Abstract

Analytical method development, validation, and transfer are key elements of any pharmaceutical development program. Analytical methods are intended to establish the identity, purity, physical characteristics and potency of drug we use. Methods may also support safety and characterization studies or evaluation of drug performance. Further, quantification of active pharmaceutical ingredients (API) of herbal origin using analytical techniques viz., HPTLC/HPLC is very important for the standardization of herbal drugs and for the quality control of raw materials used for the development of medicine as well as in quality assurance of the prepared formulations. Thus novel method development and validation of herbal drugs is very interesting field of study for both academics and industries. Besides, the quality of herbal medicine i.e. the profile of the constituents in the final product has implications in efficacy and safety. Due to complex nature and inherent variability of the constituents of plant based drugs, it is difficult to establish quality control parameters and modern analytical techniques are expected to help in circumventing this problem. Nowadays, various analytical techniques such as Liquid Chromatography Mass Spectrometry (LC-MS), Gas Chromatography Mass Spectrometry (GC-MS), High Performance Liquid Chromatography (HPLC), High Performance Thin Layer Chromatography (HPTLC) are extremely useful for qualitative and quantitative evaluation of herbal drugs. Other analytical techniques such as Volumetric Analysis, Capillary Zone Electrophoresis, Voltametry, Gravimetry and spectrophotometry are also frequently used for quality control and standardization of herbal drugs. Out of these techniques, some of them like LC-MS; GC-MS are fast and sensitive but are highly expensive. On the other hand, techniques like spectrophotometry, capillary zone electrophoresis, voltametry show low resolution owing to poor reproducibility. The cost effectiveness of HPLC and HPTLC (compared to LC-MS and GC-

MS) makes them first choice for the qualitative and quantitative analysis of herbal drugs. Further one can achieve fast, specific, sensitive, reproducible and accurate results using HPTLC and HPLC.

WHO recommends and encourages the use of traditional herbs/remedies because huge amount of raw material is easily available and these herbs are comparatively safe due their low toxic effects. Many herbs have been evaluated in clinical studies and are currently being investigated phytochemically to understand their anti-tumor actions against various cancers. Thus, cancer patients who are further burdened by drug-induced toxic side effects of allopathic drugs, could take help from the complementary and alternative medicine hoping for a better cure. Some herbal anticancer drugs which have been used for quantification include vincristine, vinblastine, podophyllotoxin, etoposide, strychnine and brucine. The leaves of anticancer plant C. roseus contains vincristine and vinblastine which shows antineoplastic properties. These anticancer drugs work by inhibiting mitosis (cell division) in metaphase. Similarly, the rhizomes of P. hexandrum plant yield cytotoxic lignan podophyllotoxin which possesses anti-tumour activity by inhibition of the microtubule assembly. This podophyllotoxin, is also used for the semi-synthesis of anti-neoplastic drug, etoposide. Etoposide's antineoplastic activity is achieved through DNA strand breakage, which likely results from the formation of a complex involving drug, DNA, and the DNA, unwinding enzyme topoisomerase II. Similarly, the seeds of S. nux-vomica plant yield strychnine and brucine which shows antineoplastic properties. Crude extract of S. nux-vomica has been reported to exhibit an inhibitory effect on reverse transcriptase of RNA tumour virus (I), protein kinase, and HIV-1 protease. Further plant podophyllum hexandrum yield antinapthodianthrone derivative hypericin which is used for the treatment of inflammation and depression. Hypericin exhibit anti-inflammatory effect by inhibiting the release of leucotrienes.

The seeds of ammi visanaga plant yield furanochromones derivative khellin. Khellin inhibits calcium influx without any difference related to the specific calcium channels. These actions on calcium influx and intracellular mobilization can contribute to its vasorelaxant action. The main objectives of the present research are to develop fast sensitive, specific, economical, reproducible and accurate chromatographic and plant extraction methods for sample preparation and quantitative analysis of above mentioned drugs using High Performance Thin Layer Chromatography (HPTLC) and High Performance Liquid Chromatography (HPLC). Briefly, the present thesis describes the method development and validation of analytical methods using HPTLC and HPLC. The present thesis comprises six chapters as summarized below.

Chapter 1: Introduction

This chapter deals the importance of herbal drugs, their analysis, brief description of chromatography and its type. A brief description of the importance of method development and validation, their regulatory guidelines and parameters for validation has also been given.

Chapter 2: Literature Review

This chapter deals with the pharmacological as well as analytical work of last 10 years or more on reported drugs namely vincristine, vinblastine, podophyllotoxin, etoposide, strychnine, brucine, hypericin and khellin.

Chapter 3: Rationale and Objectives

This chapter gives us an insight of the requirements as well as advantages of the reported HPLC and HPTLC methods using herbal active pharmaceutical ingredients (API) namely vincristine, vinblastine, podophyllotoxin, etoposide, strychnine, brucine, hypericin and khellin. The objectives of the present study has also been reported.

