A REVIEW ON BIOANALYTICAL METHOD DEVELOPEMENT FOR PHYTOCONSTITUENTS

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ABSTRACT:

Medicinal plants provide a wealth of information about and experience with treating a wide range of illnesses, thanks to their biologically active components and medicinal characteristics. The physiologically active molecules found in plants and plant products, such as vitamins, fibre, antioxidants, and chemicals that decrease cholesterol, constitute an integral aspect of a healthy human diet. There is a lot of data on this issue, but the nutritional value of plants is still not established. In the past, many nutrients found in plants and substances that improve health were found to have worth via trial and error. By the beginning of century, many plant metabolites could be quantitatively and qualitatively measured thanks to the use of nuclear magnetic resonance, infrared spectroscopy, and chromatography. Plants have long been employed by Ayurvedic practitioners to treat a wide range of diseases. Consequently, following Ayurvedic assertions on plants, scientists are now concentrating on studying and describing different plants and plant components for their potential to treat various ailments. The challenges of element separation, identification, and quantification in complex plant extracts are not going away any time soon. The good news is that there is a vast array of detectors, specialist stationary phases, and separation methods available today that can help you overcome any separation difficulty with the right combination of speed, sensitivity, and selectivity. Numerous analytical methods have been established for the purpose of identifying and quantifying phytochemicals; these methods include chromatography, hyphenated techniques, capillary zone electrophoresis, and spectrophotometric approaches.

KEYWORDS: Medicinal plants; HPLC; Review; Flavonoids; Phenolic acids; Terpenes; Terpenoids; Carotenoids; Alkaloids; Coumarins; Alkamides; Polyacetylenes

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INTRODUCTION:

Bioavailability, bioequivalence (BE), pharmacokinetics (PK), quantitative evaluation of drugs, concentrations, and metabolites; new drug development; basic pharmaceutical & biotechnology science research; therapeutic drug monitoring; and many more fields rely on methods to determine the concentration of drug in biological fluids. [1,2] In many different types of sectors, including those dealing with food, medicine, the environment, and forensics, high-pressure liquid chromatography (HPLC) is utilised as an analytical tool due to its reliability and great selectivity. [3]. Bioanalytical method validation was the subject of 2001 recommendations from the USFDA and more recent proposals from the EMEA. In order to prove a method is suitable and dependable for its envisioned bioanalytical uses, it must first be validated by conducting specific laboratory investigations. How valid the validation procedure is determines the acceptance of the analytical data. Essential investigations requiring regulatory action for consent, studies like BE or PK, may need comprehensive verification of the bioanalytical methodologies. When it comes to advance approaches utilised for sponsor's internal decision-making, authentication may not be as strict. Though, whenever previously validated technique is modified, more authentication might be required. [4,5]

To determine the analytical method's appropriateness, it is necessary to assess the effects of the many changes that are often implemented. The necessary changes that have evolved over time to back up specific investigations for diverse phases of validation in order to prove validity of the approach.

Bioanalytical technique validation is performed while:

- Create and execute an innovative bioanalytical technique.
- With the purpose of studying an innovative medicinal substance.
- Adjustments to a current method for metabolite measurement.[6]
- Sharing bioanalytical procedures across different labs or analysts.
- The analysis procedure has been changed.
- Biological matrix changes (from human plasma to urine, for example).
- Changes to procedures for processing samples [7]

Because accurate interpretation of toxicological findings as per dependability of analytical data, this is an significant concern in clinical & forensic toxicology. If the findings aren't trustworthy, the defendant might face unwarranted legal consequences or the patient could get the wrong therapy. Therefore, validation is crucial, especially for standard analytical processes. The importance of quality management and certification has grown int analytical toxicology in last several years, and is particularly relevant in this context.

Rising demands for method validation in peer-reviewed scientific papers are a reflection of this trend as well. Forensic (and clinical) toxicologists should engage in robust worldwide discussion over this issue until they reach a consensus on the parameters for bioanalytical technology validation trials and the parameters that should be accepted. Pharmacokinetic (PK) research for pharmacological registration has been the site of comparable discussions for the better part of a decade. [8]

1. NEED OF BIONALYTICAL METHOD VALIDATION

Bioanalytical methods and processes are generally considered to be state-of-the-art because of their constant development and improvement. Importantly, the characteristics of each bioanalytical method differ depending on the analyte. This calls for the potential necessity to establish analyte-specific validation criteria. The end purpose of the research may also change the approach's suitability. In order to achieve inter laboratory reliability, it’s essential to analyse bioanalytical method(s) at every site and give suitable validation info for various sites when undertaking sample analysis at more than one location for a single project. [9]

2. BIONALYTICAL METHOD DEVELOPMENT AND VALIDATION

There are essentially three phases to an analytical method's life cycle: development, validation, and implementation.

