CHROMATOGRAPHY METHOD USE IN HERBAL DRUG STANDARDISATION

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ABSTRACT
Standardisation is a process by which it is confirm that products are true beyond any doubt in respect of quality, efficacy, performance and safety. Importance of standardization is its chemical values of the individual drugs in the finished products can be used as preliminary reference standards for market samples of the single drugs. Awareness and general acceptability of the use of herbal drugs in today’s medical practice become greater in size worldwide. For the standardization, the extracts are subjected to chemical test, physical studies (ash and extractive values, pH, reducing sugar, alcohol content and etc.) and chromatographically studies (TLC, HPTLC and HPLC etc.). Heavy metal contamination also determined in the formulation by atomic absorption spectrometer (AAS). Stakeholders in herbal medicine require establishing quality parameters for collection, handling, processing and production of herbal medicine and parallel employing such parameters to make certain the safety of the global herbal market. The processes of good quality assurance and standardization of herbal medicines and products were also discussed. To achieve the authentic herbal drug chromatography is one of the key factors for that. Herbal formulations have reached extensive acceptability as therapeutic agents for several diseases we hope our research findings will help the Ayurveda industry for formulating/marketing standard drug with international acceptance.

KEYWORDS: Herbal medicine, Standardization, Quality control and Chromatographic technique.

INTRODUCTION
Long established herbal medicine and their preparations have been mostly used for the thousands of years in developing and developed countries owing to its natural origin and minimum side effects or dissatisfaction with the results of synthetic drugs. As pointed in “General Guidelines for Methodologies on Research and Evaluation of Traditional Medicines (World Health Organization, 2000)”, “In spite of its alive and continued use over July - September
many centuries, and its admired and broadly use during the last decade, such medicine has not been officially recognized in most countries. As a result, education, training and research in this field have not been granted due consciousness and support. The numeric and standard of the safety and potency data on traditional medicine are far from sufficient to meet the criteria needed to support its use worldwide. The cause for the lack of research data are due to not only to health care policies, but also to a lack of sufficient or accepted research methodology for evaluating traditional medicine” (WHO, 2000, 2001).

The idea of standardisation and quality control of drugs can be found even in the ancient Ayurveda texts. In those days, the physician himself would ensure the raw drugs by their typical taste, colour, smell, shape and texture, prepare the medicines. But in the recent time these tests are not sufficient to give scientific explanation and quality control. The W.H.O. also has been stimulating and up lifting the traditional herbal medicines in health care programs. Therefore the standardization of the raw drugs, processing, finished products, endorsement of the claims, role of action and purity from metallic and microbial contamination are few of the major concern which have to be taken in to consideration for up lifting the world wide acceptability of herbal products and also to gain clinical success and major the therapeutic effect. Standardization through organoleptic characters and physio-chemical properties like colour, odour, taste, specific gravity, pH value, total solids, reducing sugar, non-reducing sugar, total acidity, phyto-chemical screening along with chromatographic like thin layer chromatography (TLC), High performance thin layer chromatography (HPTLC), and High performance layer chromatography (HPLC) and UV-visible spectrum, Atomic absorption spectrum (AAS) were also carried out. Except from these, the total alkaloid content and polyphenol content were also estimated in the formulation. Herbal drugs regulations in India as well as an overview of regulatory status of herbal medicine in USA, China, Australia, Brazil, Canada and Germany has been reported. According to WHO guidelines, an herbal product requires to be standardized with concern to safety before releasing it into the market. To prolong consistent in quality in herbal preparation, both from batch to batch and over time is as problematical as it is necessary and has drawn serious attention recently as challenging analytical task small scale and large scale producers of herbal products are proceed large numbers of Ayurveda proprietary medicine. Whole world has high market potential in future standardization of such medicine by advanced analytical techniques is the most essential tool for quality assurance of the same. In analytical the quantitative and qualitative determination by spectrometry and chromatography will be a choice of method development of two active marker compounds. Such proposed work with its assurance of quality of such products in herbal industry is currently having great significance. As from literature there is not reported any precise and economic simulation estimation method for Azadirachtin and Gymnemic acid by UV and HPLC, Therefore it is very interesting if scientists are focusing to develop a suitable method for the same, which gives high degree of assurance with better strength, identity and purity of both the compounds. UV and HPLC/UPLC method development and validation is important tool in analytical area. Hence in herbal analysis simultaneous estimation of herbal constituents in formulation is very important and interesting.

