Applications of Mass Spectrometry Techniques to the Elucidation of Novel Metabolic Pathways of Vitamin D and the Quantification of DNA Adducts

A dissertation presented

by

Caroline Ceailles Flarakos

to
The Department of Chemistry and Chemical Biology

In partial fulfillment of the requirements for the degree of Doctor of Philosophy

in the field of

Chemistry

Northeastern University
Boston, Massachusetts
November 2008
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ABSTRACT OF DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry in the Graduate School of Arts and Sciences of Northeastern University, November 2008
ABSTRACT

This dissertation demonstrates the widespread application of mass spectrometry to the qualitative and quantitative analysis of small molecules. Specifically, this manuscript describes the implementation of GC-MS techniques towards the elucidation of novel metabolic pathways of vitamin D and the development and validation of an LC-MS/MS analytical assay for the quantification of DNA adducts. Chapter 1 provides an introduction to mass spectrometry with an overview of the major methodologies utilized to address our research goals.

Chapter 2 highlights the superseding role of mass spectrometry in the structural characterization and quantification of vitamin D, its metabolites and other emerging analogs. After a review of the vitamin D biochemistry and the development of synthetic analogs, an overview of the current techniques for the detection and characterization of vitamin D compounds is given, with specific emphasis on the contribution made by mass spectrometry.

Chapter 3 describes the elucidation of a novel metabolic pathway of vitamin D and its effect on further metabolism of the hormone. Specifically, we demonstrated that vitamin D can be metabolized into its C-3 epimer by inversion of stereochemistry around C-3 of the A-ring. In the second part of Chapter 3, we investigated the effect of the C-3 epimerization conversion on the metabolism of 1α,25-dihydroxyvitamin D₃ (1α,25(OH)₂D₃), biologically active form of vitamin D₃. Through a comparative study
between 1α,25(OH)₂D₃ and its C-3 epimer, we confirmed that both compounds undergo the same C-24 oxidation pathway, a well described metabolic pathway leading to the catabolic inactivation of vitamin D compounds. However, the C-3 epimerization was found to slow down and partially block the metabolism of 1α,25(OH)₂-3epi-D₃ through the C-24 oxidation pathway, resulting in the accumulation of stable intermediary metabolites. These observations provided further evidence for the metabolic stability of 1α,25(OH)₂-3epi-D₃, proposed as one of the possible mechanisms responsible for its unique biological actions.

Chapter 4 focuses on the metabolism of 20-epi-vitamin D analogs and reports the elucidation of a novel pathway, namely C-1 esterification with fatty acids. Over the past decade, 20-epi analogs, in which the methyl group at C-20 is in its unnatural orientation, have been of particular interest because they have been shown to decrease cell proliferation and promote cell differentiation with a potency significantly greater than 1α,25(OH)₂D₃. In order to understand the mechanisms responsible for this enhanced potency, we investigated the metabolism of such analogs. Selected 20-epi-vitamin D analogs were reported to be metabolized via C-1 esterification with fatty acids. HPLC, GC-MS, ESI-MS and ¹H-NMR were used in a complementary fashion in order to elucidate this novel metabolic pathway.

Chapter 5 describes the development and validation of a highly sensitive LC-MS/MS assay for the quantification of DNA adducts derived from benzo[a]pyrene diol epoxide (B[a]PDE), a carcinogenic and mutagenic metabolite of benzo[a]pyrene (B[a]P). A brief
introduction to DNA adducts is provided, with specific focus on polycyclic aromatic hydrocarbons (PAHs). The synthesis and characterization of B[a]PDE-deoxyguanosine (B[a]PDE-dG) reference standards and B[a]PDE-[\(^{15}\)N\(_5\)]-dG internal standard constituted the first step in the method development and is described in detail. The development and validation of the LC-MS/MS quantitative method is then reported. In the last section of Chapter 5, the validated LC-MS/MS assay is applied to the quantification of B[a]PDE-deoxyguanosine adducts formed in human lymphoblastoid TK6 cells treated with B[a]PDE. We investigated the relationship between DNA adduct formation, toxicity, and gene expression and observed a positive dose-response correlation, providing further evidence for the potential involvement of DNA adducts in carcinogenesis.

Chapter 6 presents suggested future directions in the areas of vitamin D and DNA adducts research based on the results presented in this dissertation.
ACKNOWLEDGEMENTS

The completion of this dissertation has been a long journey and would not have been possible without the personal and practical support of numerous people.

My first, and most earnest, acknowledgement goes to my graduate advisor, Dr. Paul Vouros. During the past six years, Dr. Vouros has always been encouraging, motivating and enlightening and his patience, genuine caring and concern, and faith in me enabled me to pursue my graduate education even in the most difficult times.

I also wish to give special thanks to Dr. Satya Reddy. I have been very fortunate to work closely with Dr. Reddy for the past three years on various vitamin D research projects. He is one of the most knowledgeable persons I have interacted with during my graduate years. His excitement about science and life in general continues to inspire me every day.

I am also very grateful to the remaining members of my PhD dissertation committee, Drs. Penny Beuning, Pam Mabrouk and Philip Le Quesne. Their academic support and input and personal cheering are greatly appreciated.

Many other individuals on the faculty and staff of the Department of Chemistry and Chemical Biology assisted and encouraged me in various ways during my course of studies. I am especially grateful to Drs. Graham Jones, William Hancock, David Forsyth and Robert Hanson for all they have taught me. Specials thanks also go out to Jean Harris, Shari Khalil, and Rich Pumphrey who have always been a tremendous help no matter the task or circumstance.
My gratitude is also extended to the members of the Vouros research group, both past and present, for their years of friendship and support: Christine Andrews, Dayana Argoti, Terrence Black, Jianmei Ding-Kochling, Jimmy Flarakos, Lynn Gennaro, Jim Glick, Rose Gathungu, Adam Hall, Dan Kirby, Daren Levin, Mark Muenter, Kristen Randall, Elaine Ricicki, Susie Schiavo, Dennis Szymanski, and John Williams. Their contribution was essential to my personal and professional development.

No acknowledgments would be complete without giving thanks to my family. My parents instilled many admirable qualities in me. They have taught me about hard work, self-respect and persistence. They have always expressed how proud they are of me and how much they love me. I am grateful to them both and I too love them dearly.

My brother, Nicolas, is probably the person I owe my success to. He has always been my greatest fan and supporter, reassuring me whenever in doubt. Had he not been by my side during my first year in college, my curriculum would probably have been very different.

My final, and most heartfelt, acknowledgement goes to my husband and best friend Jimmy. Our friendship started when I joined the Vouros group and, with great surprise and joy, our close friendship evolved into a deep loving relationship. The completion of this dissertation coincides with the beginning of our married life; nothing could have brought more happiness into my life. Jimmy’s support has been central to my completion of this study; he has given me confidence and motivated me in so many ways. There are no words that can express my gratitude and appreciation for all you have done and been for me. I love you and am forever indebted to you for giving me your love and your heart.

