ISSN: 0975-766X
CODEN: IJPTFI
Available Online through
www.ijptonline.com
Research Article

METHODS FOR STRUCTURAL STUDIES OF AN ANTIBODY, SCREENING METABOLITES IN PROTEIN DRUG AND ANALYSIS OF SPENT CELL CULTIVATION MEDIA USING LC-MS

V.Krishnaiah[a] and Y.V Rami Reddy*
Department of chemistry, Sri Venkateswara University, Tirupathi - 517 502, A. P, India
Email: vkreddysvu@gmail.com

Received on 23-02-2012 *Accepted on 10-03-2012*

Abstract

We describe the development of a method in which protein oxidation by H_2O_2 followed by ultrahigh-pressure liquid chromatography (UHPLC) coupled with electrospray ionization time-of-flight mass spectrometry (ESI-ToFMS) and multivariate analysis are used to detect alterations in conformational states of proteins. In the study reported here, an IgG1 monoclonal antibody in native and denatured conformational states was oxidized by treatment with hydrogen peroxide. Peptide fragments generated by tryptic digestion were then analyzed by UHPLC-ESI-ToFMS. After reducing noise and extracting peaks from the LC–MS data using MzExplorer, software developed in-house and based on Matlab, we were able to distinguish peptides arising from the native and denatured states of the oxidized protein by principal component analysis.

Keywords: Recombinant monoclonal antibody; Protein conformation; Methionine; Peptide mapping; Ultrahig pressure liquid chromatography; Mass spectrometry; Principal component analysis.

Introduction

Human have always used substances that make them feel relaxed and stimulated. As time progressed, preparations were discovered that could be used to alleviate aches, pains and other ailments. These were all naturally occurring substances without refinement and isolation of specific compounds (drugs). Discovery and refinements of specific drugs to use

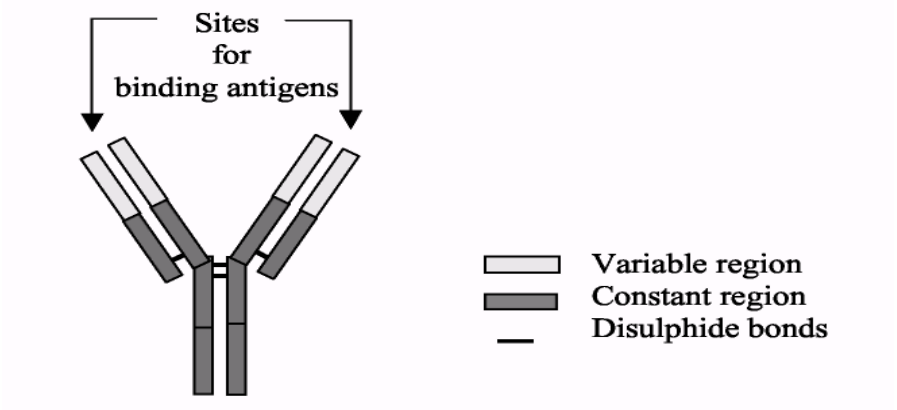
in medicine occurred and was performed later by alchemists and medical experimenters (e.g. the discovery of ethanol and its refinement to use in medicine by a Persian chemist, physician and philosopher,

There are strict requirements on today's pharmaceutical preparations in all steps of development, production, delivery etc. From the first idea to Pharmaceuticals introduction on the market, many expertise's in different field of science (e.g. physiologists, physicians, biochemists, organ chemists, analytical chemist, toxicologists and etc.) are involved and collaborate. Many different challenges have to be overcome on the way. One of the challenges that analytical chemists commonly meet is the analysis of complex samples. Detection suitable separation and detection methods can remove the bulk of the potentially interfering compounds and matrix substances; although in the real world it is often virtually impossible to eliminate all of the unwanted constituents while retaining all their constituents of interest. Further, even if the selected methods can handle the sample-related problems, there are still often problems e.g. associated with peak-shifts in LC-MS.

Results and Discussion

Protein samples

Antibodies (also known as immunoglobulin, Ig) comprise a class of antigen-specific, immunological proteins that are classified into five isotypes (IgA, IgD, IgE, IgG and IgM). IgG immunoglobulin, which are the major immunoglobulin in normal human serum, are monomeric and have molecular weights of ca. 150 kDa. They consist of four peptide chains, two heavy chains and two light chains, held together with a total of four disulfide bonds. Each IgG molecule has two antigen binding sites.

