Mass Spectrometry in Medicinal Chemistry

Edited by
Klaus T. Wanner and Georg Höfner
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Mass Spectrometry in Medicinal Chemistry

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Klaus T. Wanner and Georg Höfner
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Preface

For a long time, mass spectrometry in organic chemistry was just used for the “fingerprint” identification of different compounds. Initiated by F.W. McLafferty and K. Biemann, and largely extended by C. Djerassi, H. Budzikiewicz and D.H. Williams, sets of structure-specific fragmentation rules were established, which enabled organic chemists to interpret the chemical structures of their compounds, even highly complex natural products and drugs. Within a few years, between 1962 and 1964, five books on mass spectrometry of organic compounds were published, three of them by the Djerassi group. In this manner, Carl Djerassi made another significant contribution to medicinal chemistry, besides his research results on optical rotation dispersion and his role in the development of the “pill”. Nowadays, mass spectrometry is well established in drug research, for the characterization of new compounds, their structure elucidation and structural confirmation, the identification of drugs and their metabolites in body fluids, and in anti-doping campaigns.

Largely unperceived by medicinal chemists, in the past two decades mass spectrometry developed into a powerful tool in drug discovery, by the detection and analysis of ligand–protein interactions. One of the major breakthroughs to enable such applications was the development of new desorption – ionisation techniques for large-sized, non-volatile molecules, i.e. proteins, RNA, and DNA fragments. The importance of these new tools was honored in 2002, by the Nobel prize in Chemistry for John B. Fenn, Professor at the Virginia Commonwealth University, for his contributions to electrospray ionisation (ESI), and to Koichi Tanaka, an engineer at Shimadzu Corp., Japan, for the development of matrix-assisted laser desorption ionisation (MALDI), sharing the prize with Kurt Wüthrich at ETH Zurich, Switzerland, for his contributions to protein 3D structure elucidation by NMR. In parallel, progress in instrumentation, for better mass (more correctly, mass/charge: \( m/z \)) separation and ion detection, and coupling with HPLC separation broadened the field of potential applications.

Whereas mass spectrometry in proteomics was discussed in an earlier volume of this series (Volume 28, M. Hamacher et al. 2006, *Proteomics in Drug Research*, Wiley–VCH, Weinheim), the current monograph focuses on mass spectrometry applications in lead discovery and optimization. As discussed in more detail in the foreword of the volume editors, the chapters provide a comprehensive over-
view on all current and potential, “non-classic” applications of mass spectrometry in various areas of drug research, especially small molecule screening, fragment-based drug discovery, ligand–protein interactions, protein 3D structure characterization, and the study of pharmacokinetics.

The series editors would like to thank Klaus T. Wanner and Georg Höfner, as well as all chapter authors, for compiling and structuring this comprehensive monograph on mass spectrometry techniques. In addition, we want to thank the publisher Wiley–VCH, especially Dr. Frank Weinreich and Renate Dötzer, for their ongoing support of our series “Methods and Principles in Medicinal Chemistry”.

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November 2006
A Personal Foreword

Mass spectrometry has been a well established technique in analytical chemistry for more than five decades, but its use to characterize target–ligand interactions is comparatively new. Only the availability of modern mass spectrometers achieving sufficient accuracy and sensitivity as well as the advent of soft ionization techniques such as ESI or MALDI has paved the way for successful studies in this field. From the first investigations in the early 1990s until now a great variety of mass spectrometry-based approaches covering target–ligand interactions have been implemented in the drug discovery process, so that drug–ligand interactions can be explored from almost every perspective: it is possible to focus on the ligand, the target–ligand complex or the target (i.e. its binding site). Among the numerous advantages that qualify mass spectrometry for this purpose are two that should be emphasized: First, mass spectrometry offers the possibility to monitor the interacting partners without labelling either the ligand or the target. Second, mass spectrometry has the capability to identify structurally unknown hits, i.e. compounds binding to the target, from huge combinatorial compound libraries. Conversely, mass spectrometry can also provide an insight into the molecular structure of the binding domains on macromolecular targets.