**CONVENTIONAL METHODS FOR STANDARDIZATION OF CRUDE DRUG**

Standardization is just like a passport for the herbal drug. For this conventional it covers parameter like medico- botanical survey, identification, botanical authentication, macroscopic, examination. Performing testing of drugs as per approved Pharmacopoeial testing protocol-fully pharmacognostical profile, Identification by various chromatographic techniques, appraisal of purity by physico-chemical profile. Evaluation of strength by active marker or assay estimation and safety by heavy metal profiling, microbiological limit test
analysis, aflatoxins analysis, pesticides residue and biological activity. Macroscopic identity of medicinal plant materials is based on sensory evaluation parameters like shape, size, colour, texture, odour and taste while microscopy involves comparative microscopic inspection of powdered herbal drug. Further, advances in microscope technology have increased the accuracy and capabilities of microscopy as a mean of herbal crude material identification due to the implication of light and scanning electron microscopes (SEM) in herbal drug standardization. The phytochemical assessment for standardization purpose includes the following- Preliminary testing for the presence of different chemical groups, quantification of chemical groups of interest (e.g., total alkaloids, total phenolic, total triterpenic acids, total tannins), establishment of fingerprint profiles, multiple marker-based fingerprint profiles and quantification of important chemical constituents.

**Analytical technique use in herbal standardisation**

In industry quality control worked mainly based on three important pharmacopoeias definitions:

Identity: Is the herb the distinction?

Purity: Confirm there contaminants, e.g., in the form of other herbs which should not be there?

Content or assay: Quantitative determination of active constituents within the defined limits.

It is prominent that the content is the most critical one to finalize, because in maximum herbal drugs the active constituents are unknown. Occasional markers can be employed which are, chemically defined constituents that are of interest for control purposes, independent of whether they have any therapeutic activity or not. To found to be identity and purity- criteria such as type of preparation, sensory properties, physical constants, adulteration, contaminants, moisture, ash content and solvent residues have to be confirmed. The true originality of the crude herbal material, or the botanical quality, is of first importance to build the equality control of herbal drugs. In preliminary stage identity can be gained by macro- and microscopically examinations. For this voucher specimens are authentic reference sources. Eruption of diseases among plants may outcome in changes to the physical appearance of the plant and lead to wrong identification. High percentage of purity is closely associated with the safe use of drugs and deals with parameter such ash values, contaminants (e.g. foreign matter in the form of other herbs), and heavy metals. Although, due to the application of modern analytical methods, modern purity evaluation includes microbial contamination, aflatoxins, radioactivity, and pesticide residues is confirmed. Analytical methods like spectrometric-such as photometric analysis (UV, IR, MS, and NMR), and chromatography-Thin layer chromatography (TLC), High performance Thin layer chromatography (HPTLC), High performance liquid chromatography (HPLC), and Gas chromatography (GC) can be employed in order to organise the constant composition of herbal preparations. Quantitative analysis which is confirming the content is the most problematic area of quality control to perform, since in most herbal drugs the active constituents are not known. Sometimes markers can be used. In all other cases, where no active constituent or marker can be defined for the herbal drug, the percentage extractable matter with a solvent may be used as a form of assay, an approach often seen in pharmacopeia. The option of the extracting solvent depends on the nature of the compounds involved, and might be deduced from the tradition values. A special form of assay for the determination of essential oils is by steam distillation. When the active constituents (e.g. Senno sides in Senna) or markers (e.g. alkyd amides in Echinacea) are known, a vast array of modern chemical analytical methods such as ultraviolet/ visible spectroscopy (UV/VIS), TLC, HPLC, GC, mass spectrometry (MS), or a combination of GC and MS (GC/MS), can be employed.
CHROMATOGRAPHY TECHNIQUES IN HERBAL DRUG IDENTIFICATION AND CHARACTERIZATION

Thin layer chromatography (TLC)

TLC was the appropriate, versatile method of choice for herbal analysis before, Instrumental chromatography methods like GC and HPLC were established. Yet TLC is regularly used for the analysis of herbal medicines because several pharmacopoeias such as Indian herbal pharmacopoeia (IHP), Ayurveda pharmacopoeia (AP), American Herbal Pharmacopoeia (AHP), Chinese drug monographs and analysis (CDMA), Pharmacopoeia of the People’s Republic of China (PPRC), etc. Because, TLC is used as a simple method of first screening with a semi quantitative evaluation together with other chromatographic techniques as there is relatively less change in the simple TLC separation of herbal medicines than with instrumental chromatography.