Chapter 4: Experimental

This chapter deals with the details of chemicals, reagents used to quantify hypericin and khellin using HPLC and vincristine, vinblastine, podophyllotoxin, etoposide, strychnine, brucine using HPTLC. The details of the preparation of standard solutions, extraction of drugs from plants, instrumentation and method validation parameters has also been given.

Chapter 5: Results and Discussion

This chapter describes the results of validation parameters as well as optimization of the reported HPLC methods for hypericin, khellin and HPTLC methods for vincristine, vinblastine, podophyllotoxin, etoposide, strychnine, brucine.

For hypericin, quantification was performed by HPLC using acetonitrile: methanol: 10 mM ammonium acetate (pH 5.0) in the ratio 54: 36: 10 (v/v/v) as mobile phase with UV detection at 590 nm. The method was validated as per the ICH guidelines for linearity, precision (inter-day, intra-day and inter-system), robustness, accuracy, LOD and LOQ. The relationship between the concentration of standard solutions and the peak response was linear within the concentration range of 10-80 µg mL^{-1} for hypericin. For the proposed method LOD and LOQ were calculated using signal to noise ratio method and found to be 3.1 µg mL^{-1} and 9.6 µg mL^{-1} for hypericin. The % RSD value for method precision was found to be 0.36-1.35% for hypericin. Accuracy of the method was checked by recovery studies conducted at three different levels and the average percentage recovery was found to be 100.13%.

For khellin, quantification was performed by HPLC using methanol: water (75: 25, v/v) as the mobile phase with UV detection at 247 nm. The method was also validated for precision (inter-day, intra-day, inter-system), robustness, accuracy, LOD and LOQ. The relationship between the concentration of standard solutions and the peak response was linear within the concentration

range of 10 µg mL^{-1} to 80 µg mL^{-1} for khellin. The % RSD value for method precision was found to be 0.63-1.97% for khellin. Accuracy of the method was checked by recovery studies conducted at three different levels and the average percentage recovery was found to be 100.53% for khellin.

For vincristine and vinblastine, simultaneous estimation of vincristine and vinblastine was performed by HPTLC on a silica gel plate using toluene-methanol-diethylamine (8.75: 0.75: 0.5, v/v/v) as the mobile phase. The method was validated as per the ICH guidelines for precision (inter-day, intra-day, inter-system), robustness, accuracy, LOD and LOQ. The relationship between the concentration of standard solutions and the peak response was linear within the concentration range of 100 ng $spot^{-1}$ to 4000 ng $spot^{-1}$for vincristine and 200 ng $spot^{-1}$ to 4000 ng $spot^{-1}$ for vinblastine. The method precision was found to be 0.77-1.78 (%RSD) and 1.24-2.13 (% RSD) for vincristine and vinblastine, respectively. Accuracy of the method was checked by recovery study conducted at three different levels and the average percentage recovery was found to be 100.21 % for vincristine and 99.99 % for vinblastine, respectively.

Podophyllotoxin was quantified in the roots of Podophyllum Hexandrum whereas Etoposide was quantified in a marketed formulation. The method involved densitometric evaluation of both Podophyllotoxin and Etoposide after resolving it by HPTLC on silica gel plate with dichloromethane-methanol-formic acid (9.5: 0.5: 0.5, v/v/v) as the mobile phase. The method was validated for precision (inter-day, intra-day, inter-system), robustness, accuracy, LOD and LOQ. The relationship between the concentration of standard solutions and the peak response was linear within the concentration range of 150 ng $spot^{-1}$ to 2400 ng $spot^{-1}$ for Podophyllotoxin and 200 ng $spot^{-1}$ to 2000 ng $spot^{-1}$ for Etoposide. Instrumental precision was found to be 1.03-1.80 (%RSD) and 0.79-1.99 (%RSD) for Podophyllotoxin and Etoposide, respectively. Accuracy

of the method was checked by recovery studies conducted at three different levels and the average percentage recovery was found to be 100.6 % for Podophyllotoxin and 100.4 % for Etoposide, respectively.

For strychnine and brucine, simultaneous estimation was performed by HPTLC on silica gel plate using chloroform-methanol-formic acid (8.5: 1.5: 0.4 v/v/v) as the mobile phase. The method was validated as per the ICH guidelines for precision (inter-day, intra-day, inter-system), robustness, accuracy, LOD and LOQ. The relationship between the concentration of standard solutions and the peak response was linear within the concentration range of 50-1000 ng $spot^{-1}$ for strychnine and 100 - 1000 ng $spot^{-1}$ for brucine. The method precision was found to be 0.58-2.47 (% RSD) and 0.36-2.22 (% RSD) for strychnine and brucine, respectively. Accuracy of the method was checked by recovery studies conducted at three different concentration levels and the average percentage recovery was found to be 100.75% for strychnine and 100.52% for brucine, respectively.

All these five methods (two HPLC and three HPTLC) mentioned above are rapid, simple, specific, sensitive, reproducible and accurate and these methods can be used for the routine analysis of the reported API's and for the quality control of raw materials of these plants and several unani and ayurvedic formulations containing them as ingredients.

Chapter 6: Summary and Conclusion

This chapter deals with the summary of all the reported drugs and their conclusion.