It is the intention of this book to give an overview of the opportunities that mass spectrometry provides in medicinal chemistry, focusing primarily on the early drug discovery process. Therefore, particular emphasis is put on screening procedures for low relative molecular mass drug candidates supplemented by other approaches suitable to elucidate target–ligand interactions and the field of pharmacokinetic investigations. Instead of giving a complete summary of this topic, which would be clearly beyond the scope of a single book, selected approaches are presented reflecting the diversity of possible strategies.

For those readers who are not yet familiar with mass spectrometry, the introduction provides an explanation of the basics of mass spectrometry and its instrumentation as well as practical aspects and applications in bioanalysis. Next, a block of three chapters shows different affinity selection procedures suitable to identify hits from combinatorial compound libraries. This subject, being metaphorically speaking a search for a needle in a haystack, is of outstanding relevance for “big pharma”. The techniques described here offer real high throughput capabilities and are implemented already in the routine industrial screening
process. The next three chapters present more techniques also dealing with small molecule screening. One approach combines the biological assay directly with the analytical method using microcoil reactors integrated in a HPLC system to study target–ligand interactions. Another is based on the unique features of frontal affinity chromatography and has already proved its potential in several screening projects. The last one is a very simple but also very effective approach that enables binding assays with native, i.e. nonlabelled markers in analogy to conventional radioligand binding assays. Although ESI clearly dominates mass spectrometric screening procedures, MALDI and other ionization techniques based on laser desorption can also be utilized for this purpose. This is documented in the following chapter summing up recent advances in this field. In a further chapter the challenging concept of fragment-based drug discovery is presented which makes use of dynamic equilibrium processes in order to accumulate fragments with rather moderate affinity to a target binding site by forming a covalent bond to a linker. Even though this concept is basically a synthetic approach, its success is unambiguously connected to the use of mass spectrometry. The topic of target–ligand interactions presented in the preceding chapters is rounded off by two chapters showing mass spectrometric strategies benefiting from hydrogen deuterium exchange at the target. In one approach the deuterium uptake by the target as a function of the test compound is quantified in order to deduce binding affinity or stoichiometry. The other approach describes the possibility to characterize protein structure and conformational changes of proteins as well as how to localize the physical interactions between target and ligand, based on the exact assignment of target incorporated deuterium atoms in proteolytically generated peptide fragments. The last chapter touches on the issue of pharmacokinetics where mass spectrometry traditionally plays a prominent role. The fact that these mass spectrometric investigations can help to avoid failures in later clinical trials further illustrates the immense value of mass spectrometry for the drug discovery process.

As editors we would also like to take the opportunity to cordially thank all authors for their contributions. We hope that the applications collected in this book will give the reader an idea of the capabilities of mass spectrometry when used in the early stages of drug discovery. Considering that mass spectrometry only began to have an impact on early drug discovery in the past decade, we can expect that this process will be further accelerated in the near future by the rapidly proceeding evolution of mass spectrometry as an analytical tool to screen bioactivity.

Munich, November 2006
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Part I
Introduction to MS in bioanalysis
1
Mass Spectrometry in Bioanalysis – Methods, Principles and Instrumentation

Gérard Hopfgartner

1.1 Introduction

Mass spectrometry started about 100 years ago with the work of Sir J.J. Thomson. His interest was the quantitative measurement of the mass and charge of the cathode rays (electrons). For that purpose he constructed the first mass spectrometer (parabola mass spectrograph) and he received in 1906 the Nobel Prize for Physics in recognition of his work [1]. In the next decades the major focus in the development and application of mass spectrometry was dedicated to the studies of isotopes [2]. In 1918 Dempster [3] developed an instrument in which a strong magnetic field was produced, between two semicircular iron plates, to separate positive ion rays with great resolving power. He also described the bombardment of chemical compounds with electrons forming positive ions. This technique is known today as electron impact ionization and is still widely used in modern mass spectrometry. In the early 1940s the first commercial instruments based on magnetic deflection and electron impact ionization became available. These instruments were mostly applied for the analysis of hydrocarbons in petroleum products. Beyond instrumental development the end of the 1950s saw the application of mass spectrometry for structure elucidation of natural products and the studies of fragmentation patterns. At the same time the concept of several mass analyzers was described, such as time of flight or ion cyclotron resonance.

