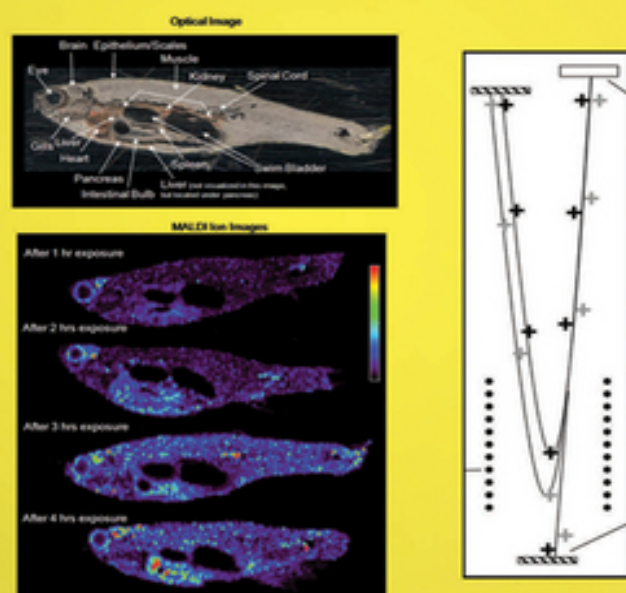


Wiley Series on Mass Spectrometry
Dominic M. Desiderio and Nico M. M. Nibbering, Series Editors

Mass Spectrometry for Drug Discovery and Drug Development



Edited By
WALTER A. KORFMACHER

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MASS SPECTROMETRY FOR DRUG DISCOVERY AND DRUG DEVELOPMENT

Edited by

WALTER A. KORFMACHER

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*This book is dedicated to the most important people in my
life:*

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PREFACE

This book was written as part of a series of books on the utility of mass spectrometry (MS) for various scientific fields. The emphasis for this book is the description of the application of MS to the areas of new drug discovery as well as drug development. MS is now used as the main analytical tool for all the stages of drug discovery and drug development. In many cases, the way MS is applied to these endeavors has changed significantly in recent years, so there is a need for this book in order to provide a reference to the current technology. Thus, the readers of this book would be pharmaceutical scientists including medicinal chemists, analytical chemists, and drug metabolism scientists. This book will also be of interest to any mass spectrometry scientist who wants to learn how MS is being used to support new drug discovery efforts as well as drug development applications.

The book has 15 chapters that are written by experts in the topic that is described in the chapter. The first chapter provides a current overview of the various types of MS systems that are used in new drug discovery and drug development. This chapter will be useful to those still learning about MS as well as experts who want to understand the latest MS technology. One of the major changes in the MS field has been the emergence of high-resolution mass spectrometry (HRMS) as a tool not only for qualitative analyses, but also for quantitative analyses. This change has the potential to produce a true paradigm shift. In the future, it can be predicted that many quantitative bioanalytical assays will shift from using the selected reaction monitoring (SRM) technique with high-performance liquid chromatography-tandem mass spectrometry (HPLC-

MS/MS) to HPLC-HRMS. Discussions of why and how this will happen can be found in the second, third, and fourth chapters of this book. This shift from HPLC-MS/MS to HPLC-HRMS has the potential to radically change how MS is used in both new drug discovery and drug development. In addition to these three chapters, the final chapter in the book looks at the new topic of quantitative analysis of peptides and asks whether one should use SRM or HRMS for these assays.

Metabolite identification has been a major focus of MS for several decades. Chapter 5 describes the current MS technology that is used for metabolite identification including new software tools that have made this task easier. One of the newer applications of MS is the quantitative and qualitative analysis of biological drugs; this new topic is described in the sixth chapter along with a discussion of the MS analysis of proteins and peptides. Another important part of drug development is the characterization of impurities and degradation products; the utility of MS for this task is described in the seventh chapter. Medicinal chemists are at the center of all new drug discovery and drug development activities; Chapter 9 describes how MS is used to support the efforts of medicinal chemists in this effort.

