICE IN ANALYTICAL METHOD DEVELOPMENT AND VALIDATION

The first stage of the programme was to study how analytical method development and validation is typically carried out at present, and to formulate this into a simple step by step approach. Such a template[17] was not only used as the foundation of this research programme but could also serve as a simple systematic guide for other practitioners and those new to the field. Furthermore, it was recognized that this protocol should satisfy the requirements of the most strategically important regulatory bodies. These requirements were critically evaluated, identifying the key similarities and, more importantly, differences between the validation requirements of the FDA, USP, and ICH.[18] The aim of the field was to take forward to apply the identified best practices to studies of a diverse range of pharmaceutical substances, products, and compounds used in pharmaceuticals and diagnostics.[19–26] Everyday, many analysts face the need and challenge to develop and validate HPLC, LC-MS, and GC methods. Whereas individual's approaches may exhibit considerable diversity, a best practice method development and validation follows the systematic approach (Figure 1). This is a highly successful approach to a method development and validation process. Before embarking on the development of a new method, always search the literature to see if a suitable one already exists. If a suitable one is found, it will still be necessary to perform some method optimisation and validation to prove that the method can be successfully adapted for its intended use.

In the feasibility phase, the analyst will determine whether the assigned task can be successfully accomplished by using available resources.

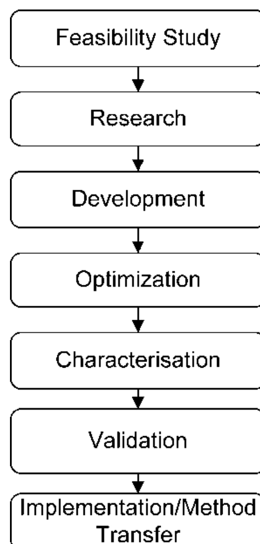

Figure 1. Systematic approach in analytical method development and validation.

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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. Development is the translation of research findings into a new analytical method and the systematic use of knowledge or understanding gained from research directed toward the analytical methods, including the design and development of prototypes and processes. Robustness studies must be considered in this phase. Robustness: Measure of a method's capacity to remain unaffected by small but deliberate variations in method parameters. The development phase must also include system suitability testing and stability of analytical solutions, as well as mobile phase.

In optimization study, a developed method can be further improved to gain greater confidence on the generation of analytical data; the search for the best solution among alternatives, or the extreme value of a variable. The current developed approach emphasis the allocation of greater resources during the development and optimization phases. This allows the analyst to have more confidence in 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 test 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 qualified. In all cases proper validation documentation should be archived to support the

qualification process. All 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 and its acceptance criteria should be statistically analyzed by appropriate experts in order to test its validity.

Timely implementation/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. 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. Unfortunately, very limited information on method transfer can be found in the literature. Typically in any organization, before the method transfer, scientists from both sites need to go through the method details very carefully. As a best practice and successful transfer of analytical method, prepare a method transfer validation protocol that is agreed by the both sites, approved, and executed.

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METHOD DEVELOPMENT AND MODERN ANALYTICAL TECHNIQUES

Method development is not always a simple task since there are a substantial number of parameters in HPLC, which may influence the final results that are obtained. Specially, when the required method does not exist in the literature, the analyst needs advanced knowledge and experience on both analytical equipment and drug substance, or drug product, that need to be analyzed. In this situation, applying a systematic approach, as discussed above, can make a task simple, and reduces resources of the company.

Reversed-phase chromatography is probably the most commonly used separation mechanism in liquid chromatography and consists of a non-polar stationary phase (normally octadecyl, C_{18} or octyl C_8 chains) bonded to a solid support that is generally micro particulate silica gel (non-polar). The mobile phase is polar and, therefore, the sample compounds are partitioned between the mobile and the stationary phases. The separation is normally performed using aqueous mobile phase containing different percentages of organic modifiers (e.g., methanol, ethanol, acetonitrile, or THF) to increase the selectivity between species. Solute retention is also influenced by eluent

pH, which affects the dissociation level of the analyte and, therefore, its partition between the mobile and stationary phases.