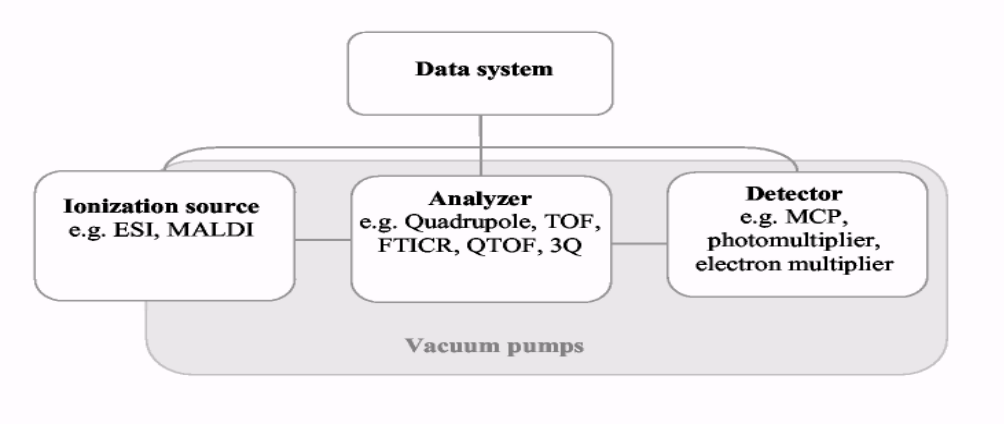


Sample preparation prior to LC/MS analysis

Sample matrices can pose considerable challenges in attempts to identify, quantify and/or characterize compounds of interest by liquid chromatography-mass spectrometry (LC/MS), or other analytical techniques, since interfering substances in them can dramatically reduce the resolution of LC, decrease the ionization efficiency of MS and increase the resulting chemical noise, thereby increasing the limit of detection. Therefore, sample preparation is generally required to facilitate the isolation and concentration of compounds of interest from various matrix components, and hence the separation and detection of the analytes. Sample preparation techniques that may be applied include chemical and/or physical processes, e.g. oxidation and filtration, respectively.

Mass spectrometry

Generation of ions in gas phase from compound/complex of interest is a prerequisite for any mass spectrometry (MS) experiments. Mass spectrometers are typically composed of several parts: an ion source, an analyzer, a detector, data handling system. Vacuum pumps (rotary vacuum pumps in combination with turbo-molecular pumps) are also needed to provide high vacuum to avoid ion collision. Each of these parts is discussed below, focusing on mass spectrometry of large molecules. Ions generated in the ion source are transferred, separated and resolved in the mass analyzer (e.g. a quadrupole or time of flight system, see below). The ions that survive this journey and reach the detector (e.g. a photomultiplier, MCP) will be detected. The type of detector used should, of course, be compatible with the type of analyzer.



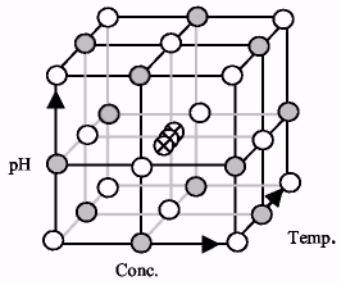
EXPERIMENTAL

The aim of Design of Experiment (DoE) [84] is to obtain as much information as possible from as few experiments as possible. DoE is a good way to identify interactions between significant factors and optimal values of variables of the investigated system. The chosen experimental domain must span the important known variation. So, some knowledge about the experimental system is required in order to obtain a reasonably complete and reliable model. However, an ideal design is not always viable for practical reasons, particularly when a living organism is involved; since some settings of some variables will be impossible to apply in real life. In order to confounding by the non-controlled phenomena, randomization of the experiments (collecting samples, instrumental analysis etc.) is needed.

Important aspects to consider in each DoE are: experiments, factors, responses, the experimental domain and experimental design. A factor (X) is a controllable variable that is varied in a predefined interval (low and high level settings, denoted by "-" and "+", respectively, with or without a center-point, denoted "0"). A response (Y) is a dependent variable that is measured. The experimental domain is the overall domain spanned by all the factors under investigation. There are many different types of experimental designs, and those used for screening purposes differ from those used for optimization purposes. The aim of screening is to sift the important factors from the non-important ones, while the aim of optimization is to find settings of the important factors that will yield an optimum response. Therefore, screening designs generally include fewer experiments than optimization designs for "response surface modeling" (RSM). The experiments to perform, in terms of the permutations of varied factors, are selected according to a "factorial design", of which there are various types. Examples of such designs for three-level RSM, including Box Benhken and Central Composite Face (CCF) full or fractional factorial designs are shown in Figure 7. Different types of experimental design are described further elsewhere [85-86]. Experiments included in the Box Benhken and CCF designs are indicated by white circles and grey circles, respectively. Center points are experiments in which all the design factors are at their mean values. The factors (here pH, temperature and concentration) are varied in each experiment, and the response (signal) depends on the factors. The run order is randomized, since this is the best way to minimize systematic errors due to handling, instrument drift, etc. In some cases randomization cannot be applied, for example if changing design factors are very time-consuming, costly and/or difficult. This may be considered when analyzing the results and seeking systematic patterns. In experiment 1, the factor settings for

pH and temperature are at their lowest respective levels in the experimental domain, while the concentration is at the middle of the interval. The signal (response) is then measured and soon.

A)



B)

		Factors (X)			Response (Y)
Exp.No	RunOrde	pH	Tem	Conc	Signa
1	13	-	-	0	?
2	2	+	-	0	?
3	7	-	+	0	?
4	12	+	+	0	?
5	6	-	0	-	?
6	14	+	0	-	?
7	15	-	0	+	?
8	4	+	0	+	?
9	10	0	-	-	?
10	1	0	+	-	?
11	9	0	-	+	?
12	11	0	+	+	?
13	5	0	0	0	?
14	3	0	0	0	?
15	8	0	0	0	?