While the first coupling of gas chromatography and mass spectrometry had been reported in the late fifties [4] one had to wait for almost another 20 years before the direct interfacing of liquid chromatography with mass spectrometry (LC-MS) was described by Arpino et al. [5]. With the direct liquid interface (DLI) the effluent of the chromatographic column was directly introduced in the electron impact source. Contrarily to gas chromatography coupled to mass spectrometry (GC-MS), LC-MS did do not catch on as rapidly. One of the reasons was that the MS interface could only handle LC flow rates of a few microliters per minute. Another limitation was that electron impact or chemical ionization was not suit-
able for very thermolabile and high molecular weight compounds. It took almost
10 years before the LC-MS analysis of larger molecules, using continuous flow
fast atom bombardment (FAB), was first reported [6, 7]. For small molecules it
was thermospray (TSP) [8] and particle beam (PB) [9] which allowed the routine
use of LC-MS. Thermospray formed in most cases ammonium adducts, while
particle beam yielded electron impact spectra. Within a few years thermospray
was rapidly replaced by atmospheric pressure ionization techniques.

Quadrupole mass spectrometers [10] or quadrupole ion traps are today the
most widely used mass spectrometers. The physical bases were described in the
early 1950s by Paul and Steinwedel. For his work Paul received the Nobel Prize
in 1989 [11]. Triple quadrupole mass spectrometers have become very popular
instruments for qualitative and quantitative analysis. Yost et al. [12] built in 1978
the first instrument and it took four years before this type of instrument was
commercialized. The coupling with liquid chromatography or gas chromatogra-
phy is well established and benchtop ion traps or quadrupoles are nowadays part
of the standard equipment of many analytical laboratories.

For the analysis of macromolecules and in particular for proteins a major mile-
stone was achieved with the development in 1987 of matrix assisted laser desorp-
tion ionization by Karas and Hillenkamp [13] and in 1988 of electrospray ioniza-
tion by J. Fenn (Nobel Prize in 2002) [14].

Over the past decade progress in mass spectrometry and its hyphenation with
separation techniques has made these tools essential in life sciences. The present
chapter will describe current ionization techniques as well as mass analyzers.

1.2
Fundamentals

Mass spectrometry is a sensitive analytical technique which is able to quantify
known analytes and to identify unknown molecules at the picomoles or femto-
moles level. A fundamental requirement is that atoms or molecules are ionized
and analyzed as gas phase ions which are characterized by their mass ($m$) and
charge ($z$). A mass spectrometer is an instrument which measures precisely the
abundance of molecules which have been converted to ions. In a mass spectrum
$m/z$ is used as the dimensionless quantity that is an independent variable. There
is still some ambiguity how the x-axis of the mass spectrum should be defined.
Mass to charge ratio should not no longer be used because the quantity measured
is not the quotient of the ion’s mass to its electric charge. Also, the use of the
Thomson unit (Th) is considered obsolete [15, 16]. Typically, a mass spectrometer
is formed by the following components: (i) a sample introduction device (direct
probe inlet, liquid interface), (ii) a source to produce ions, (iii) one or several
mass analyzers, (iv) a detector to measure the abundance of ions, (v) a computer-
ized system for data treatment (Fig. 1.1).