Get the reference standards ready, validate them, and analyse samples regularly.

Bioanalytical method and assay technology development; and

Using established bioanalytical procedures for the analysis of common drugs and establishing run/batch approval criteria.[10]

3. IMPORTANT PUBLICATIONS ON VALIDATION (1991 TO PRESENT)

To help bioanalytical chemists, Karnes et al. researched on bioanalytical procedure validation in 1991.11 After a year, the "Analytical Methods Validation: Bioavailability, Bioequivalence, and Pharmacokinetic Studies" meeting in Washington, DC was documented in a report by Shah et al. (meeting Report). 12 Specific acceptance criteria

and the parameters of bioanalytical processes to be assessed were agreed upon during this conference. Researchers in the field of bioanalysis looked to this study for direction in the years that followed. Although this seminar covered a lot of ground, no one really came up with any solutions to real-world problems like experimental designs or statistical assessment. In 1994, the Conference Report was subjected to statistical experiments by Karnes HT,mann et al. about the stipulated acceptance standards for precision & accuracy. 13 Their research led them to doubt these standards could be used in real-world scenarios. Published between 1995 and 1997 were many articles by Dadgar et al. 20, 15, Wieling et al. 16, Bressolle et al. 17, and Causon 18, all of which dealt with practical issues related to bioanalytical technique validation, including experimental designs and statistical methodologies. In a superb 1998 review, Hartmann et al. addressed both the theoretical and practical challenges of validating bioanalytical chromatographic methods. 19 Lastly, during the Washington conference update, everyone got together to talk about what's happened since the last meeting. In a subsequent publication (Conference Report II) 20, Shah et al. reiterated the findings; the FDA later used this work as a basis for their own regulations. 21 Additionally, clinical chemistry and Journal of Chromatography B 22 are among the journals that have developed their own standards for validation. Although their main focus is on pharmaceutical product analysis rather than bioanalysis, they do provide helpful background on certain important issues and ideas pertaining with validation of analytical approaches. Approved in 1994 & 1996, respectively, the former included theoretical context and terminology (number 23) and methodology and practical considerations (number 24). Any one of them may be downloaded for free from the ICH website. Goal of this review is to summarise and evaluate studies on validation of (bio)analytical techniques that have been previously cited, and to discuss their possible effects on clinical and forensic toxicology.

4. TERMINOLOGY

While surveying the literature on method validation, the first problem that emerged was the wide variety of language employed by the authors. This topic is well covered in two publications by Hartmann et al. [13]. The assessment 19 pushed for a general conformity to the ICH 23 nomenclature, with the exception of accuracy, which called for a more specific definition (cf. 4.3.). Nevertheless, stability—an essential quality for validating bioanalytical methods—was not defined in the ICH terminology. Additionally, any bioanalysis interferences (e.g., from metabolites) are not taken into consideration by the ICH definition of selectivity. Both criteria, however, were appropriately defined in Conference R. Shah Report II. [12]

5. VALIDATION PARAMETERS

Everyone agrees that quantitative techniques need to have their stability, accuracy (bias, precision), limit of quantification, selectivity, calibration model, and calibration examined as part of their validation process. Additional qualities to think about are robustness, repeatability, detection limit, and recovery. [11, 13, 20, 17]

Table 1: US FDA guidelines for bioanalytical method validation [22]

Bioanalytical validation methods	**US FDA guidelines**
Selectivity	Blank samples of relevant biological matrix must be collected from a minimum of 6 different locations for analysis. It is important to check the interference and selectivity of each blank at the LLOQ level.
Accuracy	Six separate measurements per concentration are required for accurate measurement. For the purpose of determining accuracy, it is advised to use minimal of 3 concentrations within predicted concentration range. A deviation of up to 20% is acceptable for LLOQ, but otherwise mean must be within ±15% of actual value. The degree of accuracy is determined by how far the mean values are from the actual values.
Precision	In order to ensure accuracy, it is recommended to do at least five determinations per concentration. Three concentrations within the estimated concentration range should be considered at least. With the exception of the LLOQ, where the precision should not surpass 20% of the CV, the CV should not be more than 15% across all concentration levels.
Recovery	Experiments on recovery should be conducted at lower, medium & higher concentrations using unextracted standards which stand in for 100% recovery.