Mass spectrometry has progressed extremely rapidly during the last decade: production, separation, and detection of ions, data acquisition, data reduction, etc. This has led to the development of entirely new modern instruments and applications.

The combination of chromatographic separations with mass spectrometric detection is considered an indispensable tool for problem solving in analytical chemistry and, increasingly, for routine analytical methods. Mass spectrometric detection brings an added level of information, complementary to the chromatographic process that improves the certainty of identification and the specificity of detection. Mass spectral information can generally be obtained from a sample size typical of common analytical methods. In the last 10 years, research efforts in the field of HPLC-MS have changed considerably. HPLC-MS has rapidly matured to become a very powerful and useful analytical tool that is widely applied in many areas of chemistry, pharmaceutical sciences, and biochemistry. Investigation into the coupling of HPLC and MS began in the early 1970s. In the first 20 years, most of the attention had to be given to solving interface problems and building new technology. However, most scientists with HPLC-MS today are only concerned with application of the commercially available techniques in their field of interest. Technological problems in interfacing appear to be solved, and from the wide variety of interface developed over the years, basically only two remain, i.e., electrospray ionization (ESI) and atmospheric-pressure chemical ionization (APCI), which are both atmospheric-pressure ionization (API) techniques. With ESI and APCI, HPLC-MS has been implemented in analytical strategies in many application areas, e.g., environmental analysis, drug development within the pharmaceutical industry, characterization of natural products, and the characterization of biomolecules like peptides, proteins, oligosaccharides, etc.

The selection of the appropriate HPLC conditions, whether reversed-phase liquid chromatography, ion-pairing chromatography, capillary electrophoresis, or ion chromatography, and of the most sensitive ionization mode, ESI or APCI, depends upon the polarity and acidity of the analyte. The ESI is best applied to the highly polar nature of the analyte and APCI ionizes most efficiently compounds with low to moderately high polarities and, in this respect is complementary to electrospray, which gives the best sensitivity for ionic compounds. Both interfaces, ESI and APCI, can be operated in positive and negative ion mode. Often, an appropriate selection for a given analyte can be made by considering that ESI transfers ions from solution into the gas phase, whereas APCI ionizes in the gas phase. As a rule of thumb, analytes occurring as ions in solution may be best analyzed by ESI, while non-ionic analytes may be well suited for APCI.

As for the other detection principles discussed above, all of these contribute significantly to the present day success of hyphenation in HPLC. There is

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no doubt that, also, today, HPLC-PDA UV plays an important role (detection and peak-purity) in many research and development studies, and for a wide variety of routine analyses.

EXPERIMENTAL

Chemicals and Reagents

4-hydroxybenzoic acid ester (Batch #1005425) was obtained from Lancaster Synthesis (Morecambe, England). HPLC grade acetonitrile was obtained from Merck (Darmstadt, Germany). Deionized distilled water was used throughout the experimental study.

HPLC Instrumentation

HPLC analysis was performed using a Waters Alliance 2690 Separations model with a 996 Waters PDA detector system (Waters, Elstree, UK). The second HPLC system was used for intermediate precision studies and consisted of Perkin Elmer (Norwalk, CT) equipped with a model series 200 UV Visible detector, series 200 LC pump, series 200 autosampler, and series 200 peltier LC column oven, using a Symmetry C_{18} column (3.9 × 150 mm, 5 μm) at ambient temperature. The mobile phase was acetonitrile/water (65:35, v/v). The mobile phase was filtered through a 0.45 μm membrane filter and degassed before use. The flow rate was set at 1.0 mL/min. UV detection was performed at 254 nm and volume of sample injected was 20 μL.

Preparation of Standard and Sample Solutions

HBAE (100 mg) was accurately weighed and added to a 100 mL volumetric flask before being dissolved in acetonitrile. A 2.0 mL aliquot of stock solution was diluted to 100 mL in the mobile phase, yielding a final concentration of 20 μg/mL. Standard solutions for the evaluation of HBAE linearity were prepared over a concentration range of 5.0–40 μg/mL, to 25, 50, 75, 100, 150, and 200% in the mobile phase.