An area of continuing interest is the application of MS to surface analysis in order to understand the distribution of drugs and metabolites as well as proteins and peptides on tissue slices from laboratory animal studies and sometimes human clinical tissue samples. Chapter 8 describes the new technique called liquid extraction surface analysis (LESA) that is used for tissue profiling. Chapter 10 discusses MS imaging for proteins and peptides, while Chapter 11 describes the use of MS imaging for drugs and metabolites. Together, these three chapters provide a comprehensive

overview of how MS imaging is being used for various drug discovery and drug development applications.

The rest of the book covers various specific topics that are important parts of the drug discovery and drug development process. Chapter 12 deals with the important topic of screening for reactive metabolites. This topic has received increased attention in recent years because of concerns that reactive metabolites may lead to drug safety issues. Two new topics are covered in Chapters 13–14. Chapter 13 describes the use of MS for siRNA applications and Chapter 14 covers the various ways MS is used in the field of metabolomics. The last chapter in the book, Chapter 15, takes a look at the new field of quantitative analysis of peptides using MS techniques.

Overall, this book provides a comprehensive picture of the latest MS technology and how it is being used throughout the various stages of new drug discovery and drug development. I want to thank the authors of each chapter for their efforts and careful attention to detail. I also want to thank Nico Nibbering and Dominic Desiderio, the editors of this MS series, for inviting me to be the editor of this volume. Finally, I want to thank my family for their support of this effort, with special thanks going to Madeleine, my wife.

WALTER A. KORFMACHER

OVERVIEW OF THE VARIOUS TYPES OF MASS SPECTROMETERS THAT ARE USED IN DRUG DISCOVERY AND DRUG DEVELOPMENT

G rard Hopfgartner

1.1 INTRODUCTION

Since J.J. Dempster published one of the first reports on the detection of volatile organic compounds using electron impact ionization in 1918, significant progress in ion sources and mass analyzers has been achieved. The aim this chapter is to focus on the most commonly used techniques in drug metabolism studies for quantitative or qualitative analysis, and also to discuss some of the “niche” techniques. In terms of the ionization techniques, atmospheric pressure ionization (API) sources including electrospray (ESI), atmospheric pressure chemical ionization (APCI), and atmospheric pressure photoionization (APPI) have revolutionized the analysis of low molecular weight compounds (LMWCs) by high-performance liquid chromatography-mass spectrometry (HPLC-MS). In addition, matrix-assisted laser desorption/ionization (MALDI) was originally developed for the characterization of biopolymers,

but is also attractive for the analysis of LMWCs and for mass spectrometry imaging (MSI) of drugs and their metabolites in tissues. Ambient ionization techniques have also gained interest for the same type of applications. Finally, inductively coupled plasma (ICP) mass spectrometry has also been explored as an alternative detector to ^{14}C -labeled drug for drug metabolism studies.

Triple quadrupole MS systems have become the workhorse for quantitation and, in combination with linear ion traps (LITs), are very attractive for qualitative/quantitative workflows. Ions traps are still used as standalone mass spectrometers but more and more in combination with others types of mass analyzers. A new paradigm shift will certainly come from high-resolution, accurate mass systems such as time-of-flight (TOF), ion cyclotron resonance, and Orbitraps, which will allow the application of novel approaches in mass spectrometry for drug metabolism studies. Due to the complexity of the samples, additional orthogonal separation power is always required and ion mobility mass spectrometry could play a more important role in the near future. One of the key problems in HPLC-MS is that the response is compound dependent; accelerator mass spectrometry (AMS) is one option that can be used to overcome this limitation and to provide the ultimate sensitivity in human studies.

