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SPECTROSCOPIC & CHROMATOGRAPHIC METHODS

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Abstract

The aim of the present study was to investigate the use of spectroscopic methods, near-infrared (NIR) and Raman spectroscopy, in elucidating phenomena taking place during wet granulation. More specifically, a processing-induced transformation, hydrate formation, which takes place during wet granulation, was studied. In addition, the use of near-infrared spectroscopy in the process monitoring of high-shear wet granulation was studied by comparing it to impeller torque measurements, which is an established process monitoring method. The measurements were performed off- or at-line. Moreover, the difficulty to grasp the large data amounts produced by different process monitoring methods was addressed by combining the data and visualizing it with projection methods. Two different approaches were investigated, principal components analysis and self-organizing maps, which are linear and non-linear methods, respectively. The development of the pharmaceuticals brought a revolution in human health. These pharmaceuticals would serve their intent only if they are free from impurities and are administered in an appropriate amount. To make drugs serve their purpose various chemical and instrumental methods were developed at regular intervals which are involved in the estimation of drugs. These pharmaceuticals may develop impurities at various stages of their development, transportation and storage which makes the pharmaceutical risky to be administered, thus they must be detected and quantitated. For this analytical instrumentation and methods play an important role.

Keywords: *Spectroscopic, Chromatographic, Methods, Analytical, Instruments, Pharmaceuticals, Quality, Chromatography, etc.*

INTRODUCTION:

Polyphenols belong to a chemical class of flavonoids which are widely distributed in vegetables and plants. They possess a number of biological activities and thus received much attention in phytotherapy research. The phospholipid complexes were originally coined as “Phytosomes”. No literature has been reported on the reactivity of polyphenols towards phospholipids depending upon refluxing time, cooling temperature, nature of phospholipids and the polyphenols in mixture form or in an isolated form. We aim to compare different analytical techniques for quantification of biomarkers and their phospholipid complexes. In this study biomarkers were taken at standard concentrations and its phospholipid complex were prepared. LC-MS/MS, HPLC, FTIR and NMR analytical techniques were used to

quantify, characterize and monitor the complexes. Methods used for complexation of biomarkers gave good yields. 1H-NMR and FT-IR were reported for confirmation of phospholipids complex with natural polyphenols but the present study highlighted the need of spectrometry and chromatographic system in order to get better clarity regarding the formation of phospholipids complexes. NMR spectra can only give us the information if the complex has formed or not but doesn't give the required information which flavonoid has complexed. Mass spectrometry revealed good precision and gave superior results over NMR of the respective complexes formed. More over the purity of these complexes which were formed during the reaction can be understood by the chromatographic system. This work found novelty in terms of revealing the molecules involved and their efficiency in the process of complexation.

REVIEW OF LITERATURE:

The process of drug development starts with the innovation of a drug molecule that has showed therapeutic value to battle, control, check or cure diseases. The synthesis and characterization of such molecules which are also called active pharmaceutical ingredients (APIs) and their analysis to create preliminary safety and therapeutic efficacy data are prerequisites to identify the drug candidates for further detailed investigations. In the field of pharmaceutical research, the analytical investigation of bulk drug materials, intermediates, drug products, drug formulations, impurities, degradation products, and biological samples containing the drugs and their metabolites is very important. From the commencement of official pharmaceutical analysis, analytical assay methods were included in the compendial monographs with the aim to characterize the quality of bulk drug materials by setting limits of their active ingredient content. In recent years, the assay methods in the monographs include titrimetry, spectrometry, chromatography, and capillary electrophoresis; also the electro analytical methods can be seen in the literature. From the stages of drug development to marketing and post marketing, analytical techniques play a great role, be it understanding the physical and chemical stability of the drug, impact on the selection and design of the dosage form, assessing the stability of the drug molecules, quantitation of the impurities and identification of those impurities which are above the established threshold essential to evaluate the toxicity profiles of these impurities to distinguish these from that of the API, when applicable and assessing the content of drug in the marketed products. The analysis of drug and its metabolite which may be either quantitative or qualitative is extensively applied in the pharmacokinetic studies. This review highlights the role of various analytical techniques and their corresponding analytical methods in the analysis of pharmaceuticals.

1. MATERIALS, CHEMICALS AND REAGENTS: All the biomarkers and phospholipids (Phosphatidylcholine – 60%) were purchased from Sigma (USA). All technical grade solvents were procured from SD fine chemicals (India).