RESULTS AND DISCUSSIONS

Method Validation

Prior to method validation in the pharmaceutical and diagnostic industries, analytical equipment must be qualified (installation, operational and performance qualification), as well as software validated in compliance with the U.S.

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Code of Federal Regulations (FDA 21 CFR Part 11). Best practices in method development and validation is equally important in the analysis of active and inactive components in formulated products. In this study, a simple and robust HPLC assay method for determining the content of HBAE was validated.

Linearity and Range

The linearity of the method should be tested in order to demonstrate a proportional relationship of response versus analyte concentration over the working range. The linearity range for evaluation depends on the intended use of the analytical method. The ICH guidelines specified a minimum of five concentration levels, along with certain minimum specified ranges. For an assay, the minimum specified range is from 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. Additional suggestions for the appropriate range are available in other literatures.[27–32] 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. The y-intercept should be less than a few percent of the response obtained for the analyte at the target level. The percent relative standard deviation (RSD), intercept, and slope should be calculated.

In the present study, linearity was studied in the concentration range 5.0–40 μg/mL (25–200% of nominal concentration, $n = 3$) and the following regression equation was found by plotting the peak area (y) versus the HBAE concentration (x) expressed in μg/mL: $y = 29935x + 51338$ ($r^2 = 1.000$). The correlation coefficient (r^2) obtained for the regression line demonstrates the excellent relationship between peak area and concentration of HBAE (Table 1). The range is derived from linearity studies and depends on the intended application of the test method. It is established by confirming that the assay procedure provides an acceptable degree of linearity, accuracy, and precision when applied to samples containing amounts of analyte within, or at the extremes, of the specified range of the test method. The range is normally expressed in the same units as the test results obtained by the method. In this study, the data obtained during the linearity and accuracy studies was used to assess the range of the assay method. The precision data for this assessment was the precision of the three replicate samples analyzed at each level in the accuracy studies. The valid analytical range of the method is that range of concentrations, which pass the linearity and accuracy criteria, and yields an RSD of $<2\%$. The linearity data described earlier demonstrates acceptable linearity for HBAE over the range of 80 to 120% of the target concentration.

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Table 1. Method validation results

Validation steps	Parameter	Acceptance criteria	Results
Repeatability ($n = 10$)	Retention time (min) RSD (%)	≤ 2	0.09
	Peak area RSD (%)	≤ 2	0.13
	Peak height RSD (%)	≤ 2	0.16
Linearity ($n = 3$)	Correlation coefficient (r^2)	>0.998	$R^2 = 1.000$
	Equation for regression line		$Y = 29936x + 51337$
LOD	s/n ratio	s/n = 3:1	(s/n 3.3), 2.5 ηg mL
LOQ	s/n ratio	s/n = 10:1	(s/n 10.2), 5.5 ηg mL

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Accuracy/Recovery Studies

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. Accuracy is usually determined in one of four ways. First, accuracy can be assessed by analyzing 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 well characterized procedure that is known to be accurate. The third approach is based on the recovery of known amounts of analytes. This is performed by spiking analytes 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. 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 spiked analytes. This approach is used if it is not possible to prepare a blank sample matrix without the presence of the analyte. In this respect, the mean recovery should be 100 $\pm$ 2% at each concentration over the range of 80–120% of the target concentration. The ICH recommends collecting data from a minimum of nine determinations over a minimum of three concentration levels covering the specified range (e.g., three concentrations, three replicates each).

In the present study, a number of different solutions were prepared with known added amounts of HBAE and injected in triplicate. Percent recoveries of response factor (area/concentration) were calculated. The results of accuracy studies are shown in Table 2, and it is evident that the method is accurate within the desired recovery range. The RSD values obtained for the recovery of HBAE at 50, 75, 100, and 150% of target are 0.15, 0.19, 0.14, and 0.12%, respectively. Each value was the result of three individual sample preparations and analyses. These data support a method range of 80 to 120% of the target concentration.