À vous tous, Merci du fond du Coeur!
To my brother and my parents
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<tr>
<td>AA</td>
<td>Aromatic amine</td>
</tr>
<tr>
<td>amol</td>
<td>amole(s) (= 10^{-18} mole(s))</td>
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<tr>
<td>APCI</td>
<td>Atmospheric pressure chemical ionization</td>
</tr>
<tr>
<td>B[a]P</td>
<td>Benzo[a]pyrene</td>
</tr>
<tr>
<td>B[a]PDE</td>
<td>Benzo[a]pyrene diol epoxide</td>
</tr>
<tr>
<td>CE</td>
<td>Capillary electrophoresis</td>
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<tr>
<td>CI</td>
<td>Chemical ionization</td>
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<td>CLPBA</td>
<td>Chemiluminescence protein binding assay</td>
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<tr>
<td>CPBA</td>
<td>Competitive protein binding assay</td>
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<tr>
<td>ct-DNA</td>
<td>Calf-thymus DNA</td>
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<tr>
<td>dA</td>
<td>Deoxyadenosine</td>
</tr>
<tr>
<td>dG</td>
<td>Deoxyguanosine</td>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>EA</td>
<td>Ethyl acetate</td>
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<td>EI</td>
<td>Electron ionization</td>
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<td>EIA</td>
<td>Enzyme immunoassay</td>
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<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>ESI</td>
<td>Electrospray ionization</td>
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<tr>
<td>fmol</td>
<td>fmole(s) (= 10^{-15} mole(s))</td>
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<td>GC</td>
<td>Gas chromatography</td>
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<tr>
<td>GC-MS</td>
<td>Gas chromatography – Mass spectrometry</td>
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<td>HAA</td>
<td>Heterocyclic aromatic amine</td>
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<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<td>IS</td>
<td>Internal standard</td>
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<td>LC-MS</td>
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<td>LIF</td>
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<td>m/z</td>
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<td>NaBH₄</td>
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<td>Polycyclic aromatic hydrocarbon</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
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<td>VDR</td>
<td>Vitamin D receptor</td>
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Chapter 1:

Introduction to Mass Spectrometric Techniques
1.1. Introduction

Over the years, mass spectrometry (MS) has evolved from being a specialized technique used exclusively by “mass spectrometrists” to one which is used by a wide range of professionals from forensic chemists to art authentication experts. Mass spectrometry has been an essential tool in the process of structure elucidation and is used in any state of the art analytical laboratory.

There are three primary reasons for the emergence of mass spectrometry as such an indispensable analytical technique. First, mass spectrometry has the ability to provide consistently reproducible intact or fragment molecule data with a high degree of specificity while requiring minimal sample preparation. In conjunction with the simplification of the instrument operation and data processing software, MS instruments have become more user-friendly. The intuitive nature of the current graphic user interfaces has permitted the relatively inexperienced yet diligent scientist to operate mass spectrometers with a high degree of proficiency. Lastly, the discovery and development of a wide variety of ionization techniques such as electrospray (ESI), nanoelectrospray (nESI), and electron ionization (EI) have permitted the interfacing of well established separation techniques such as high performance liquid chromatography (HPLC) and gas chromatography (GC) with relative ease.

Today, mass spectrometry is almost exclusively used in some hyphenated mode such as GC-MS or LC-MS. Hyphenated techniques are essentially orthogonal techniques which involve the chromatographic separation of component mixtures followed by MS
separation of these same components based on the mass-to-charge ratios ($m/z$) of their corresponding ions.

To perform MS analysis, we must use an instrument which is typically comprised of five parts as illustrated in **Figure 1.1**:

- Sample inlet system
- Ionization technique
- Mass analyzer
- Ion detector
- Data system

**Figure 1.1.** The components of a mass spectrometer.
A thorough description of all ionization techniques, mass analyzers and detectors would be beyond the scope of this introduction. Therefore, we will only review selected MS components as they relate to the applications described in this dissertation.

1.2. Sample introduction and ionization techniques

The major strength of MS is the ability to simply couple the ion source directly to the column effluent of well established chromatographic separation techniques such as GC or LC. It is imperative that a particular separation technique is partnered with an appropriate ionization technique.

1.2.1. Electron Ionization (EI)

EI is by far the most common and perhaps standard form of ionization for gas chromatography mass spectrometry (GC-MS) applications requiring structural identification and database searching. In EI, the analyte vapor is subjected to bombardment by energetic electrons (typically 70 eV) emitted from a filament. Most electrons are elastically scattered, others cause electron excitation of the analyte molecules upon interaction, while a few excitations cause the complete removal of an electron from the molecule. The last type of interaction generates a radical cation, generally denoted as \( M^+ \), and two electrons:

\[
(1) \quad M + e^- \rightarrow M^+ + 2e^- 
\]

The \( M^+ \) ion is called the molecular ion and its \( m/z \) ratio corresponds to the molecular mass of the analyte. The electrons can further bombard the analyte causing a hard
ionization that fragments the molecule. EI is characterized by its reproducibly distinct fingerprint-like spectra which can easily serve as search queries against vast spectral libraries. These searches often produce candidate “hits” based on % probability matches ranging from 1-100, with 100 being a perfect match.

1.2.2. Chemical Ionization (CI)

Although not as common, chemical ionization (CI), a softer ionization technique, can also be used with GC-MS. In chemical ionization, a reagent gas – typically methane or ammonia – is introduced into the mass spectrometer. Depending on the ionization mode (positive CI or negative CI) chosen, this reagent gas will interact with the electrons and analyte and cause a 'soft' ionization of the molecule of interest to yield a distinct [M+H]⁺ ion. Chemical ionization fragments the molecule to a lower degree than the hard ionization of EI and therefore yields fewer fragments. One of the main benefits of using chemical ionization is that a mass fragment closely corresponding to the molecular weight of the analyte of interest is produced.

1.2.3. Electrospray Ionization (ESI)

The most widespread LC-MS ionization technique is electrospray ionization (ESI). Fenn and co-workers first introduced the technique in 1984, based on work previously conducted by Dole and co-workers. ESI is considered an electrophoretic process which occurs in three steps: (1) ion generation, (2) droplet evaporation yielding highly charged droplets, (3) conversion of these droplets to gas phase ions.
Figure 1.2 depicts the formation of positive ions on the surface of the liquid in the presence of an electric field. This causes repulsion between the positive surface ions, at the needle tip, resulting in the formation of the Taylor cone\textsuperscript{14}. Repulsion of the surface ions increases with liquid evaporation leading to very high charge density on the droplet. This eventually leads to surface instability (Rayleigh instability)\textsuperscript{15} and results in the ejection of smaller subdroplets. The process continues until one charge in the gaseous state is formed. This continuous current is maintained similar to an electric circuit with the ESI tip surface being replenished with the oxidation of negatively charged species in order to maintain a charge balance between the tip and the interface. The formation of ions in the gaseous state via ESI is extremely reproducible and is the primary reason for its use in small molecule quantitative mass spectral measurements.

Figure 1.2. Schematic representation of electrospray process. (Reproduced from 16).
Fenn’s initial experiments described how he suspended charged droplets of protein in solution and quickly stripped away the solvent leaving only the bare ion. ESI does not use any heat to initiate the droplet evaporation process which permits labile molecules such as proteins and peptides to enter the ion source essentially intact with minimal fragmentation of precursor ions. In addition to providing reproducible ionization, ESI operates under atmospheric pressure conditions and at mobile phase flow rates ranging from 50 μL/min to 1 mL/min, thereby making ESI a suitable ionization technique to interface to HPLC.

ESI may be used in two modes yielding either positive or negative ions. Positive ion formation occurs with the analyte abstracting a proton from the acidic mobile phase components yielding an [M+H]^+ ion. Similarly, negative ion formation is induced by the basic mobile phase components abstracting a proton from the analyte and yielding an [M-H]^- ion.

To enhance ionization efficiency, volatile mobile phases are essential. Organic modifiers such as methanol and acetonitrile and volatile aqueous buffers such as ammonium acetate and formate are used to improve desolvation and reduce mobile phase surface tension thereby improving the droplet formation and ionization efficiency. These aqueous buffers must be kept at concentrations lower than 50 mM to reduce any potential ion suppression. Ion suppression may occur as a result of charge competition between the buffer ion and the analyte of interest. It leads to a non-linear response of the analyte signal vs. concentration. Reducing ion suppression effects is therefore particularly important when performing quantitative analysis.
ESI’s key qualitative strength resides in its ability to generate multiply charged ion distributions critical for the characterization of peptides, proteins or oligonucleotides\textsuperscript{17-21}. High molecular weight biomolecules can support multiple charges when they are ionized, thereby reducing the overall $m/z$ ratio of the precursor ions and permitting their detection within the typical mass range ($m/z < 3000$) of conventional mass spectrometers (e.g., quadrupole or ion trap).