Preparation of biomarker mixture for HPLC: Three standard polyphenols catechins, quercetin and myricetinprepared in methanol to give a concentration of 10 µg/ml. All the solutions were stored in the dark at 4°C. HPLC analysis indicated these solutions were stable within 2 months. Standard quercetin, catechins and myricetin solution was mixed in equiproportion (1 ml each of 10 µg/ml solution) to give 10 µg/ml solution of standard mixture of polyphenols. The final solution of standard polyphenol mixture was made in the range of 20-15000 ng/ml. These three polyphenols were chosen because these three polyphenols possess different solubility in various solvents as only myricetin is soluble in water; catechin is soluble in methanol and ethanol and not in water and quercetin is soluble in acetone. Methanolic extract was prepared for the polyphenols to successfully quantify using suitable analytical tools.

High Performance Liquid Chromatographic (HPLC) conditions: The chromatographic system (Jasco) consisting a PDA detector was used. A reverse phase Qualisil BDS-C column (4.6 mm f x 25. mm) packed with 5 µm diameter particles was used. In order to estimate the three polyphenols simultaneously a gradient phase chromatographic methods was developed. In the course of experiments, several ratio of acetonitrile: water in isocratic system was studied and the effect of o-phosphoric acid to regulate the pH value was examined.

2 LC INSTRUMENTS AND ANALYTICAL CONDITION: Polyphenols analysis by LC-ESI-MS/MS were carried out using an Agilent 1100 series LC and LC/MSD Trap VL mass spectrometer (Agilent Technologies, USA) equipped with electrospray ionization (ESI) interface. In order to obtain optimum ionizing conditions, the reference solution was used were both Atmospheric Pressure Chemical Ionization (APCI) and electrospray ionization interface were tested in positive and negative ion modes by scanning between m/z 200550 per second. The column temperature was maintained at 25°C. Quantification was achieved using selected ion monitoring system (SIM) mode of ion. The flow rate was 0.5 ml/min.

Preparation of standard solution and working reference solution for LC-MS/MS: Standard solution was prepared by dissolving biomarkers like quercetin, catechin and myricetin in methanol. The concentration prepared was in the range of 5 ppm-200 ppm both for the biomarkers and the extract. Biomarkers like quercetin, catechin and myricetin were taken in combination to prepare biomarker mixture as working reference solution for simultaneous determination of these three polyphenols.

Preparation of phospholipid complex of biomarker mixture: Biomarkers were taken into a solution of phospholipid in dichloromethane (DCM) in 2:1 proportion (1gm 25 mg of phospholipids and 450 mg of biomarker mixture). Two types of reactions conditions were maintained during preparation of phospholipid complex. After refluxing for certain time the mixture was filtered and kept for evaporation under vacuum. The residue was re-dissolved in the DCM and added slowly to a non-solvent n-hexane. The resultant mixture was kept on cooling at room temperature (R-1). In second method all the steps were repeated except the last step where the resultant mixture was kept on cooling in refrigerator for overnight (R-2) (Table-1).

Table 1: Reaction condition of phospholipids complex

Reaction condition	Refluxing time	Cooling condition
R-1	30 min	Room temperature
R-2	60 min	Refrigeration

Recording of mass spectra: In mass spectra an ESI total ion chromatogram (TIC) scans was performed and analysed.Mass spectrometry was performed with an Agilent 6460 LC/MS/MS triple quadruple (Agilent Technologies, Santa Clara, CA) and an electrospray ionization source (ESI). To reduce the surface contact with the interior of the system, samples were directly infused via the Agilent 1290 automated injection system, with the column adaptation bypassed; 20 lL of each sample were infused at 100 lL/min. Between each run, the syringe and insert tubing were cleaned using buffer and chloroform to avoid sample to sample contamination. Phospholipids were identified by detection of specific leaving groups from precursor ions after collision-induced dissociation (CID) for example, the leaving group of fragmented phosphatidylcholine (PC).

Monitoring of phospholipid complexes reaction condition by HPLC:A gradient HPLC method for the polyphenols catechin, quercetin and myricetin was taken into consideration in monitoring the reaction condition during preparation of phospholipids. Phospholipids complex was not soluble in acetonitrile unlike polyphenols. Several solvent has been tested to dissolve the phospholipid complex and finally dioxane has been selected because its miscibility with water. Immiscibility of solvent and insolubility of complex in the solvent can leads to precipitation of complex in the mobile phase leading to clogging on the column thereby with the dioxane this problem was conquered.