Calibration curve	All samples, including the blank and zero samples, should be processed using a matrix that does not include any internal standards. The anticipated range, including LLOQ, should be covered by six to eight non-zero samples.
LLOQ	The reaction to analyte should be five times that of a blank response. An identifiable, distinct, and repeatable analyte peak with a 20% precision & accuracy of 80-120% is required.
Freeze–thaw stability	3 freeze-thaw cycles must be used for assessing stability of analyte. Thaw at room temperature after storing at the recommended temperature for 24 hours; separate aliquots for low and high concentrations. Refreeze for another 12 to 24 hours when fully thawed. Before analysing the third cycle, this cycle has to be done twice more. The third cycle should include analysis of the standard deviation of error after two more repetitions.
Short-term stability	After defrosting at room temperature, three portions of lower & higher concentrations must be maintained at room temperature for four to twenty-four hours before analysis. It should not exceed 15% in terms of percentage variation.
Long-term stability	Subjected to the identical circumstances as the research samples, at least three aliquots of each concentration were taken. Examine in three different instances. Make sure the storage duration is longer than the time it takes to go from collecting the first sample to analysing the final one.
Stock-solution stability	A minimum of six hours at room temperature is required to assess stability of medication stock solutions in comparison to internal standard. It should not exceed 15% in terms of percentage variation.
QC samples	At each test run, QC samples should be included in triplicate at 3 different concentration levels: one around 3× LLOQ, one in the mid-range, and one close to the high end. In each case, at least 4 out of 6 should fall within a 15% margin of the nominal value. Any two of the six might be beyond the 15% range, but they can't be at the same concentration. Either six QCs in total or five percent of the total unknown samples should be considered minimum.

6. IMPORTANT ASPECT OF PHYTOCHEMICALS

a. Major procedures of structural determination

The basic procedures for determining the structures of phytochemicals are shown in Figure 1. Researchers' routines, expertise, and access to relevant technologies may greatly facilitate the structural identification of phytochemicals. [25]