Thin-layer chromatography is a technique where a solute undergoes distribution between two phases, a stationary phase acting mechanism of separation through adsorption and a mobile phase in the form of a liquid. The adsorbent is a relatively thin, uniform layer of dry finely powdered material applied to a glass, plastic or metal sheet or plate. Glass plates are most commonly used. Separation may also be performed on the basis of partition or a combination of partition and adsorption, which are depending on the particular type of support that is solubility of the active constituents in given solvent, its preparation and its use with different types and composition of solvent. Identification can be effected by observation of spots of identical Rf value and about equal magnitude obtained, respectively, with an unknown and a reference sample chromatographed on the same plate. A visual comparison of the size and intensity of the spots usually serves for semi-quantitative estimation. TLC has the advantages of many-fold possibilities of detection in analysing herbal medicines. The use of TLC to analyze the herbal medicines is still popular.

Gas chromatography (GC)

Gas chromatography (GC), also known as gas liquid chromatography (GLC), Its principle for separation is partition in which separation of mixtures into components by a process which depends on their distribution of the components between a stationary phase or support material in the form of a liquid, solid or combination of both and a gaseous mobile phase. It is well-known that many pharmacologically active components in herbal medicines are volatile chemical compounds. Thus, the analysis of volatile components by gas chromatography is best option in the analysis of herbal medicines. The GC analysis of the volatile oils has a number of benefits. Firstly, the GC for the volatile oil gives a reasonable “fingerprint” which can be employed to find out the source of the plant. The composition and adequate concentration of the organic compounds in the volatile oil are specific of the particular plant and the availability of impurities in the volatile oil can be readily found out. Secondly, the extraction of the volatile oil is relatively straightforward and can be standardized and the components can be more accurately quantify using GC-MS analysis. The proper quantities of the components can be employed to monitor or assess certain characteristics of the herbal medicines. Changes in composition of the volatile oil may also be giving the indication of oxidation, enzymatic changes or microbial fermentation. The benefits of GC clearly lie in its high sensitivity of detection for almost all the volatile chemical compounds. This is predominantly fact for the usual FID detection and GC-MS. Moreover, the high selectivity of capillary columns legalise separation of many volatile compounds simultaneously inside the qualified short times. Therefore, over the past decades, GC is accepted as a useful analytical tool in the research field of herbal medicines. Particularly, with the use of hyphenated GC-MS instrument, well-grounded information for the identity of the compounds is available as well. But, the most alarming disadvantage of GC is that it is not suitable for its analysis of the samples of polar and non-volatile
compounds. For this, it is necessary to use tedious sample work-up which may include derivatization. Therefore, the liquid chromatography becomes another necessary tool for us to apply the comprehensive analysis of the herbal medicines. The first fully automated on-line GC-IR system was developed by Scott et al. Each eluted solute was adsorbed in a cooled packed tube, and then thermally regenerated into an infrared vapour cell. Subsequent to the IR spectrum being obtained, a small sample of the vapour was drawn from the IR cell into a low-resolution mass spectrometer and the mass spectrum was also taken. High -performance liquid chromatography (HPLC).

In Chromatographic technique high performance liquid chromatography (HPLC), also known as high pressure liquid chromatography, is essentially a form of column chromatography in which the stationary phase consists of small particle (3-50 μm) packing contained in a column with a small bore(2-5mm). Here, one end of column which is attached to a source of pressurized liquid eluent (mobile phase). HPLC functioning basically on three forms of separation that are ion-exchange, partition and adsorption.