When using ESI, sample introduction can be performed via two methods: direct infusion or online coupling to a separation technique such as HPLC\textsuperscript{6}. Typically, direct infusion involves the incremental introduction of a dissolved analyte solution via syringe pump at a rate of 1-20 μL/min into the ESI source. The sample must be sufficiently concentrated and pure, enabling the user to detect the analyte ion in the presence of interfering solution ions. Direct infusion is primarily used to identify the precursor ions, product ions and conduct fragment characterization of the analyte prior to separation. It is also used to optimize signal intensity and other operating conditions. Sample introduction involving online coupling to HPLC will be discussed in more depth in the hyphenated MS techniques section.

\textit{Nanoelectrospray Ionization (nESI)}

Nanoelectrospray ionization, also known as nanospray, is a low flow version of electrospray introduced by Mann and Wilm in 1996\textsuperscript{22}. It was developed to mitigate one of the disadvantages of electrospray, ion suppression. Nanospray has several advantages relative to conventional electrospray\textsuperscript{11,12,23-25}. In nanospray, the limited surface area yields
a higher charge-to-volume ratio (ca. 20-fold), greatly increasing the surface activity of the droplet. This permits the droplet to support more intra-droplet charge competition without distorting the linearity of the analyte response vs. concentration. Furthermore, smaller droplets allow easier expulsion of analyte ions from the droplet surface during coulombic explosion resulting in higher signal intensity. Practically, the physical location of the spray tip resides 1-2 mm from the orifice – much closer than in conventional ESI – of the mass spectrometer giving a higher percentage of the desolvated ions the opportunity to enter the mass analyzer region.

Extremely low flow rates are needed (ca. 10-100 nL/min) in order to operate under nESI conditions. These low flow rates can be achieved either by splitting the flow from a conventional chromatographic system or by utilizing nano-HPLC instrumentation. Nanospray is often difficult to automate since the reproducibility of HPLC pumping systems has been questionable and expensive. Therefore, most laboratories prefer to operate in microspray mode, a similar technique which operates at flows of 100-500 nL/min and is commonly used with capillary columns. The higher flow rates of microspray relative to nanospray make reproducible gradients easier to achieve while retaining the benefits of low flow electrospray.

1.2.4. Atmospheric Pressure Chemical Ionization (APCI)

Unlike ESI, a liquid phase ionization process, APCI is a gas phase ionization technique based on the early work of Field and Munson. During their experiments, they observed that some molecules introduced into an electron impact source at high pressure would generate protonated molecular ions such as [M+H]+ rather than the conventional M+. 
molecular ion. The spectra generated from these stable molecular species provided less structural information, however yielded ions which could easily be used to determine the molecular weight of the analyte.

In the APCI interface, illustrated in Figure 1.3, the column effluent is nebulized into a heated vaporizer tube where the solvent evaporation is almost complete. The gas-vapor mixture enters the APCI source at atmospheric pressure where ionization is initiated by electrons generated at a corona discharge needle. Subsequently, the ions generated are sampled into the high vacuum of a mass spectrometer for mass analysis.

Figure 1.3. Schematic representation of an APCI source. (Reproduced from 31)
The APCI interface was first developed by Horning’s group in the early 1970s\textsuperscript{32-37}. Henion and co-workers\textsuperscript{38,39} initially worked with a direct liquid probe and eventually developed a prototype heated nebulizer APCI source\textsuperscript{40,41} in 1986. APCI is a robust and reproducible technique and is the standard source for analyzing small pharmaceutical-based molecules under 800 Da in molecular weight. It provides a relatively large dynamic range and is simple to use and easy to maintain. Furthermore, the ions formed with APCI are predominantly singly charged making identification of the protonated molecule unambiguous.

Four reactions govern the gas phase ionization process occurring in APCI\textsuperscript{42}. These reactions were initially studied by Horning and Munson and have been adapted below for positive and negative APCI ion formation.

Proton Transfer (positive):

(2) \[ \text{N}_2 + e^- \rightarrow \text{N}_2^{++} + 2e^- \]

(3) \[ \text{N}_2^{++} + \text{H}_2\text{O} \rightarrow \text{N}_2 + \text{H}_2\text{O}^{++} \]

(4) \[ \text{H}_2\text{O}^{++} + \text{H}_2\text{O} \rightarrow \text{H}_3\text{O}^+ + \text{HO}^- \]

(5) \[ \text{H}_3\text{O}^+ + \text{M} \rightarrow (\text{M}+\text{H})^+ + \text{H}_2\text{O} \]

Proton Abstraction (negative):

(6) \[ \text{OH}^- + \text{M} \rightarrow (\text{M}-\text{H})^- + \text{H}_2\text{O} \]

(7) \[ \text{CH}_3\text{O}^- + \text{M} \rightarrow (\text{M}-\text{H})^- + \text{CH}_3\text{OH} \]
Secondary reactions of low energy electrons leading to proton abstraction can also occur.

\[
\begin{align*}
(8) \quad & \text{O}_2 + e^- \rightarrow \text{O}_2^- \\
(9) \quad & \text{O}_2^- + 2\text{M} \rightarrow 2(\text{M-H})^- + 2 \text{HO}^-
\end{align*}
\]

\[
(10) \quad \text{O}^- + \text{M} \rightarrow (\text{M-H})^- + \text{HO}^-
\]

Primary ion formation (Equation 2) occurs in the positive ion mode by removal of a valence electron from the nitrogen gas by the corona discharge needle\textsuperscript{32}. These reactions yield secondary ions and lead to the ultimate formation of hydronium ions \(\text{H}_3\text{O}^+\) in the APCI source. Under atmospheric pressure conditions, these hydronium ions are responsible for proton transfer (Equation 5) to the analyte of interest (\(\text{M}\)) and subsequent formation of the perennial protonated molecule detected in the mass spectrometer as \((\text{M}+\text{H})^+\). Similarly, in the negative ion mode the hydroxide or methoxide ions in the source chamber react with \(\text{M}\) to abstract a proton (Equations 6 and 7) yielding the de-protonated molecule \((\text{M-H})^-\). In addition, less abundant radical ions can also contribute to de-protonation as illustrated in Equations 8-10.

1.3. Mass analyzers

1.3.1. Triple quadrupole

Triple quadrupoles represent the most widely used mass analyzers today. Their popularity is primarily due to their ease of use, wide mass range, unique specificity and good linearity for quantitative applications. Quadrupoles are equipped with two pairs of parallel hyperbolic or circular metallic rods. One pair of rods is set at a positive electrical
potential while the other pair is set at a negative potential. A combination of dc and rf voltages is applied to create a dc/rf field, permitting only ions of preselected m/z ratio(s) to traverse the field and reach the detector.

**Theory of mass selectivity in the quadrupolar field**

There are several important parameters which allow a preselected ion to negotiate a path through the quadrupole field. These parameters are: dc (U), rf amplitude (V), rf frequency (ω) and the distance between the quadrupole rods (r). The oscillatory motion of the ions makes it easy for an ion of inappropriate resonance frequency to destabilize, touch the rods and become grounded. Therefore, adjusting U and V can create a quadrupole field which will allow only ions of a given m/z ratio to appropriately resonate giving a stable trajectory through the quadrupole. **Figure 1.4** depicts the configuration of a quadrupole mass analyzer. The motion and trajectory of ions are also depicted as they traverse the rods on their way to the detector.