1.2 IONIZATION TECHNIQUES

1.2.1 Electrospray

Electrospray is currently one of the most commonly used ionization techniques; in ESI, either singly or multiply

charged gas phase ions are generated at atmospheric pressure by electrically charging a liquid flow. It is based on a condensed phase process where preformed solutions ions are transferred to the gas phase. ESI for mass spectrometry was developed by John Fenn and coworkers in an attempt to analyze large biomolecules by mass spectrometry [1]. Charged droplets are generated by applying a strong potential of several kilovolts (2–6 kV) to a liquid stream. An electric field gradient is generated, which induces the deformation of the liquid into a conical shape called the Taylor cone. Then the solution forms a charged aerosol. After size reduction of the droplets by evaporation at atmospheric pressure, ions escape from the droplets and are sampled into the mass analyzer. The concept of applying high potential to a metal capillary to generate ions at atmospheric pressure followed by mass spectrometric detection has also been reported by Alexandrov et al. [2, 3], and they named their method extraction of dissolved ions under atmospheric pressure (EDIAP).

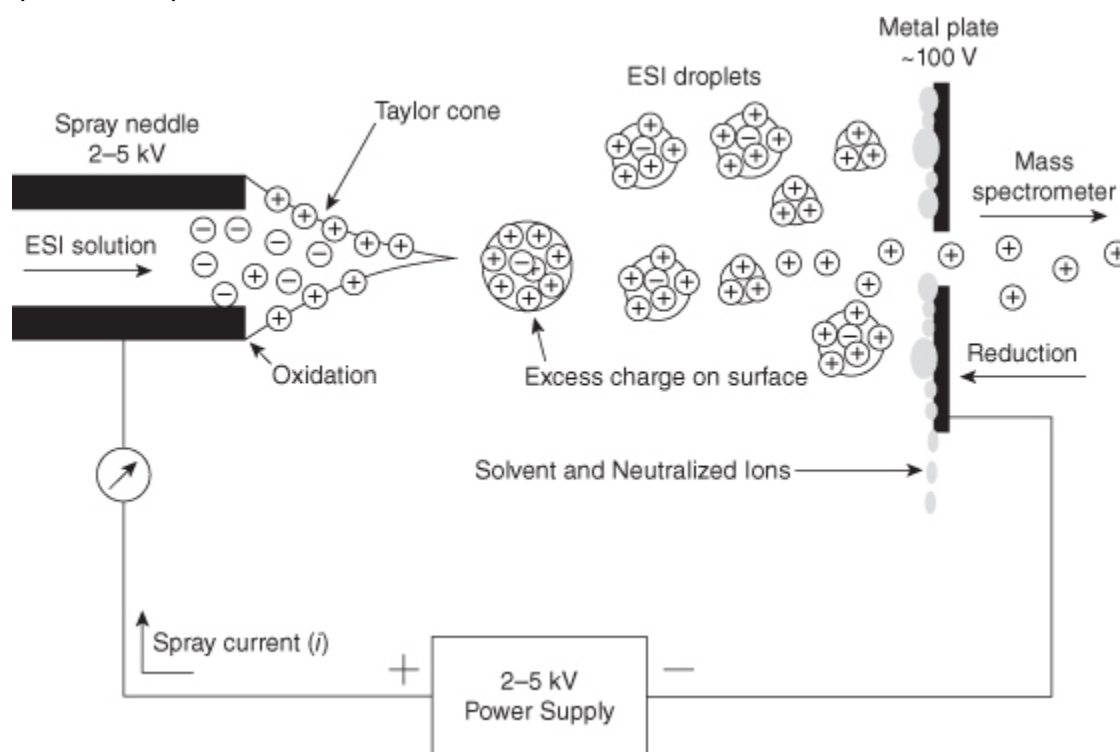
The stability of the aerosol is strongly dependent on the solvent composition, the flow rate, and the applied potential; typically, electrospray works best at the flow rate of a few microliters per minute. To achieve higher flow rates, the spray formation can be assisted by a nebulizing gas (nitrogen), which has been referred to as ionspray [4] or pneumatically assisted electrospray. Most modern instruments can handle flow rates from a few nanoliters per minute to several milliliters per minute. Various atmospheric pressure ion source geometries have been developed, using in most cases some combination of nebulizing gas and heat [5]. Pneumatically assisted electrosprays are well suited as ionization sources for liquid chromatography at various flow rates. It has been stated that ion spray mass spectrometry behaves like a concentration-sensitive detector [6], where the reduction of liquid chromatography column internal

diameter should result in an increase of the MS response considering that the same amount of analyte is injected. The actual behavior of ESI sources is very dependent on the ion source geometry and the instrumental settings.