HPLC is a well-grounded for the analysis of herbal medicines because it is simple to work and its use is not limited by the volatility or stability of the sample compound. In general, HPLC can be applied to analyze almost all the compounds in the herbal medicines. Therefore, over the past decades, HPLC has awarded as the most large scale application in the analysis of herbal medicines. Reversed-phase (RP) columns role in herbal medicine analysis is very popular for the analytical separation. First important condition for the optimal separation for the HPLC involves many factors, like the different compositions of the mobile phases, their pH adjustment, pump pressures, etc. For a good experimental design the optimal separation above parameters are very important in order to obtain better separation. Some new techniques have been recently developed in research field of liquid chromatography.

High performance thin layer chromatography (HPTLC).

HPTLC is the common fingerprint method for herbal analysis. Principle of HPTLC is as TLC. But parallel advantage of HPTLC is that it has full concept which covers a broad standardized methodology based on scientific principle as well as the use of validated methods for qualitative and quantitative analysis. HPTLC full fill all quality requirements of today’s analytical labs, even in a fully regulated environment. Poly herbal drugs are easily identified by HPTLC for example four species of herbal medicines were identified easily by HPTLC of the resins with this technique. Authentication of various species of Ginseng and Radix Puerariae is possible, as well as the evaluation of stability and consistency of their preparations from different manufactures. HPTLC fingerprint is mainly employed to study the compounds with low or moderate polarities. As reported many fungal polysaccharide acid hydrolyses established a fingerprint by using automated multiple development. HPTLC technique in large scale employed in pharmaceutical industry in process development, identification and detection of adulterants in herbal product and helps in identification of pesticide content, mycotoxins and in quality control of herbs and foods. Syzygium Jambolanum was quantitatively evaluated in terms of stability, repeatability, accuracy and phyto constituents such as glycoside (Jamboline), tannin, allergic acid and gallic acid by HPTLC. HPTLC was used for detection, monitoring and quantification of bacoside A and B in Bacopamonnieria and its formulations. The standardization of Cannabis Stavia was done by estimating the content of cannabinoids in urine sample using HPTLC. HPTLC was used to estimate with aferine A, a constituent of with aniasomniferous in herbal extract and polyhedral formulations. HPTLC method has been reported for quantitative estimation of swetiamarin in...
different marketed polyherbal formulations and small fruits, big fruits and fresh fruits variety of \textit{E. Littoral}.\textsuperscript{18} Chandanasava known to be effective in karsya (malnutrition) was standardised by organoleptic study, physico-chemical analysis\textsuperscript{19}. Liquid chromatography-mass spectroscopy (LC-MS)

LC-MS has become method of choice in many stages of drug development\textsuperscript{20}. Chemical standardization of an aqueous extract of the mixture of the 20 herbs provided 20 chemical compounds serving as reference markers using LC-MS\textsuperscript{21}. Further, LC-MS analysis of amino glycosides showed that these drugs are highly soluble in water, exhibited low plasma protein binding, and were more than 90\% excreted through the kidney. Further this technique helps in analysis of amino glycosides in plasma samples with ion pairing chromatography\textsuperscript{22}. Two HPLC methods, one combined with a photodiode array detector (LC/UV) and another with mass spectrometry (LC/MS), were reported for the analysis of aristolochic acid I and II in herbal medicines.

Liquid chromatography- nuclear magnetic resonance (LC-NMR)