The positive and negative rod pairs act as high and low mass filters respectively. Since the dc/rf ratio is constant, the quadrupoles are operated under constant resolution. Although quadrupole instruments operate at unit resolution, they are still considered low-resolution instruments.
Figure 1.4. Schematic representation of a quadrupole mass analyzer. (Reproduced from http://www.waters.com (accessed September 2008))

The region of the quadrupole field which is stable for a given $m/z$ is often called the stability region. Mathematical relationships known as the Mathieu equations are used to predict which $m/z$ values are permitted to obtain a stable trajectory through a quadrupolar field.

\[
\text{Q (rf component)} = \frac{4zeV}{mr^2\omega^2} \quad \text{(11)} \\
\text{A (dc component)} = \frac{8zeU}{mr^2\omega^2} \quad \text{(12)}
\]
**Equation 11** represents the rf dependent parameter where variables $e$, $m$, $r$, $\omega$, $z$, and $V$ are: charge, mass, radius between rods, rf frequency, number of charges, and rf potential, respectively. Similarly, **Equation 12** represents the dc dependent parameter, where $U$ represents the dc potential. Mathieu also developed an intuitive stability diagram to illustrate his mathematical relationships. **Figure 1.5** shows the ion stability diagram and the stable scan line (constant $dc/\text{rf}$). The axes are assigned as A and Q for the dc and rf voltages, respectively. Along the scan line, we have stable ion trajectories yielding high ion transmission. Ion paths that deviate from this scan line result in unstable trajectories giving no ion transmission. According to the Mathieu equations, increasing $m/z$ allows for a larger stability region area. This phenomenon is also illustrated in **Figure 1.5**, the area of the “triangle-like” region increases as $m/z$ increases. The slope of the scan line also has a significant effect on the resolution. As the slope decreases, so do the resolution and selectivity but this allows more ions to travel through the quadrupole field increasing ion transmission. Similarly, increasing the slope of the scan line increases the resolution and reduces transmission. If the slope of the scan line is high enough so that it does not cross any of the stability regions, then there is no ion transmission. If the quadrupole is operated in rf only mode ($A=0$), the scan line remains on the Q axis and ions can be focused without mass selectivity (no mass resolution).
**Triple quadrupole configuration and scan modes**

Quadrupoles may be linked in series to improve specificity and signal-to-noise ratio of ion output signals. **Figure 1.6** depicts a schematic representation of a triple quadrupole. The first quadrupole (Q1) is under high vacuum and is mass resolving. The second quadrupole (Q2) is filled with argon or nitrogen and acts as a collision cell which allows ions to be excited and fragmented. It operates in \( rf \) only mode and is not mass resolving. The degree of fragmentation may be controlled by adjusting the pressure of the gas in the collision cell. The third quadrupole (Q3) is mass resolving and separates ions prior to reaching the multiplier. In this common configuration, Q1 and Q3 are the only mass...
discerning sets of quadrupoles. Q2, rf-only quadrupole, focuses ions without selecting a specific m/z.

**Figure 1.6.** Schematic representation of a triple quadrupole instrument.

A variety of scan modes can be set to extract the maximum amount of information during a particular sample analysis (**Figure 1.7**). The *full scan* mode can scan from 50 to 4,000 Da depending on the manufacturer. It is the most universal yet least efficient mass scan mode available for a triple quadrupole (efficiency = duty cycle x transmission, where the duty cycle is defined as the percentage of analyte ions formed during ionization that enter the mass analyzer and are actually detected). The *selected ion monitoring (SIM)* mode involves the isolation of an m/z value in Q1 with subsequent detection. The SIM mode yields the highest ion intensity, however it may lack sufficient specificity and often possesses a high baseline noise. In the *product ion scan* mode, a precursor ion is isolated in Q1 before being fragmented in Q2, all product ions are then detected in Q3. In the *precursor ion* mode, all precursors sharing a common fragment ion are detected. The *neutral loss scan* mode is based on the detection of all precursor ions sharing a common neutral fragment. Lastly, the most selective scan mode, also yielding the highest signal-
to-noise ratio, is the selected reaction monitoring (SRM) mode. This scan mode is based on the isolation of a precursor ion in Q1 followed by its fragmentation in Q2 but, unlike the product ion mode, only one pre-determined product ion is isolated in Q3 for detection. The SRM mode therefore allows for the monitoring of a specific transition. It is the scan mode which has made mass spectrometry a benchmark in pharmaceutical quantitative analysis.

Figure 1.7. Triple quadrupole MS and MS/MS operational scan modes.
1.3.2. 3D-Quadrupole Ion Trap

Quadrupole ion traps exist in both 2D linear or 3D varieties and use a constant dc and radio frequency (rf) oscillating electric fields to trap ions. We will only discuss the 3D ion trap. The ion trap has many complementary features compared to a triple quadrupole and is extremely useful in the structural elucidation of unknown molecules.

The invention of the 3D quadrupole ion trap is attributed to Paul and Steinwedel who were issued a patent in 1960 based on previously published work. Years later, Stafford and co-workers adapted the technology, for the Finnigan corporation, into a benchtop production instrument. Wolfgang Paul shared the Nobel Prize in Physics in 1989 for his conception of this novel instrumentation.

Three hyperbolic electrodes, consisting of a ring and two endcaps, form the core of the ion trap and confine ions in a limited space with oscillating electric fields (Figure 1.8).

**Figure 1.8.** Cutaway view of an ion trap mass analyzer.
Ions created by electrospray ionization are focused using an octopole transmission system into the ion trap. Ions may be gated into the trap through the use of a pulsing lens or through a combination of rf potentials applied to the ring electrode. The pulsed transmission of ions into the trap differentiates ion traps from "beam" instruments such as quadrupoles where ions continually enter the mass analyzer. The ion gate allows the trap to maximize signal while minimizing space-charge effects. Space-charge results from too many ions in the trap and may cause a distortion of the electrical fields leading to an overall reduction in performance. Many modern instruments have a form of gain control which detects uninitiated electric field distortion and closes the ion gate thus preventing the “overfilling” of the trap. The ion trap is typically filled with helium to a pressure of about 1 mTorr. Collisions with helium dampen the kinetic energy of the ions and serve to quickly focus trajectories toward the center of the ion trap, enabling trapping of injected ions. Trapped ions are further focused toward the center of the trap through the use of an oscillating voltage, called the fundamental rf, applied to the ring electrode. An ion will be stably trapped depending upon the values for the mass and charge of the ion, the radial size of the ion trap (r), the oscillating frequency of the fundamental rf (ω), and the amplitude of the voltage on the ring electrode (V). The dependence of ion motion on these parameters is described by the dimensionless parameter \( q_z \). An analogous parameter \( a_z \) describes the effect on the motion of ions when a dc potential (U) is applied to the ring electrode. The equations that govern ion trajectories within an ion trap (Equations 13 and 14) are three-dimensional variations of the Mathieu equations (Equations 11 and 12) used to explain the operation of quadrupoles\(^{48}\):
The "stability diagram" (Figure 1.9) shows a theoretical region where radial and axial stabilities overlap. Depending upon the amplitude of the voltage placed on the ring electrode, an ion of a given \( m/z \) will have \( a_z \) and \( q_z \) values that fall within the boundaries of the stability diagram and the ion will be trapped. If the \( a_z \) and \( q_z \) values at that voltage fall outside of the boundaries of the stability diagram, the ion will hit the electrodes and be lost. Commercial ion traps work along the line \( a_z = 0 \).