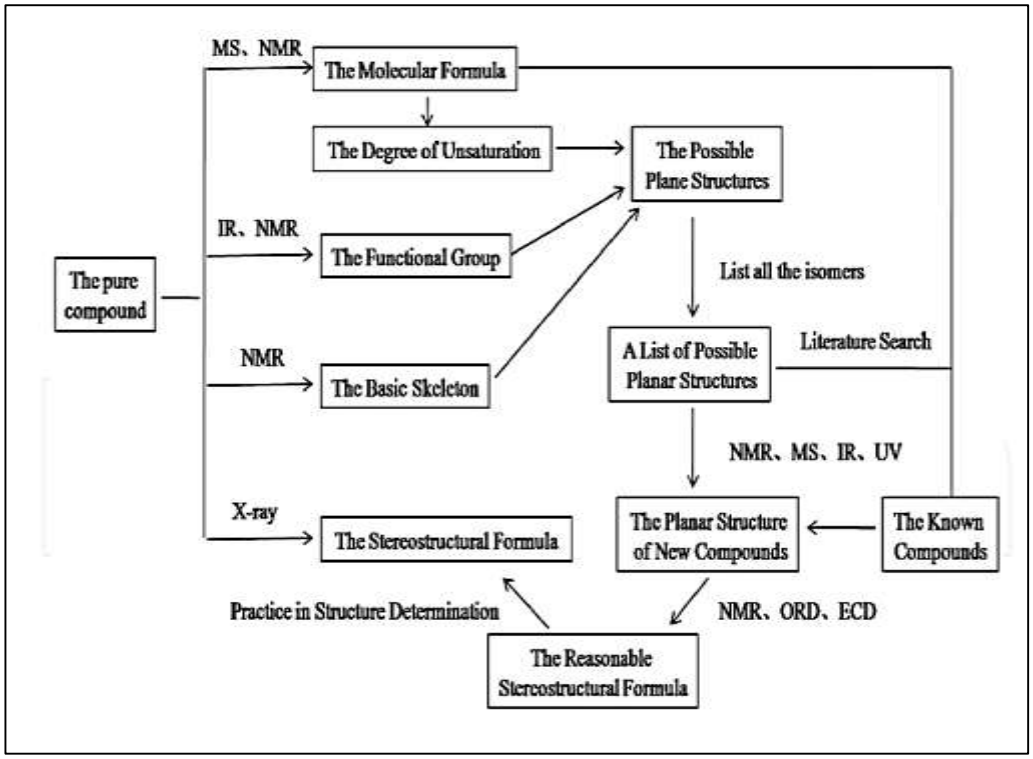

Fig 1: Procedure for studying the structure of phytochemicals

b. EXTRACTION PROCESS

Maceration, infusion, percolation, digestion, decoction, hot continuous extraction (Soxhlet), sonication, phytonic extraction, and extraction using supercritical fluid are some of the common methods used to extract medicinal plants from their roots. These methods typically use hydrofluorocarbon solvents. Aromatic plants may be extracted using hydrodistillation techniques, hydrolytic maceration with subsequent distillation and expression, or enfleurage. A few examples of modern methods for extracting aromatic compounds from plants include molecular distillation, microdistillation, thermal microdistillation, protoplast extraction, headspace trapping, and solid phase micro-extraction.[26]

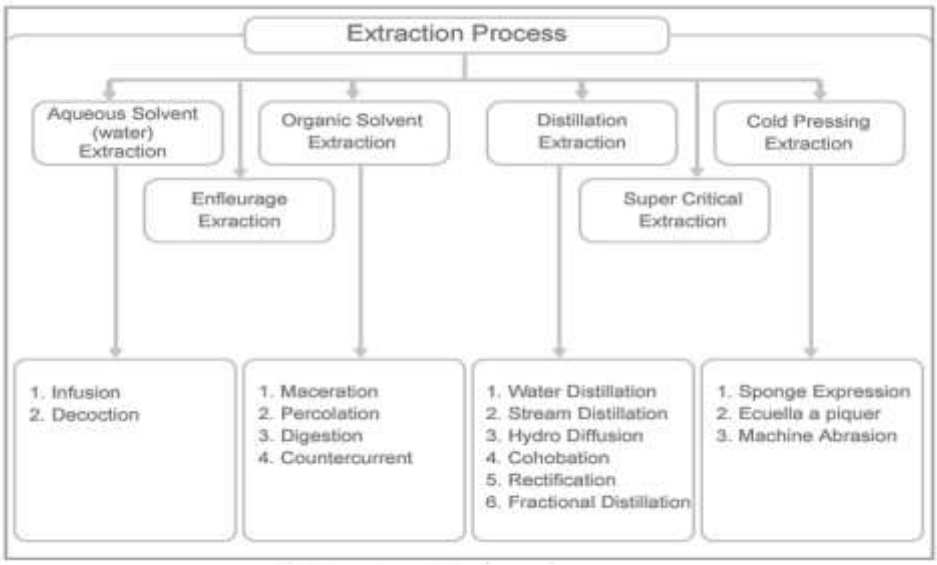

Fig 2: method of extraction process

Table 2: Solvents used for active component extraction [27]

Water	Ethanol	Methanol	Chloroform	Ether	Acetone
Anthocyanins	Tannins	Anthocyanins	Terpenoids	Alkaloids	Phenol
Starches	Polyphenols	Terpenoids	Flavonoids	Terpenoids	Flavonols
Tannins	Polyacetylenes	Saponins		Coumarins	
Saponins	Flavonol	Tannins		Fatty acids	
Terpenoids	Terpenoids	Xanthoxyllines			
Polypeptides	Sterols	Totarol			
Lectins	Alkaloids	Quassinoids			
		Lactones			
		Flavones			
		Phenones			
		Polyphenols			

Table 3: Structural features and activities of various phytochemicals from plants [28]

Phytochemicals	Structural features	Example(s)	Activities
Phenols and Polyphenols	C_3 side chain, - OH groups, phenol ring	Catechol, Epicatechin, Cinnamic acid	Antimicrobial, Anthelmintic, Antidiarrhoeal
Quinones	Aromatic rings, two ketone substitutions	Hypericin	Antimicrobial