Table 2. Recovery studies of HBAE from samples with known concentration

Sample	Percent of nominal	Recovery (%) ($n = 3$)	RSD (%)
1	50	99.66	0.15
2	75	99.79	0.19
3	100	99.88	0.14
4	150	99.86	0.12
Mean		99.80	

Specificity

In order to design a chromatographic system for the analysis of an active component of a pharmaceutical product, it is essential to have a good knowledge of; (a) susceptibility of the drug to degradation and its degradation pathway; (b) assay interference by possible degradants or synthesis precursors; and (c) assay interference by chemicals employed in sample preparation and excipients in the formulation. Degradation products may be formed by acid/base hydrolysis, oxidation, Ultraviolet (UV) irradiation, heat, light, etc.

In the present study, initially, a reference standard of HBAE was chromatographed. Figure 2 clearly demonstrates that HBAE is well separated from any potential interference. Assay interference was investigated by injecting a placebo. No interfering peaks (Figure 3) were observed. Forced

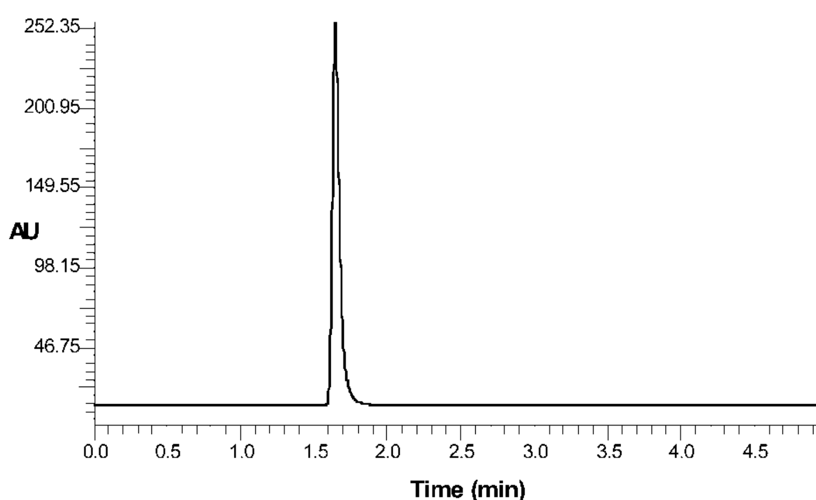

Figure 2. HPLC chromatogram of HBAE.

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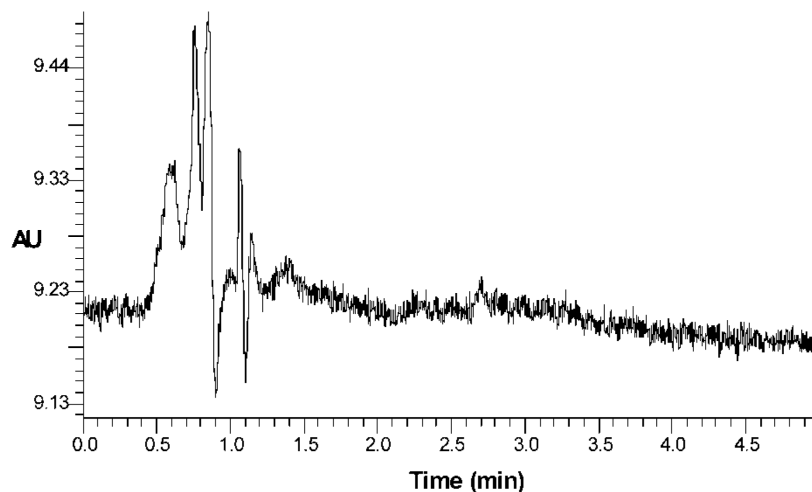

Figure 3. HPLC chromatogram of placebo.