3 THIN LAYER CHROMATOGRAPHY: In thin layer chromatography, a solid phase (stationary phase), the adsorbent, is coated onto a solid support as a thin layer (about 0.25 mm thick). In many cases, a small amount of a binder such as plaster of Paris is mixed with

the absorbent to facilitate the coating. Many different solid supports are employed, including thin sheets of glass, plastic, and aluminum. The mixture (A plus B) to be separated is dissolved in a solvent and the resulting solution is spotted onto the thin layer plate near the bottom. A solvent, or mixture of solvents, called the eluents, is allowed to flow up the plate by capillary action. At all times, the solid will adsorb a certain fraction of each component of the mixture and the remainder will be in solution. Any one molecule will spend part of the time sitting still on the adsorbent with the remainder moving up the plate with the solvent. A substance that is strongly adsorbed (say, A) will have a greater fraction of its molecules adsorbed at any one time, and thus any one molecule of A will spend more time sitting still and less time moving. In contrast, a weakly adsorbed substance (B) will have a smaller fraction of its molecules adsorbed at any one time, and hence any one molecule of B will spend less time sitting and more time moving. Thus, the more weakly a substance is adsorbed, the farther up the plate it will move. The more strongly a substance is adsorbed, the closer it will stay near the origin.

4 SPECTROSCOPIC TECHNIQUES:

Spectrophotometry: Another important group of methods which find an important place in pharmacopoeias are spectrophotometric methods based on natural UV absorption and chemical reactions. Spectrophotometry is the quantitative measurement of the reflection or transmission properties of a material as a function of wavelength. The advantages of these methods are low time and labor consumption. The precision of these methods is also excellent. The use of UV-Vis spectrophotometry especially applied in the analysis of pharmaceutical dosage form has increased rapidly over the last few years. The Light of Knowledge is an often used phrase, but it is particularly appropriate in reference to spectroscopy. Most of what we know about the structure of atoms and molecules comes from studying their interaction with light (electromagnetic radiation). Different regions of the electromagnetic spectrum provide different kinds of information as a result of such interactions.

Mass Spectrometry: Sample molecules are ionized by high energy electrons. The mass to charge ratio of these ions is measured very accurately by electrostatic acceleration and magnetic field fluctuations, providing a precise molecular weight. Ion fragmentation patterns may be related to the structure of the molecular ion.

Ultraviolet-Visible: Spectroscopy Absorption of this relatively high-energy light causes excitation of electrons. The easily accessible part of this region (wavelengths of 200 to 800 nm) shows absorption only if conjugated pi-electron systems are present.

Infrared Spectroscopy: Absorption of this lower energy radiation causes vibrational and rotational excitation of groups of atoms within the molecule. Because of their characteristic absorptions identification of functional groups is easily accomplished.

Nuclear Magnetic: Resonance Spectroscopy Absorption in the low-energy radio-frequency part of the spectrum causes excitation of nuclear spin states. NMR spectrometers are tuned to certain nuclei (e.g. 1H, 13C, 19F and 31P). For a given type of nucleus, high-resolution spectroscopy distinguishes and counts atoms in different locations in the molecule.

HPLC Coupling with Various Spectroscopic Techniques: HPLC can be combined with numerous other analytical techniques but the most important coupling principle is the one with spectroscopy. Chromatography and spectroscopy are orthogonal techniques, i.e. their types of information are very different. Chromatography is a separation method and spectroscopy is a technique which yields a 'fingerprint' of molecules. Coupling with atomic spectrometry is rarely used although it allows the detection of toxic metals in environmental samples or of metalloproteinase. Four other techniques, HPLC-UV, HPLC-FTIR, HPLC-MS and HPLCNMR are more important because excellent spectra are obtained with them, thus allowing structure elucidation.

CONCLUSION:

This study compared the pre-processing and non-linear modelling techniques for the calibration of NIR spectroscopy in the presence of light scattering effect. A rigorous statistical procedure was adopted to obtain reliable comparison results. Although none of the techniques is always the best on all datasets, OPLEC and GP are found to be the most promising in terms of low prediction error. Compared with traditional approaches (D1, D2 and SNV), the more recently developed pre-processing methods (EMSC, EISC and OPLEC) are more favourable. This is due to better modelling of the light scattering effect (such as including the wavelength terms % in the mode) and more advanced parameter estimation strategy (such as that of OPLEC). Therefore, if future research can lead to clearer understanding of the light scattering mechanism, and this information can be chemo metrically modelled, then more powerful pre-processing techniques may emerge. However, in some practical situations, the light scattering effect is coupled with other disturbances to jointly affect the linearity of the spectral measurements. For example, when NIR spectrometers are applied for *in situ* monitoring of drying processes, both light scattering and temperature variation will affect the calibration accuracy. Under these circumstances, the development of pre-processing method by modelling of all major factors may be infeasible.

In this regard, non-linear calibration techniques are preferred since they directly model the spectra-concentration relationship. Among the three non-linear models considered in this study, GP is recommended since it consistently attained lower RMSEP than ANN and LS-SVM. Finally, we have attempted to combine pre-processing and non-linear techniques; yet this strategy does not always outperform the individual techniques. This "hybrid" approach is conceptually appealing, and it will be further investigated in the future work.

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