Preprocessing of LC/MS data prior to multivariate pattern recognition modeling

The data obtained from an observation in LC/MS analysis have three dimensions: retention time (IR), m/z and intensity. An observation with a specific m/z and tR can be regarded as the identity of an ion in LC/MS data. The produced data (raw data) is a sum of a *structured data* and *noise.* The fraction of the raw data that correlates with the property of interest are *structured data (contains information);* everything else is *noise.* The amounts of data produced from LC/MS analyses have increased greatly over the years as a result of instrumental developments (which have provided higher sensitivity, accuracy and resolution). In non-targeted analysis, when every compound in a complex sample is of interest, it is more difficult to optimize the experimental methods regarding the matrix effects, thus stronger matrix effects on the resulted data are to be expected.

The reasons for pretreating the data generally are to reduce the effects of the noise, simplify the data by reducing the dimensionality of the data and to improve the predictive ability of the model. The data interpretation is more time-consuming than the data acquisition and thus has traditionally been the bottleneck in LC/MS-based metabolomic studies. The data are often pretreated before modeling the dataset. For initial preprocessing of LC/MS data software is needed to compress the raw LC/MS datasets obtained (which are of mega- or giga-byte scale) and structure the data in a suitable format for applying pattern recognition methods; NetCDF is an example of a suitable format for further MVDA of the

data. The next step is to reduce both chemical noise (caused by fluctuations in temperature, pH and concentrations etc.) and instrumental noise (which is a composite of noise from all of the instrumental components) [2]. Data points with the same m/z-value that are consistently observed may be contributions from the mobile phase. In Studies I and III an in-house program was used to produce a two-dimensional matrix from LC/MS data in NetCDF format. The software filters the data with a "two-dimensional finite impulse response filter" to improve the Gaussian shade of the peaks and reduce high-frequency noise. A peak list is then produced by position (m/z and IR) and intensity via automatic peak extraction.

Validation of the models

It is very important to be aware that mathematical models provide a simplification of complex realities, rather than truly portraying the modeled realities. Hence, validation is a crucial step of modeling in chemo metrics, since it provides indications of the reliability of conclusions that can be drawn from the modeling. Without validation, acquired models cannot be applicable in real life. Chemometric models can be validated in either of two ways. External validation is one approach, in which new objects (test sets) that have not been used in the model are applied. The test set should be of known and suitable content. Use of test sets is recommended when large numbers of samples are available, and 30-50 % of the samples can be used as a test set, while the rest of the objects can be used in the training set for modeling. Internal validation is the other approach, in which objects that have been used in the modeling are also used in validation of the model e.g. cross-validation [96]. In cross-validation only a training set is required, but one (or a group) of objects is removed at a time. The object(s) that was/were removed is/are used to test the performance of the model on "new" data. Each time another object(s) is/are removed and used as "new" data, a new test of the predictive ability of the model is performed. The validation is performed by comparing the predicted and true values and used to estimate the goodness of the predictive ability of the model.

References

1. Swarbrick James and Boylan James.C, Encyclopedia of pharmaceutical technology, volume I, Marcel Dekker Inc. New York, 1998: 217-224.
2. Beckett AH, Stenlake JB, Practical Pharmaceutical chemistry, 1997, 4th edition,part 2, CBS publication and distributors:275-337.

3. Martindale (2007) The complete drug reference 35th edition Pharmaceutical Press, London: 866.

4. Satoskar RS, Bhandarkar SD, Ainapure SS, editors. Pharmacology and Pharmaco- therapeutics. 15 th ed. Mumbai: Popular Prakashan; 1997, 462.

5. Budavari, S, eds., In; the Merck Index. 12 th ed. New Jersey: Merck and Co., Inc; 1996, 406.

6. Harvey PS, Goa KL, Drugs, 1999, 58:469-475.

7. Erturk S, Aktas ES, Ersoy L, Fccoglu S. An HPLC method for the determination of atorvastatin and its impurities in bulk drug and tablets. J. Pharm. and Biomed.Anal., 33; 2003, 1017-1023.

8. Qutab SS, Razzaq SN, Khan IU, Ashfaq M, Shuja ZA. Simultaneous Determination of Atorvastatin Calcium and Ezetimibe in Pharmaceutical Formulations by Liquid Chromatography. J. Food and Drug Anal., 2007; vol. 15(2):139-144.

9. Shah DA, Bhat KK, Mehta RS, Shankar MB, Baldania SL, RP-HPLC method for the determination of atorvastatin calcium and nicotinic acid in combined tablet dosage form. Ind. J. Pharm. Sci., 2007; 69 (5): 700-703

10. Santisky D, Neto I, Solich P, Sklenakova H,Conceicao M, Montenengro BS, et al. Seqential injection chromathograpicdetermination of Paracetamol, caffeine and Acetylsalicylic acid in pharmaceutical tablets. JSep Sci 2004 May; 27(7-8):589.

Corresponding Author:

Y.V Rami Reddy*

Email:*vkreddysvu@gmail.com*