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degradation studies were also performed to evaluate the specificity of HBAE under four stress conditions (heat, UV light, acid, base) (Table 3). Solutions of HBAE were exposed to 50°C for 1 h, UV light using a Mineralight UVGL-58 light for 24 h, acid (1 M HCl) for 24 h, and base (1 M NaOH) for 4 h. A summary data of the stress results is presented in Table 4, which showed no changes in retention times of HBAE and no degradation peaks were detected. The peak at 1.66 min is identified as that due to HBAE, since its UV spectrum matches that of a known sample of HBAE as shown in Figure 4.

Precision Studies

Precision is the measure of the degree of repeatability of an analytical method under normal operation, and is normally expressed as the percent relative standard deviation for a statistically significant number of

Table 3. Assay (%) of HBAE under stress conditions

Stress conditions	Sample treatment	RT (min) (HBAE)	Assay (%) (HBAE)
Reference	Fresh solution	1.66	99.78
Acid	1 M HCl for 24 h	1.65	99.71
Base	1 M NaOH for 4 h	1.65	99.80
Heat	50°C for 1 h	1.66	99.82
Light	UV light for 24 h	1.65	99.79

Table 4. Demonstration of the intermediate precision of the HPLC assay

Sample	HPLC1			HPLC2		
	S1 (50%)	S2 (100%)	S3 (150%)	S1 (50%)	S2 (100%)	S3 (150%)
Operator 1, day 1	99.84	99.80	99.77	99.75	99.82	99.82
Operator 1, day 2	99.77	99.75	99.75	99.81	99.79	99.77
Operator 2, day 1	99.70	99.76	99.76	99.76	99.76	99.70
Operator 2, day 2	99.54	99.62	99.59	99.78	99.81	99.80
Mean (HPLC1 & 2)	99.71	99.73	99.73	99.78	99.80	99.76
Mean (Operators)	99.80	99.79	99.78	99.70	99.73	99.71
RSD (criteria ≤2%) HPLC 1 & 2	HPLC1 S1 + HPLC2 S1 = 0.05; HPLC1 S2 + HPLC2 S2 = 0.06; HPLC1 S3 + HPLC2 S3 = 0.05					
RSD (criteria ≤2%) operators	HPLC1 S1 + HPLC2 S1 = 0.06; HPLC1 S2 + HPLC2 S2 = 0.04; HPLC1 S3 + HPLC2 S3 = 0.05					

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samples. Precision may be performed at three different levels: repeatability, intermediate precision, and reproducibility. Repeatability (intra-day assay precision) is the results of the method operating over a short time interval under the same conditions (intra-assay precision). 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. A precision criterion for an assay method is that the instrument precision (RSD) will be ≤1%, and for the impurity assay, at the limit of quantitation, the instrument precision (repeatability) will be ≤5%. Documentation in support of precision studies should include the standard deviation, relative standard deviation, coefficient of variation, and the confidence interval. In this study, precision of the method was evaluated through the repeatability of the method (intra-assay precision) by assaying ten replicate injections of HBAE at the same concentration (20 μg/mL), during the same day, under the same experimental conditions. The RSD values of the retention time, area, and height of HBAE peak were found to be <0.20% as shown in Table 1.

Intermediate precision (inter-day variation) is the results from lab variations, due to random events, such as different days, analysts, equipment, etc. In determining intermediate precision, experimental design should be employed, so that the effects (if any) of the individual variables can be monitored. Precision criteria for an assay method is that the intra-assay precision will be ≤2%, and for an impurity assay at the limit of quantitation, the instrument precision will be ≤5%, and the intra-assay precision will be ≤10%. In this study, intermediate precision (within-laboratory variation) was demonstrated by two operators, using two HPLC systems, and evaluating the relative percent purity data across the two HPLC systems at three