ESI works best with preformed ions in solution and when preformed ions are separated from their counter ions. In 1991, Kebarle et al. [7] reported the electrophoretic nature of ESI, in which the charge balance requires the conversion of ions into electrons. Therefore, oxidation may occur at the needle ([Fig. 1.1](#)), and the interface of the mass spectrometer acts as a counter electrode.

Figure 1.1 Schematic of the electrospray process

(adapted with permission from Reference 136).



Electrospray is particularly suitable for the analysis of inorganic ions and molecules that have acidic or basic functional groups. Organic molecules are generally observed as protonated or deprotonated molecules depending on their pKa. Bases are best detected in the positive mode, while acids give good signals in the negative mode.

Therefore, for best signal, the pH of the mobile phase must be adjusted to the acidic or basic nature of the analyte. However, for peptides, it has been shown that intense signals can be observed either in the positive or in the negative mode using strongly acidic or basic solutions, respectively. These observations are reported as “wrong way round” and have been discussed by Zhou and Cook [8]. For many analytes besides the protonated or deprotonated molecules, adduct ions such as sodium or potassium adducts in the positive mode or with formate in the negative mode can be observed. Also, they can also form dimers such as $[2M+H]^+$, which are gas phase reactions [9]. Often it is almost impossible to control the intensity of sodium adducts. The formation of adducts is based on ionization by charge separation which occurs in solution and can be exploited to analyze by ESI polar compounds which are neutral or weakly acidic or basic. In the negative mode, chloride ions adducts can be formed when chlorinated solvents such as chloroform are used [10] or for the analysis of tocopherols and carotenoids where silver ions are added to form $[M+Ag]^+$ ions [11]. Analysis of analytes in highly aqueous solution is more challenging in the negative mode than in the positive mode. This is mainly due to an electrical discharge occurring at the tip of the sprayer (corona discharge) resulting in the chemical ionization of the analyte and the solvent [12, 13]. Generally, negative ESI operated at lower potential and compressed air is preferred to nitrogen as nebulizing gas.

Typical flow rates for electrospray and pneumatically assisted electrospray range from $\mu\text{L}/\text{min}$ to mL/min . Electrospray can also be operated at very low flow rates; indeed, nanoelectrospray (flow rates $<500 \text{ nL}/\text{min}$) was developed with the intention to minimize sample consumption and maximize sensitivity [14]. The infusion of a few microliters will result in a stable signal for more than

30 min using pulled capillaries [5] or chip-based emitters [15, 16]. With the infusion signal, averaging allows one to improve the limit of detection in tandem mass spectrometry. The uniqueness of nanoelectrospray is that at nL/min flow rates the droplet sizes are in the submicron range and that the complete spray is sampled into the mass spectrometer. Nanoelectrospray has become particularly important in combination with nanoflow liquid chromatography or chip-based infusion [17]. The ionization efficiency is strongly analyte dependent. Thus, in drug metabolism studies, the relative signal intensities from the sample cannot be correlated directly to the relative abundance of the metabolites. Hop et al. [18] reported that the uniformity of the ionization response could be improved, compared with ESI, by using a chip-based nanoelectrospray source. They argue that the generation of a high electric field around the nozzles produces a large excess of protons and smaller droplets, which minimizes the differences in the ionization efficiency for the analytes.