Ions oscillate in the trap with a frequency known as the secular frequency that is determined by the values for \( a_z \) and \( q_z \) and the frequency of the fundamental rf. A mass spectrum is generated by sequentially ejecting fragment ions from low \( m/z \) to high \( m/z \). This is performed by scanning the amplitude of the fundamental rf voltage to make ion trajectories become sequentially unstable. Ions are ejected through orifices in the endcap electrode and detected using a collision dynode and electron multiplier system.
Figure 1.9. Ion stability diagram in the quadrupole ion trap. (Reproduced from http://www.thermo.com (accessed September 2008))

MS/MS fragmentation can also be performed within the ion trap. During the trapping step, a supplementary potential is applied to the endcap electrodes matching the secular frequency and inducing resonance excitation. An ac voltage known as the “tickle voltage” applied to the endcap electrodes during this period causes the ion kinetic energies to increase. This leads to ion dissociation from multiple collisions with the helium damping gas. This process induces fragmentation in a manner analogous to that obtained using a triple quadrupole mass spectrometer. Since the product ions resulting from fragmentation are still within the trap they can be further isolated and fragmented again resulting in MS\textsuperscript{n} transitions where n = number of transitions. The MS\textsuperscript{n} capability
of an ion trap is extremely useful in structural elucidation studies of small molecules, peptides and proteins. Up to 12 stages of tandem mass spectrometry (MS$^{12}$) have been performed using an ion trap, greatly increasing the amount of structural information obtainable for a given molecule.

Other advantages of the ion trap mass spectrometer include its compact size, the ability to trap and accumulate ions to increase the signal-to-noise ratio and the large duty cycle in full scan operating mode.

1.4. Electron Multiplier Detector

The final element of the mass spectrometer is the detector. The detector records either the charge induced or the current produced when an ion passes by or hits a surface. The most common detector is the channel electron multiplier (Figure 1.10), a horn-shaped continuous dynode structure coated on the inside with an electron emissive material.

Each ion striking the multiplier creates secondary electrons which, in an avalanche effect, create more secondary electrons to eventually generate a current pulse. The curved shape of the multiplier is intended to reduce backscattering and reduce background interference. Channel electron multipliers represent the most common type of MS detectors due to their reliability, ease of installation, good linear dynamic range.
Figure 1.10. Outer view of an electron multiplier (upper panel) and cutaway view of the detector electrode (lower panel). (Adapted from 49)
1.5. Chromatographic Separation Modes

As mentioned earlier, mass spectrometry is often coupled to online separation techniques such as HPLC or GC. Such coupling generally involves the use of ESI or EI interfaces.

1.5.1. High Performance Liquid Chromatography (HPLC)

HPLC is one of the most widely used analytical techniques. It is used to separate and analyze compounds through the mass-transfer of analytes between stationary and mobile phases\(^{50,51}\). The interaction of the solute with the mobile and stationary phases can be manipulated through different choices of both solvent and stationary phases. HPLC can be divided into two broad categories: normal phase and reversed phase\(^{52}\).

Reversed phase chromatography

Reversed phase (RP) liquid chromatography is the most common separation mode of conventional HPLC. The separation occurs on a silica-based column possessing a covalently attached hydrophobic bonded phase. There are several available hydrophobic bonded phases ranging from C\(_1\) to C\(_{18}\) straight hydrocarbon chains bonded directly to the silanol stationary phase. Compounds are separated by injecting a plug of the sample mixture onto the column. The different components in the mixture migrate through the column at different rates based upon their ability to partition between the mobile liquid phase and the bonded stationary phase. The distribution ratio of the analyte between the stationary and the mobile phases is based on an equilibrium governed primarily by its molecular structure and chemical properties, the type of bonded phase, the composition of the mobile phase and the temperature of the system.
**Normal phase chromatography**

Normal phase (NP) liquid chromatography is another less common HPLC separation mode. In normal phase, the separation occurs on a bare silica-based stationary phase column or one with a polar bonded functional group such as cyano or amino propyl. Compounds are separated in a similar fashion as in reversed phase chromatography. However, in normal phase chromatography the mobile phase remains significantly more non-polar relative to the polar stationary phase. The selection of stationary phases for normal phase chromatography is fairly limited compared to reversed phase and is the primary reason for its comparative lack of popularity.

**Chiral chromatography**

Chiral chromatography is a subsection of both normal and reversed phase separation modes that is oriented towards the exclusive separation of chiral substances. Enantiomeric separations are achieved in chiral chromatography by the judicious use of chiral phases. Chiral selectivity is usually achieved by employing chiral stationary phases, although chiral mobile phases have been successfully employed. For any chiral separation, the stationary phase must be chosen so that the spatial arrangement of its composite atoms increases the probability of interaction to differ significantly between the isomers to be separated. An application of chiral separation will be described in Chapter 5 where a polysaccharide-coated phase column was used to resolve diastereomers. With that particular stationary phase, compounds form hydrogen bonds with the carbamate linkages between the side-chains and the polysaccharide backbone. A compound can form multiple hydrogen bonds with the linkages of different side chains,
greatly expanding the possible interactions. The polysaccharide backbone exists in a helical conformation, giving rise to steric restrictions that may inhibit access of one enantiomer to hydrogen-bonding sites. This can result in numerous potential enantioselective interactions and enhanced selectivity.

1.5.2. Gas Chromatography (GC)

Despite the emergence of HPLC, gas chromatography (GC) remains the most widely used separation technique in analytical chemistry, a position it has held for over three decades. The popularity and applicability of the technique is principally due to its unchallenged resolving power for closely related volatile compounds and the high sensitivity and selectivity offered when coupled to mass spectrometry. Unlike HPLC, samples are introduced as a vapor onto the GC column. Most gas chromatographs utilize open tubular capillary columns and the chromatographic separation partly depends on the column's dimensions (length, diameter, film thickness) as well as the stationary phase properties. On the column, the solubility of each component in the gas phase is dependent on its vapor pressure, which is in turn a function of the column temperature and the affinity between the compound and the stationary phase. Differences in vapor pressure cause the molecules of each component to partition between the mobile gas phase—generally helium—and the stationary phase. Every time a molecule enters the gas phase, it is swept towards the detector by the carrier gas flow. Consequently, compounds having different physical and chemical properties will reach the detector at different times. The use of a mass spectrometer as the detector in gas chromatography was developed during the 1950s by Roland Gohlke and Fred McLafferty. These sensitive devices were bulky,
fragile, and originally limited to laboratory settings. The development of affordable and miniaturized computers has helped in the simplification of the use of this instrument, as well as allowed great improvements in the amount of time it takes to analyze a sample. This has led to their widespread adoption in a number of fields. Applications of GC-MS include drug detection, fire investigation, environmental analysis, explosives investigation, and identification of unknown samples. GC-MS can also be used in airport security to detect substances in luggage or on human beings.

1.6. Conclusions

Mass spectrometry, coupled to HPLC or GC, is a powerful analytical technique used widely to identify unknown compounds, quantify known compounds, and elucidate the structure and chemical properties of molecules. Detection of compounds can be accomplished with very minute quantities – as little as $10^{-15}$ moles for a compound of mass 1000 Daltons – making mass spectrometry a very attractive technique for a wide range of applications.

Mass spectrometry has been the key analytical technique in the Vouros laboratory for several decades. The next few chapters will illustrate various applications of mass spectrometric hyphenated techniques (GC-MS and LC-MS) for both qualitative and quantitative analysis of vitamin D metabolites and analogs and DNA adducts. Chapter 2 will review in depth the role of mass spectrometry in vitamin D research. Chapters 3 and 4 will illustrate the elucidation of novel metabolic pathways of vitamin D and the
structural characterization of novel analogs mainly using GC-MS. Finally, chapter 5 will describe the isolation and purification of DNA adducts diastereomers using reversed phase and chiral chromatography followed by the development of a highly sensitive LC-MS/MS method for the quantitative analysis of DNA adducts in cells treated with the carcinogenic metabolite of benzo[a]pyrene.
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Chapter 2:

Vitamin D – Chemistry, Metabolism and Analysis
Vitamin D is a steroid that has long been associated with the regulation of calcium homeostasis and bone mineralization. More recently, it has become clear that receptors for vitamin D are present in a wide variety of cells, and thus the vitamin has biologic effects which extend far beyond the control of mineral metabolism\textsuperscript{1}.