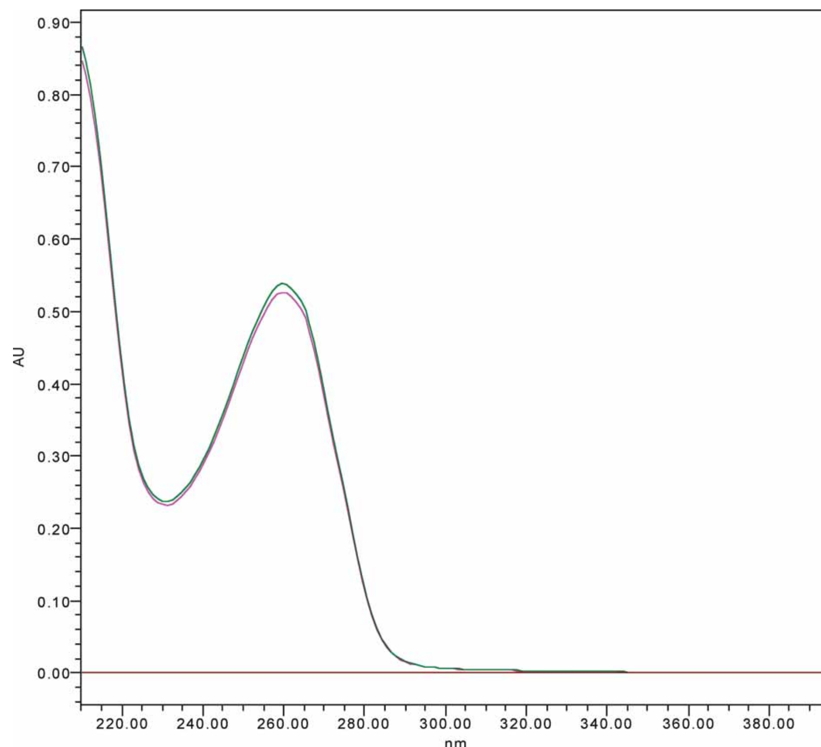

Figure 4. PDA UV match spectra of the middle of the peak corresponding to the RT of the main component HBAE and a reference sample.

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concentration levels (50%, 100%, 150%) that cover the HBAE assay method range (5.0–40 μg/mL). The mean and RSD across the HPLC systems and analysts were calculated from the individual relative percent purity mean values at 50, 100, and 150% of the test concentration. The RSD values presented in Table 4 were less than 1% for both HPLC systems and operators, and illustrated the good precision of the analytical method.

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% of the value obtained by the primary testing laboratory. For an impurity method, results obtained in multiple laboratories will be statistically equivalent, or the mean results will be within 10% (relative) of the value obtained by the primary testing lab for impurities. Reproducibility is not normally expected if intermediate precision is performed.

Limit of Detection and Quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) tests for the procedure are performed on samples containing very low concentrations of analyte. LOD is defined as the lowest amount of analyte that can be detected above baseline noise; typically, three times the noise level. LOQ is defined as the lowest amount of analyte, which can be reproducibly quantitated above the baseline noise, that gives s/n = 10. In this study, LOD for a 20 μL injection of HBAE standard (s/n = 3.3) was 2.5 ηg/mL (Figure 5, Table 1) and LOQ (s/n = 10.2) was 5.5 ηg/mL (Figure 6, Table 1) and RSD <2% ($n = 6$).

CONCLUSIONS AND SUGGESTIONS FOR FURTHER WORK

The efficient analytical method validation is a critical element in the development of pharmaceuticals. Indeed, the principle of the validation of these methods is today widely spread in all the domains of activities where measurements are made. Nevertheless, the simple question of acceptability, or not, of an analytical method for a given application, remains incompletely determined in several cases, despite the various regulations relating to good practices (GLP, GMP, ...) and other documents of normative character (ICH, USP, FDA, ...). There are many official documents describing the criteria of validation to be tested, but they do not propose any simple and systematic approach to experimental/validation activities and limit themselves most often to the general concepts.