Flavoes Flavonids Flavonols	Phenolic structure, one carbonyl group Hydroxylated phenols, C_6-C_3 unit linked toan aromatic ring Flavones + 3-hydroxyl group	Abyssinone Chrysin, Quercetin, Rutin Totarol	 Antimicrobial Antidiarrhoeal
Tannins	Polymeric phenols (Mol. Wt. 500-3000)	Ellagitannin	Antimicrobial, Anthelmintic, Antidiarrhoeal
Coumarins	Phenols made of fused benzene and α-pyrone rings	Warfarin	Antimicrobial
Terpenoids and essential oils	Acetate units + fatty acids, extensivebranching and cyclized	Capsaicin	Antimicrobial Antidiarrhoeal
Alkaloids	Heterocyclic nitrogen compounds	Berberine, Piperine, Palmatine, Tetrahydropalmatine	Antimicrobial, Anthelmintic, Antidiarrhoeal
Lectins and Polypeptides	Proteins	Mannose-specific agglutinin, Fabatin	Antimicrobial
Glycosides	Sugar + non carbohydrate moiety	Amygdalin	Antidiarrhoeal
Saponins	Amphipathic glycosides	Vina-ginsenosides-R5 and -R6	Antidiarrhoeal

7. RECENT ADVANCES IN BIO-ANLYTICAL TECHNIQUES FOR PHYTOCHEMICALS

HPTLC Analysis of Extract:'

Table 4. HPTLC Analysis of Medicinal Plants.

Sample	Matrix	extraction element	Chromatographic conditions	Detection	RT (in Min)	Reference
Syzygium cumini seed	Extraction	Ethanol	toluene: ethyl acetate: formic acid (6:6:1.5v/v/v)	271 nm	0.47 ±0.02	[67]
Abrus precatorius, Phyllanthus maderaspatensis, Nymphaea alba Linn	Extraction	Methanol	Toluene: ethyl acetate: formic acid: methanol (3:3:0.8:0.2 v/v/v)	280 nm	0.46	[68]
Woodfordia fruticosa	reflux	HPLC water	toluene: chloroform: ethyl acetate: formic acid (2:6:6:2)	254 nm, 366 nm	-	[69]
Potentilla species	extraction	Methanol	Toluene: ethyl formate: formic acid (6:4:1 v/v/v)	254 nm	-	[70]
Rosa hybrida	Extraction	-	CAN:H2O:HCOOH (50:50:5)	270 nm	-	[71]

HPTLC Analysis of Ayurvedic / Traditional formulation:

Table 5: HPTLC Analysis of Ayurvedic formulation

Ashwagandharishta	decoction	Methanol	Toluene: ethyl acetate: formic acid: methanol (9:9:3:0.6 v/v/v/v)	285 nm	0.46 ± 0.02	[72]
Triphala churnam	Extraction	Methanol	Toluene: Ethyl Acetate: Formic Acid: Methanol (3:3:0.8:0.2 v/v)	280 nm	0.47	[73]
Dhatrinisha churna	Extraction	methanol	toluene: ethyl acetate: methanol: formic acid (16: 14: 1: 4 v/v)	330 nm	-	[74]
Arjunarishta	Decoction	methanol	Toluene: ethyl acetate: formic acid: methanol 9:9:3:0.6 (v/v)	285 nm	0.46	[75,75]
Manjisthadi churna	Extraction	Methanol	toluene: ethyl acetate: methanol: formic acid (10:9:6:5 v/v)	280 nm	0.72	[77,78]

HPLC Analysis of Herbal Product:

Table 6: HPLC Analysis of Ayurvedic formulation

Roots of Salacia species, leaves of Lagestroemia parviflora and fruit rind of Garcinia indica	Extraction	methanol	Phenomenex C18 column (250mm × 4.6mm id, 5µm pore size)	A: acetonitrile B: buffer solution (0.03% v/v Phosphoric acid) 45:55 v/v	SPD-M20A - PDA	6.09	[93]
Strawberry jam, Blueberry jam and Raspberry jam	Decoction	homoge-nized in methanol	L-column ODS (5 µm, 250×4.6 mm id)	5 mM potassium dihydrogen phosphate solution (pH 2.5)-acetonitrile (41:9 v/v)	DAD	-	[94]
Muscadine grapes	(Acetone: H2O: acetic acid, 70:29.7:0.3, v/v)	-	Zorbax Stablebond Analytical SB-C18 column (4.6 mm ×250 mm, 5 µm	A (0.5% formic acid aqueous solution) and mobile phase B (methanol)	DAD	-	[95]

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CONCLUSION

Degradation of plant phytochemicals and subsequent variation in extraction methods can make reproducibility in the results impossible. It is important to establish and adhere to the optimum extraction methods in order to produce batches with consistently high quality, ideally within the narrowest practical range.In this study, we covered the many extraction methods and bioanalytical techniques that are now accessible for the analysis of phyto-constituents found in herbs.

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