### 2.1. Vitamin D Chemistry and Nomenclature

There are two main forms of vitamin D, namely vitamin D\textsubscript{2}, also referred to as ergocalciferol, and vitamin D\textsubscript{3}, also known as cholecalciferol, the natural form of vitamin D in animals and man. The molecular structures of vitamin D\textsubscript{2} and D\textsubscript{3} are closely related to those of the classical steroid hormones. As shown in Figure 2.1, vitamin D\textsubscript{3} is a 27-carbon derivative of cholesterol; vitamin D\textsubscript{2} is a 28-carbon molecule derived from the plant sterol ergosterol. Besides containing an extra methyl group, vitamin D\textsubscript{2} differs from vitamin D\textsubscript{3} in that it contains a double bond between carbons 22 and 23. Technically, vitamin D is classified as a secosteroid, where the B-ring of the steroid structure has been broken. This important structural aspect is reflected in the official names of the molecules. According to the IUPAC rules, the secosteroid vitamin D\textsubscript{3}, by relation to cholesterol, is named 9,10-seco-(5Z,7E)-5,7,10(19)-cholestatriene-3\textbeta-ol while the official name of vitamin D\textsubscript{2} is 9,10-seco-(5Z,7E)-5,7,10(19),22-ergostatetraene-3\textbeta-ol. The studies presented in the next two chapters focus on vitamin D\textsubscript{3} and its analogs. Therefore, when reference is made to vitamin D, the lack of subscript usually implies vitamin D\textsubscript{3}.
Contemporary views categorize vitamin D₃ as a prosteroid hormone rather than a true vitamin: the main source of vitamin D₃ is its endogenous biosynthesis from the precursor 7-dehydrocholesterol, present in the skin². Upon exposure of the skin to sunlight (UV-B), the precursor undergoes photolysis which results in the opening of the B-ring of the steroid structure, followed by thermo-isomerization to yield vitamin D₃ (Figure 2.2).
Figure 2.2. Biosynthesis of vitamin D$_3$. 
2.2. Vitamin D Activation

Vitamin D₃ itself is biologically inert. Its bioactivation involves the sequential action of specific hydroxylase enzymes, members of the cytochrome P450 family, and leads to the synthesis of its biologically active metabolite, 1α,25-dihydroxy-vitamin D₃ (1α,25(OH)₂D₃).

Upon dietary ingestion or biosynthesis, vitamin D₃ enters the circulatory system and is transported to the liver. In the liver, it is hydroxylated to form 25-hydroxy-vitamin D₃ (25(OH)D₃)³⁴, the major circulating form of vitamin D₃ and immediate precursor of the fully active and hormonal form of the vitamin, 1α,25(OH)₂D₃. The C25-hydroxylation, initial step in vitamin D activation, occurs quickly and is almost unregulated. Therefore, serum 25(OH)D₃ is often used as a biomarker for the assessment of vitamin D status. 25-hydroxy-vitamin D₃ is further metabolized in the kidney and other tissues, yielding 1α,25(OH)₂D₃ – the most potent form of vitamin D₃ (Figure 2.3)⁵⁻⁷. The formation of 1α,25(OH)₂D₃ in the kidney is tightly regulated by the parathyroid hormone and, through negative feedback, by 1α,25(OH)₂D₃ itself. Other regulators include calcium, phosphate, growth hormone and prolactin. In normal human plasma, 1α,25(OH)₂D₃ circulates at approximately 1000-fold lower concentrations that 25(OH)D₃ and is generally present at 20 to 65 pg/mL.
Figure 2.3. Bioactivation pathway of vitamin D₃.
1α,25(OH)2D3, also known as calcitriol, undergoes further metabolism in its target tissues through modification of the side-chain and the A-ring as illustrated in Figure 2.4. The C-24 oxidation pathway, the main side-chain modification pathway, is initiated by hydroxylation at C-24 of the side-chain and yields the end product calcitroic acid8,9. The C-23 and C-26 oxidation pathways, the minor side-chain modification pathways, are initiated by hydroxylation at C-23 and C-26 of the side-chain and together lead to the formation of the end product, 1α,25(OH)2-23,26-lactone10-13. In a vitamin D-supplemented state, excess 25(OH)D3 may also be converted to other side-chain metabolites via the C-23 and C-24 oxidation pathways leading to their terminal physiological inactivation.

Although these side-chain oxidative pathways yield metabolites that are considered “non-functional”, the presence of these compounds in circulation could pose serious problems in the analysis of 25(OH)D3 and 1,25(OH)2D3 as will be highlighted in Section 2.5.

The C-3 epimerization pathway, A-ring modification pathway discovered in the 1990s, leads to the inversion of configuration of the C-3 hydroxyl group from β to α, yielding 1α,25(OH)2-3epi-D3. First reported in human keratinocytes by Reddy et al. in 1994, this pathway has since been observed in human colon carcinoma cells, bovine parathyroid cells, rat osteosarcoma cells, and various cultured cell lines14-17. 1α,25(OH)2-3epi-D3 was also isolated as a circulating metabolite of 1α,25(OH)2D3 in rats treated with pharmacological doses of 1α,25(OH)2D318. The C-3 epimerization pathway is now well accepted as a common metabolic pathway for the major metabolites of vitamin D3. Recent studies reported the metabolism of 25(OH)D3, 24R,25-dihydroxy-vitamin D3.
(24R,25(OH)₂D₃), and 1α-hydroxy-vitamin D₃ (1α(OH)D₃) into their respective C-3 epimers. It has been proposed that the C-3 epimerization pathway of 1α,25(OH)₂D₃, like the previously well established C-23 and C-24 pathways, plays an important role in activating or inactivating 1α,25(OH)₂D₃. This will be further highlighted in Chapter 3.
Figure 2.4. Metabolic pathways of 1α,25(OH)₂D₃. (Adapted from 22)
2.3. Vitamin D Biological Actions

The active metabolite of vitamin D₃ – 1\(\alpha\),25-(OH)₂D₃ – exerts most of its physiological and pharmacological actions through its nuclear receptor, the vitamin D receptor (VDR), regulating the transcriptional machinery of a variety of cell types.\(^{23,24}\) 1\(\alpha\),25(OH)₂D₃ is now well known to play important roles in the regulation of dozens of genes, including those associated with calcium-phosphate homeostasis, immune responses, cell proliferation, differentiation and apoptosis.\(^{7,25,26}\) The vitamin D receptor (VDR) itself has been detected in numerous target cells,\(^1\) indicating potential therapeutic applications of VDR ligands in osteoporosis, cancer, secondary hyperparathyroidism and autoimmune diseases such as psoriasis, rheumatoid arthritis, type 1 diabetes and multiple sclerosis.\(^{27,28}\)

### 2.3.1. Calcemic functions

Traditionally, vitamin D₃ has been shown to be one of the most important biological regulators of calcium metabolism by stimulating the absorption of dietary calcium through the intestine and participating in its incorporation into the skeleton.\(^6,7,29\) Without adequate levels of 1\(\alpha\),25(OH)₂D₃ in the blood, the body cannot absorb and use calcium, which is essential for such vital functions as the electrochemical signaling between brain cells. The reduced absorption of dietary calcium and mineral phosphorus through the intestine may also lead to poor skeletal integrity. In children, vitamin D deficiency results in the once common disease known as rickets and characterized by bowed legs and deformed ribs. In adults, vitamin D deficiency may lead to osteoporosis. In addition, there is accumulating evidence from prospective and retrospective studies that the
administration of vitamin D or exposure to sunlight decreases bone loss in the elderly and is associated with a decrease in the risk of hip or other osteoporosis-related fractures\textsuperscript{30,31}.