This one is conjugation technique which gives better identification and characterisation in herbal formulation. LC-NMR improves speed and sensitivity of detection and found useful in the areas of pharmacokinetics, toxicity studies, drug metabolism and drug discovery process\textsuperscript{23-25}. The identification of adulterants in a Chinese herbal medicine was done by LC-NMR technique. Gas chromatography (GC) and Gas chromatography-mass spectroscopy (GC-MS) instruments have been used for identification of large number of components present in natural and biological systems. As reported the identification and quantification of chemical constituents present in polyherbal oil formulation (Megni) consisting of nine ingredients, mainly \textit{Myristica fragrans}, \textit{Eucalyptus globulus}, \textit{Gaultheria procumbens} and \textit{Mentha piperita} was analyzed by GC-MS method. A head space solid-phase micro extraction method was reported for analysis of the volatile compounds in a traditional Chinese medicine (TCM), \textit{Rhioxma Curcumae Aeruginosae}. Thirty-five volatile compounds were separated and identified. An effective, fast and accurate capillary gas chromatography method was employed for determining organ chlorine pesticide residues in \textit{Scutellaria baicalensis}, \textit{Salvia miltiorrhiza}, \textit{Belamcanda chinensis}, \textit{Paoniae lactiflora}, \textit{Arisaema erubescens}, \textit{Anemarrhena asphodeloides} and \textit{Platycodon grandiflorum}. The SPE extract was separated by capillary column (30 m x 0.25 mm i.d. x 0.25 micron) using electro chemical detector. The split ratio obtained was 1:2.2 using the carrier gas N\textsubscript{2} (99.999\%) with the flow rate of 1.4 mL/min. The injector temperature was 220 °C and the detector temperature was 330 °C. The column temperature was increased by the rate of 20 °C /min from 100 °C to 190 °C (hold for 1.0 min), then to 235 °C by the rate of 4 °C /min and hold for 7 min at 235 °C. The good linearities were obtained for thirteen organ chlorine pesticides. The detection limits between 0.064-0.61 micro/L, average recoveries between 87.3\%-102.3\% and relative standard deviations of 1.3\%-6.8\% were obtained.
Table No.1: History of important events in herbal drug standardization

<table>
<thead>
<tr>
<th>S.No</th>
<th>Year</th>
<th>Important events</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>1983</td>
<td>The first National Health Policy 1983 claims that India’s is the richest source of herbs and the drugs should 23</td>
<td>26</td>
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<td>2</td>
<td>1995</td>
<td>A separate Department for Indian Systems of Medicine and Homeopathy (ISM and H) now known as AYUSH 24 (Ayurveda, Yoga, Unani, Siddha, Homoeopathy) was established in March 1995 to promote indigenous systems.</td>
<td>27</td>
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<td>3</td>
<td>1996</td>
<td>World Health Organization has recommended the drug control agency to regulate the quality and safety 17 profile of herbal products.</td>
<td>28</td>
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<td>4</td>
<td>1999</td>
<td>World Health Organization (WHO) had given a detail protocol for the standardization of herbal drugs comprising of a single content.</td>
<td>29</td>
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<td>5</td>
<td>2002</td>
<td>The Indian Herbal Pharmacopoeia. Mumbai, Indian Drug Manufacturer’s Association, 2002.</td>
<td>30</td>
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<tr>
<td>6</td>
<td>2002</td>
<td>Analytical approaches like Herboprint use three-dimensional HPLC and attempt to develop tools for 27activity-based standardization of botanicals.</td>
<td>31</td>
</tr>
<tr>
<td>7</td>
<td>2003</td>
<td>Department of Indian Systems of Medicines and Homoeopathy (ISM and H) established in 1995 renamed into Department of Ayurveda, Yoga and Naturopathy, Unani, Siddha and Homoeopathy (AYUSH).</td>
<td>32</td>
</tr>
<tr>
<td>9</td>
<td>2004</td>
<td>WHO guidelines on safety monitoring of herbal medicines in pharmacovigilance systems.</td>
<td>30</td>
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<tr>
<td>10</td>
<td>2004</td>
<td>In Canada, the Natural Health Products Regulations (NHPR) [13] under the Food and Drugs Act.</td>
<td>11</td>
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<tr>
<td>12</td>
<td>2006</td>
<td>The manufacture of herbal medicines 7</td>
<td>7</td>
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<tr>
<td>15</td>
<td>2009</td>
<td>AYUSH department with collaboration with Quality Council of India ( introduced certification scheme 36 for AYUSH drug products</td>
<td>13</td>
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<tr>
<td>17</td>
<td>2011</td>
<td>An EU directive passed in 2004 erects &quot;disproportionate&quot; barriers against herbal remedies by requiring 38 them to be &quot;licensed&quot; before they can be sold. It's called the Traditional Herbal Medicinal Products Directive (THMPD), Directive 2004/24/EC.</td>
<td>15</td>
</tr>
</tbody>
</table>

CONCLUSION
Chromatographic technique is one of the best options for the identification and quantification either in conjugation with spectroscopy or itself. It is a choice a method for herbal standardisation.

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CONFLICT OF INTEREST
We declare that we have no conflict of interest.

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