Most mass analyzers operate under high vacuum or at low pressure, so that the
charged particles do not deviate from their trajectories due to collision with resid-
ual gas and thus never reach the detector. Mass spectrometers can be grouped into different types of operation mode: continuous mode (magnetic sector, quadrupole), pulsed mode (time of flight), and ion trapping mode (quadrupole traps, Fourier transform ion cyclotron, orbitrap). In the source, positive or negative ions are produced either under vacuum or at atmospheric pressure. Depending on the ionization technique, either molecular ions \((\text{M}^+)\) with an odd electron number or protonated ions \(([\text{M}+\text{H}]^+)\) with an even electron number are formed. In the mass spectrum when no fragmentation occurs, in general the most intense peak represents the molecular ion, the protonated molecule or a molecule with an adduct ion followed by ions containing the heavier isotopes. \(M_r\) is the mass of one molecule of a compound, with a specified isotopic composition, relative to one-twelfth of the mass of one atom of \(^{12}\text{C}\). An important aspect is that many atoms have naturally occurring isotopes which can be differentiated by mass spectrometry. Molecules analyzed by organic mass spectrometry contain in general carbon, hydrogen, nitrogen, oxygen and sulfur. These elements have stable isotopes (Table 1.1) which have different atomic mass. Therefore, under certain conditions and for a given molecule, the isotopic contribution can be measured by mass spectrometry.

For example, carbon is composed of two naturally occurring isotopes: \(^{12}\text{C}\) for 98.9% and \(^{13}\text{C}\) for 1.1% abundance, respectively. For cyclohexane \((\text{C}_6\text{H}_{12})\) the \(\text{M}^+\) ion composed exclusively of \(^{12}\text{C}\) and \(^1\text{H}\) atoms is observed at a nominal mass of \(m/z\) 84. The nominal mass is the integer of the sum of the masses calculated from the most abundant naturally occurring isotopes. The monoisotopic

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**Fig. 1.1** Principle of a mass spectrometer, the outcome of an analysis is a mass spectrum with \(m/z\) in the x-axis and ion intensities in the y-axis. The ion intensities can be given in percentages (relative intensity) or in counts or in counts per second (absolute intensity). The most abundant peak at \(m/z\) 578.6 is called the base peak.
peak represents the exact mass of an ion or a molecule calculated from the most abundant isotope of each element. The relative intensity of this ion compared to the others ions is 100%. A weaker isotopic peak \( (M^{+} + 1) \) is observed at \( m/z \) 85 with an abundance of 6.5% corresponding to one \(^{13}\text{C} \), five \(^{12}\text{C} \) and 12 \(^{1}\text{H} \) atoms. An even weaker peak (0.2% abundance) is visible at \( m/z \) 86 \( (M^{+} + 2) \) corresponding to two \(^{13}\text{C} \), four \(^{12}\text{C} \) and 12 \(^{1}\text{H} \) atoms. In this example, the contribution of deuterium can be neglected. For large molecules with increasing the number of carbon atoms, a shift of the maximum of the isotopic distribution towards higher masses can be observed, as depicted in Fig. 1.2. Above several hundred atoms of carbons, mostly a Gaussian distribution is observed. The consequence is that, in particular for protein analysis, only the relative molecular mass and not the monoisotopic mass is observed since either the monoisotopic masses can no longer be resolved or the intensity of the peak is too weak. The average mass is the calculated mass of an ion based on the relative atomic mass of each atom.

The isotopic contribution of various atoms is additive. For low molecular weight compounds, the isotopic contribution originates mainly from the carbon atom as long as no other element with a second isotope of significant abundance is present. For a molecule of \( M_r \) 192 the intensity of the \( m/z \) 194 ion represents 12% of the \([M+H]^+\) peak \( (m/z \) 193; Fig. 1.3A). Chlorine (Cl) has two intense isotopes: \(^{35}\text{Cl} \) and \(^{37}\text{Cl} \) (76% and 24% abundance, respectively). Replacing one \(^{1}\text{H} \) by a Cl atom results in a change of the isotopic distribution of the molecule.

<table>
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<tr>
<th>Element</th>
<th>Atomic mass</th>
<th>Symbol</th>
<th>Isotopic mass</th>
<th>Abundance (%)</th>
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