### 2.3.2. New non-calcemic functions

The vitamin D endocrine system, besides playing pivotal roles in calcium homeostasis and bone mineral metabolism, is now recognized to promote a diverse range of fundamental biological functions including induction of cell differentiation, inhibition of cell growth as well as immunomodulation. Many of these activities suggest potential therapeutic applications for 1\(\alpha\),25(OH)\(_2\)D\(_3\) and possibly other metabolites in a wide range of target tissues and cell types\textsuperscript{25,26}.

#### 2.3.2.1. Control of cell proliferation and cell differentiation

**Psoriasis**

The skin is one such tissue where a broader role of vitamin D is being intensively explored. Besides producing vitamin D\(_3\), epidermal cells (keratinocytes) make 1\(\alpha\),25(OH)\(_2\)D\(_3\), contain 1\(\alpha\),25(OH)\(_2\)D\(_3\) receptors (VDR) and respond to 1\(\alpha\),25(OH)\(_2\)D\(_3\) with changes in proliferation and differentiation\textsuperscript{32-35}. The ability of the epidermis to produce 1\(\alpha\),25(OH)\(_2\)D\(_3\) from 25(OH)D\(_3\) and to catabolize the vitamin D\(_3\) metabolites quickly offers several possibilities for the topical administration of vitamin D compounds in the treatment of skin disorders. For instance, the vitamin D\(_3\) active hormone was found to exert an anti-psoriatic effect, which has been attributed to its ability to inhibit the hyperproliferation and promote the differentiation of keratinocytes in psoriatic skin\textsuperscript{36,37}.


**Cancer**

Most recently, the discovery of the VDR in a broad range of tumors and malignant cells (colon, prostate, breast) has led to a series of studies on the role of vitamin D in tumor cell growth regulation, and possibly treatment of cancer\(^1,38-40\). The relationship between cancer, diet, and vitamin D has been addressed in several reports\(^41-46\). In addition to the epidemiological studies and demonstration of vitamin D receptor in tumor cells, there has been an increasing amount of biological data supporting a role of vitamin D in cancer. Multiple studies have shown that, at high concentrations \((10^{-9}-10^{-7}\text{M})\), \(1\alpha,25(\text{OH})_2\text{D}_3\) inhibits the growth of tumor cells *in vitro* and promotes apoptosis by interfering with the cell cycle. \(1\alpha,25(\text{OH})_2\text{D}_3\) was also reported to induce cell differentiation. These *in vitro* findings were followed by *in vivo* studies demonstrating the effects of \(1\alpha,25(\text{OH})_2\text{D}_3\) on various types of cancers. Antiangiogenic and antimetastatic effects may also play a role in the tumor suppressive activity of vitamin D\(_3\). The data obtained so far on the distribution of the VDR in a broad range of tumors and the inhibition of cell growth, angiogenesis and metastasis all hold promise for the development of treatment strategies based on vitamin D\(_3\) use in a wide range of cancers\(^47,48\).

**2.3.2.2. Immunomodulation**

Cells involved in innate and adaptive immune responses – including macrophages, dendritic cells, T cells and B cells – also express the VDR, and can both produce and respond to \(1\alpha,25(\text{OH})_2\text{D}_3\). The net effect of the vitamin D system on the immune response is an enhancement of innate immunity coupled with multifaceted regulation of adaptive immunity. Moreover, epidemiological evidence indicates a significant
association between vitamin D deficiency and an increased incidence of several autoimmune diseases, which represents a sound basis for a further exploration of the potential role of 1α,25(OH)2D3 or other VDR ligands in the management or prevention of several immuno-mediated disorders, from rheumatoid arthritis to multiple sclerosis and possibly also type 1 diabetes28,49,50.

2.3.3. Vitamin D toxicity

Despite its pleiotropic applications, a major limitation to 1,25(OH)2D3 therapy is its potent calcemic and phosphatemic activities, leading to hypercalcemia or hyperphosphatemia51,52. When used at supraphysiological levels, this may result in the formation of renal stones, soft tissue calcification and can even be lethal. Unfortunately, this toxic side effect has limited the use of natural vitamin D metabolites for both mineral-related as well as novel indications. This reality stimulated a search for alternative approaches that could reduce or alleviate the hypercalcemic side effect and led to the design of novel analogs of vitamin D that only weakly stimulate bone calcium mobilization or intestinal calcium absorption, while retaining the desired biological activity.
2.4. Vitamin D Analogs

2.4.1. Rational Design of Analogs

The fundamental challenge for organic and medicinal chemists working on designing vitamin D analogs as new drugs is to incorporate structural changes leading to new compounds that are still efficacious but safer and more selective than the natural hormone.

A great number of vitamin D analogs have been synthesized over the last two decades by altering the structure of both the side-chain and the A-ring of 1α,25(OH)2D3 \textsuperscript{25,53}. Such alterations include the incorporation of unsaturations or new functional groups or the lengthening of the side-chain. Several non-steroidal analogs of 1α,25(OH)2D3 with unique biological activity profiles were also designed\textsuperscript{54}. These novel analogs lack either the full five-membered D-ring or the full six-membered C-ring or both C- and D-rings of the C-D-ring skeleton of 1α,25(OH)2D3. The structures of selected vitamin D analogs are shown in Figure 2.5.
Figure 2.5. Structures of $1\alpha,25(OH)_2D_3$ and selected analogs. (Adapted from 53)
2.4.2. Therapeutic Applications

A recent review by Brown and Slatropolsky discusses in detail the development of vitamin D analogs and their therapeutic applications\textsuperscript{53}. Several analogs are currently under development or in clinical trials for the treatment of various types of cancers, autoimmune disorders and many other diseases. Selected examples are summarized in Table 2.1.

<table>
<thead>
<tr>
<th>Vitamin D Analogs</th>
<th>Therapeutic Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>22-oxa-1α,25(OH)\textsubscript{2}D\textsubscript{3} (Oxacalcitriol or OCT)</td>
<td>Secondary hyperparathyroidism (2-HPT) Breast cancer Prostate cancer</td>
</tr>
<tr>
<td>19-nor-1α,25(OH)\textsubscript{2}D\textsubscript{3}</td>
<td>2-HPT Leukemia</td>
</tr>
<tr>
<td>1α(OH)D\textsubscript{3}</td>
<td>2-HPT Leukemia</td>
</tr>
<tr>
<td>1α(OH)-3-epi-D\textsubscript{3}</td>
<td>2-HPT</td>
</tr>
<tr>
<td>1α(OH)-24-ethyl-D\textsubscript{3}</td>
<td>Colon cancer</td>
</tr>
<tr>
<td>1α,24(OH)\textsubscript{2}D\textsubscript{3}</td>
<td>Breast cancer Psoriasis</td>
</tr>
<tr>
<td>1α,25(OH)\textsubscript{2}-26,27-F\textsubscript{6}D\textsubscript{3}</td>
<td>2-HPT</td>
</tr>
<tr>
<td>1α,25(OH)\textsubscript{2}-16ene-23yne-D\textsubscript{3}</td>
<td>2-HPT Leukemia Prostate cancer</td>
</tr>
<tr>
<td>1α,25(OH)\textsubscript{2}-16ene-23yne-26,27-F\textsubscript{6}D\textsubscript{3}</td>
<td>Prostate cancer Breast cancer Colon cancer</td>
</tr>
<tr>
<td>1α,25(OH)\textsubscript{2}-16ene-5,6-trans-D\textsubscript{3}</td>
<td>Leukemia</td>
</tr>
<tr>
<td>Calcipotriol (see structure in Figure 2.5)</td>
<td>Psoriasis</td>
</tr>
<tr>
<td>EB 1089 (see structure in Figure 2.5)</td>
<td>Colon cancer Breast cancer Prostate cancer</td>
</tr>
<tr>
<td>ED-71 (see structure in Figure 2.5)</td>
<td>Osteoporosis</td>
</tr>
</tbody>
</table>

Table 2.1. Selected vitamin D analogs under development or in clinical trials and their therapeutic applications.
2.5. Vitamin D Analysis – The Role of Mass Spectrometry

As described earlier, the metabolic activation and inactivation of vitamin D result in a plethora of vitamin D metabolites. To this large set of vitamin D compounds we must append the newly synthesized analogs. The detection and measurement of such a large class of compounds constitute an important challenge for the analytical chemist\(^5\). The vitamin D analysis can be divided into two major applications: qualitative analysis and quantitative analysis. Mass spectrometry has played and continues to play a crucial role in both areas\(^6\); GC-MS remains an excellent tool for qualitative analysis while recent advances in LC-MS have benefited quantitative applications.