Hirabayashi et al. [19] described an alternative to ESI called sonic spray. In their device the liquid is sprayed using a high-velocity nebulizing gas. Ions are produced without the application of heat or an electric potential typically at sonic gas velocity. For the analysis of labile compounds and noncovalent complexes the use of a cold spray ionization source was also described [20]. The solution is sprayed into a liquid nitrogen cooled electrospray source. The operating temperature is in the range (ca. -80 to 10°C) that minimizes fragmentation of the analytes compared with conventional electrospray.

The qualitative or quantitative outcome of an electrospray analysis may be strongly dependent on the settings of the experimental parameters such as solvents, flow rate, electrode, electric field, and additives, as well as the nature of the analytes (metal ions, LMWCs, polymers,

oligonucleotides, peptides, or proteins). Therefore, understanding the mechanisms of how gas phase ions are formed from ions in solution is important, and reviews have been carried out by Kebarle and Verkerk in this regard [21]. Two major mechanisms have been proposed—(1) the ion evaporation model (IEM) proposed by Iribarne and Thomson [22] and (2) the charge residue model (CRM) described by Dole et al. [23]—and have been a subject of extensive discussion [4, 24, 25].

In 1968, Dole et al. [23] reported the electrospray analysis of diluted solutions of synthetic polymers, in the negative mode, into air at atmospheric pressure, where the macroion current was detected by a Faraday cage after the light ions have been repelled from the beam by negative voltages on a repeller grid. At that time, there was no evidence of any possible solution to the “vaporization problem,” for large polyatomic molecules such as proteins without extensive fragmentation and decomposition [26]. Regarding the formation of gas phase ions, Dole’s proposition was that evaporation of solvent would increase the surface-charge density until it reached the Rayleigh limit at which the forces due to Coulombic repulsion and surface tension become comparable. The hydrodynamic instability results in the formation of a jet of smaller droplets repeated until complete dispersion of the liquid. Ultimately, the droplets become so small that they contain one single solute molecule, and this molecule becomes an ion, thus a “charge residue,” when it retains some of the droplet charge as the last of the solvent is vaporized.

Iribarne and Thomson [22], interested in the study of small ions, proposed in 1976 the atmospheric pressure IEM consistent with the scenario described by Dole et al. [23] in that a sequence of evaporation and Coulombic explosions leads to droplets of 10^{-6} cm where charge densities are so high that the resulting electrostatic field at their surface is

high enough ($>10^9$ V/m) to push solute ions into the gas phase. The high electric field responsible for the ion evaporation generated by the size reduction of the droplets by heat becomes competitive with further solvent evaporation. In the experiments conducted by Iribarne and Thomson, charged droplets were generated by pneumatic nebulization and the electric field was applied across the plume of evaporating spray to extract small ions and is sometimes referred to as aerospray [27]. Most published work suggests that the two models strongly depend on the nature of the analyte and that most molecules follow the IEM proposed by Iribarne and Thomson, while large macromolecules undergo mostly the charge residue mechanism. A recent study by Nguyen and Fenn [28], where the authors demonstrated the benefit of adding solvent vapor to bath gas, showed that in electrospray—at least for singly and doubly charged peptides—most gas phase ions are likely produced by the IEM rather than by the CRM. Further work indicates that CRM is preceded by IEM, in particular when buffers such as ammonium acetate or triethyl acetate are used [29], or that more nuanced emission mechanisms appear, ranging from pure ion evaporation (PIE) for small ions to pseudo-Rayleigh ion release (PRIR), a mechanism that yields charge states that are nearly indistinguishable from the CRM, for large ions [30].

Blades et al. [7, 12] showed in the early 1990s, when using stainless steel capillaries, the presence of nickel Ni(II) and iron Fe(II) ions in the electrospray solution. They compared the electrospray process with that of an electrolysis cell where an electrochemical (EC) oxidation reaction occurs at the tip of ESI capillary. In general, most of the analytes investigated are not, under standard conditions, affected by the electrochemical process that occurs in electrospray and is most pronounced at very low