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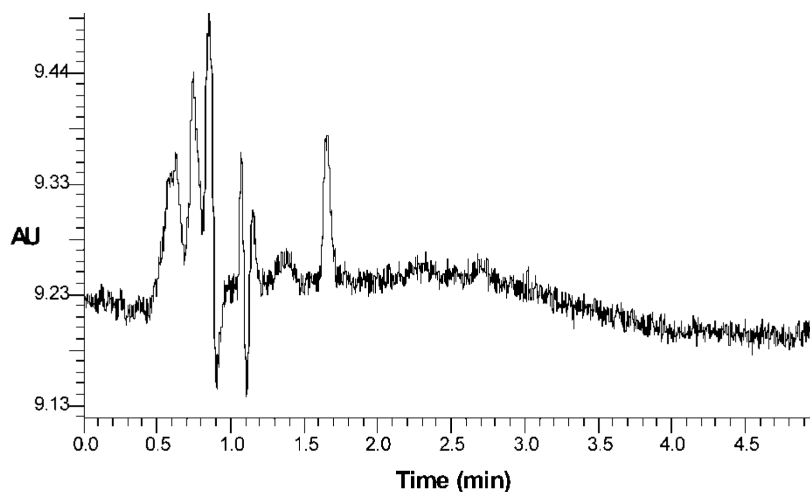

Figure 5. HPLC chromatogram for limit of detection of HBAE. Sample concentration 2.5 ng/mL.

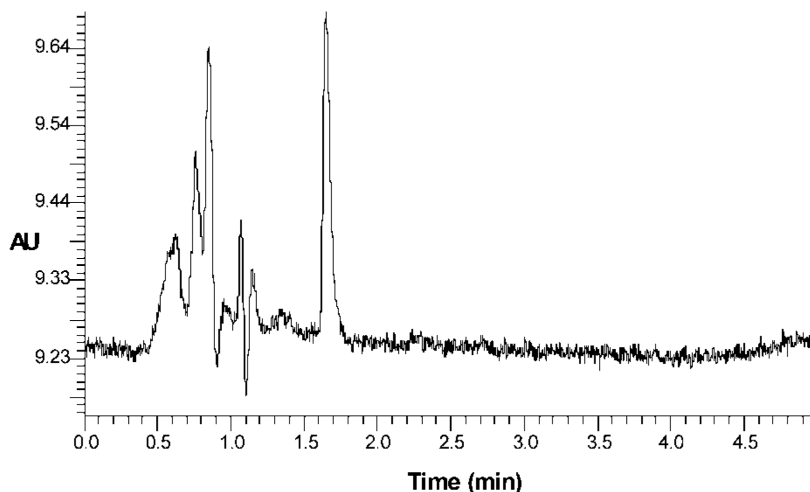

Figure 6. HPLC chromatogram for limit of quantitation of HBAE. Sample concentration 5.5 ng/mL.

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The concept of validation covered in the literature is mostly associated with development and validation of chromatographic methods. The description of equipment qualification is also discussed in the literature to a lesser extent.[33–35] However, the description of instrument qualification generally does not include the need to validate the computer aspect of the instrument (software and computer hardware), which should be considered an important part of the qualification package prior to method validation.

Another one of the critical issues that have not been addressed by the consensus reports is when method validation is necessary. In the current highly cost conscious environment, the balance of costs and benefits is an issue. The literature and regulatory agencies contain diverse approaches to performing method validation as discussed above; there is a need for a single guideline worldwide on performing method validation. ICH should expand their effort on method validation globally for more input and set the minimum standard "one world–one standard," which ensures patient safety. Alternative guidelines to ICH are not preferred; the world should stay with ICH to achieve global harmonization. This is because it is easier to revise existing guidelines that are already in operation. The guideline should cover step by step approaches from drug development to marketing authorization. The guideline should also cover all the prevalidation requirement activities, such as analytical instrument qualification that is another aspect of method validation. The benefits to the regulated industry of achieving the desired state (globally harmonized) will ensure, better quality, less recalls, less supplements, and facilitate new technology and continuous improvement. Focus will be on critical quality attributes and controls and will reduce the

regulatory burden of post approval changes. The benefits to the regulators are that they will be receiving relevant information concerning product understanding, allow consideration of product design, critical process control, and critical quality attributes, etc., for regulatory decision and, hence, reduce the burden upon regulatory resources. USP and FDA have accepted ICH documents and have updated general chapters, but old methods do not meet the criteria (e.g., TLC) and are currently not being updated. A major challenge to many pharmaceutical industries of today who still use old validated methods is that they need to upgrade/revalidate in order to meet current regulatory standards. The USP28-NF23 contains over 4000 monographs and over 180 general chapters. Approximately 200 drug substances, excipients, and drug product monographs are needed. Approximately 800–1200 current monographs need to be updated. USP works with the European and the Japanese Pharmacopoeias to harmonize excipients, monographs, and general chapters. The goal is to achieve regulatory interchange ability.

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Current Status of Compendial Monographs/Specifications

Monograph Development

Anyone can be a sponsor of a monograph. The sponsor develops and submits monographs to USP based on their company's timeline. Most of company's timelines are approximately three years prior to patent expiry. The monograph typically resembles the specification and methods, which are filed and approved by the FDA. The USP submits the monograph to the appropriate expert committee for review.

Monograph Revision

Monographs in the USP are "live" documents and are constantly being revised. Anyone can submit a revision to an official monograph. Limits are continually being changed, tightened, and/or widened.

Challenges of the Current System

The USP expert committee may challenge the specifications and methods in the monograph submission. Methods may be changed, this presents a problem since limits and methods are linked. Despite the fact that the methods and specifications are reviewed and approved by the FDA, the expert committee may ask for additional information to justify the proposed specifications and methods.

Consideration for the Future

So . . . where are we going? Should we start with a blank piece of paper and develop a “desired state” for pharmacopoeias and the public standards? Does it make sense to retrofit for compendial standards from 1820 to the “21st century? The testing of compendial standards and the specification process has remained nearly unchanged. Adoption for the future is a must and reassessment, reevaluation, and evolution is necessary. It is critical for the USP to be engaged with the changes and paradigm shift occurring at FDA. Specifications must be based on FDA approved materials. The purpose of a monograph needs to be reassessed. The role of the monograph in release, stability, and marketing surveillance needs to be reevaluated.

Evolution and Application of General Chapters

How will they be impacted by the changes and be applied (enforced) in the future? How will general chapters be developed to adapt to new paradigm and existing chapters be modified? Will there be dual standards in the USP to accommodate the new approaches, and how will content uniformity be addressed? Should it be addressed in the USP, or should the current chapter remain and companies left with the option of different specifications/ methods based on agreements with the FDA? Chapters should not duplicate efforts underway in other areas (e.g., American Society for Testing and Materials (ASTM) International Standards).

Global Harmonization

The path that the USP takes must be carefully considered. Since the USP has been engaged with the Pharmacopoeial Discussion Group (PDG) on harmonization of general chapters and monographs, it is important to move forward in collaboration with the other Pharmacopoeias and not be in isolation. We must not “undo” the work that has been done by the PDG. The USP should work with the PDG to ensure that the current harmonized items are not negatively impacted and also work prospectively to harmonize new concepts.

Also, input from the pharmaceutical industry and other users of their volumes, is, therefore, essential in the provision of information as to what is most needed in the prioritization and harmonization of the work programs. In a continually changing environment, the PDG looks to industry to produce suggestions as to what issues need to be addressed in the formulation of its work programs. Industry professionals are, therefore, urged to take a keen and active interest in the work of the PDG, to monitor its progress by

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reading the appropriate forums and to let it know where major problems are occurring.

In Overall Conclusion

The status quo is no longer adequate, evolution is necessary. In order to prepare for the future, we must now critically evaluate the role of monographs and general chapters and consider what changes must occur. These must be linked to the changes occurring at the FDA and industry. The USP must engage the FDA, industry, and PDG in the evolution process.

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