2.5.1. Qualitative analysis (MS-based methods)

Even though LC-MS is the present gold standard in mass spectrometry, GC-MS remains the technique of choice for the qualitative analysis of vitamin D compounds, covering applications ranging from the elucidation of vitamin D metabolic pathways to the structural characterization of new metabolites or synthesized analogs. The study of vitamin D metabolism is expected to be helpful in understanding the physiological role of the metabolites and developing new drugs.

2.5.1.1. Vitamin D metabolism – Elucidation of metabolic pathways

Currently, isolation by liquid chromatography and further analysis by GC-MS is considered the most complete means of metabolite identification. Normal phase HPLC has been shown to be very efficient in the separation of the hydroxylated metabolites of vitamin D but, as new metabolites are being isolated, the combination with a second,
orthogonal chromatographic system (reversed phase) is often required to confirm the homogeneity of each collected fraction or further resolve the metabolites of interest. UV absorbance provides adequate detection due to the chromophore properties of the 5,6-cis-triene system of the vitamin D skeleton. As most metabolic modifications occur either on the side-chain or the A-ring, the triene system generally remains intact and, as a consequence, most metabolites exhibit characteristic UV spectra with an absorbance maximum at 265 nm and an absorbance minimum at 228 nm. As is the case with other drug metabolites, while liquid chromatography is an excellent means for product isolation, positive identification of a compound cannot be achieved strictly by retention time alone. Co-elution with synthetic standards does provide some confirmatory evidence, but this too, can be deceiving. Therefore, chromatographic separation and isolation of metabolites generally only constitutes a preliminary step in their structural characterization.

For the past few decades, GC-MS has been a standard in the characterization of vitamin D metabolites following HPLC isolation. Electron impact, the classic ionization technique used in GC-MS, remains the most widely used method for the qualitative analysis of vitamin D compounds. The relatively hard ionization technique nicely suits such analysis where extensive structural information is required for unequivocal characterization. Although not a requirement, compounds are generally derivatized into their trimethylsilyl (TMS) ethers prior to GC-MS analysis. In addition to improving volatility and thus sensitivity and yielding unique fragment ions, the specific
derivatization of all hydroxyl groups is an efficient means of tracking structural changes due to metabolism.

**Figure 2.6** depicts the typical EI-MS fragmentation pattern of derivatized vitamin D compounds. Characteristic fragment ions detected in the high m/z region are the molecular ion $M^+$ and fragment ions resulting from the successive losses of TMSOH groups – loss of 90 mass units for each hydroxyl group being cleaved. In addition, provided the secosteroid structure remained intact during metabolism, the mass spectrum of TMS derivatives of vitamin D metabolites generally exhibits characteristic fragment ions resulting from either the cleavage of the side chain ($m/z$ 131 $\rightarrow$ C17-C20 cleavage) or cleavage across the A-ring ($m/z$ 217 and $m/z = M^+ - 131$).

![EI-MS spectrum of the TMS derivative 1α,25(OH)$_2$D$_3$.](image)

**Figure 2.6.** EI-MS spectrum of the TMS derivative 1α,25(OH)$_2$D$_3$.

If an authentic standard is available, the structure of the isolated metabolite can be elucidated and/or confirmed by direct comparison with the standard. As will be emphasized in the next section, the comparison must be based on both GC retention time and mass spectral characteristics. In the absence of an authentic standard, structure
elucidation may be achieved by conducting a thorough interpretation of the unknown’s mass spectral profile and comparing it with that of the parent compound in order to identify structure alterations. In more complex cases where the structure of the unknown cannot be readily elucidated, additional specific chemical modifications may be performed on the isolated metabolite in order to test for the presence of a characteristic functionality and thus gain more structural information. Sodium metaperiodate (NaIO₄) oxidation and sodium borohydride (NaBH₄) reduction are two common examples of such chemical modifications that allow for the detection of specific metabolic transformations, such as the incorporation of a hydroxyl group or further oxidation into a keto group. Sodium borohydride reduction is used to detect a keto functionality. Upon mixing of NaBH₄ with the unknown metabolite, any keto group is reduced to a hydroxyl group. On the other hand, NaIO₄ cleaves the bond between two carbons when each carbon bears a hydroxyl group or one carbon bears a hydroxyl carbon and the other bears a keto group. Therefore, the susceptibility of the isolated metabolite to NaIO₄ cleavage provides evidence that an additional hydroxyl group or keto group on the side chain is vicinal to a hydroxyl group already present prior to metabolism. The interpretation of the fragmentation profile of the chemically modified metabolite generally offers additional information on the structure of the unknown, such as the nature and position of the metabolic modification(s).

In summary, the structure of a newly isolated metabolite can, in most cases, be elucidated based on its molecular ion, fragmentation pattern and sensitivity to borohydride reduction or periodate oxidation.
Elucidation of the C-3 epimerization pathway – Characterization of stereoisomers

A little over a decade ago, an important breakthrough in the vitamin D field occurred with the discovery of a new pathway. Reddy et al. reported the metabolism of 1α,25(OH)₂D₃ in neonatal keratinocytes into a novel metabolite¹⁸. The new metabolite, identified as 1α,25-dihydroxy-3-epi-vitamin D₃ (1α,25(OH)₂-3-epi-D₃) only differs from the parent hormone in the stereochemistry of C-3. Its isolation and structural characterization therefore presented a significant challenge. C-3 epimerization has been shown to play a major role in the hormone activation and inactivation. Indeed, 1α,25(OH)₂-3-epi-D₃ binds to the nuclear vitamin D receptor with lower affinity than 1α,25(OH)₂D₃ and exhibits a reduced calcemic activity. On the other hand, 1α,25(OH)₂-3-epi-D₃ is equipotent to 1α,25(OH)₂D₃ at suppressing parathyroid hormone secretion in bovine parathyroid cells and at inhibiting keratinocyte proliferation. Soon after the first isolation of 1α,25(OH)₂-3-epi-D₃ in human keratinocytes, the C-3 epimerization was reported in human colon carcinoma cells, bovine parathyroid cells, rat osteosarcoma cells, and various cultured cell lines¹⁴-¹⁷.

GC-MS played a major role in the elucidation of the C-3 epimerization pathway, first metabolic pathway involving modification of the A-ring. Preliminary HPLC-UV analysis alone revealed that the newly isolated metabolite was slightly less polar than the parent molecule, 1α,25(OH)₂D₃. Likewise, when subjected to GC-MS analysis, the new metabolite eluted at a time that did not match that of 1α,25(OH)₂D₃. However, its mass spectrum yielded the same molecular ion and virtually identical fragment ions as 1α,25(OH)₂D₃, suggesting that the two compounds were stereoisomers (Figure 2.7). A
closer look at the mass spectral characteristics revealed subtle differences with respect to intensity ratios between 1α,25(OH)₂D₃ and the new metabolite and allowed to locate the position of the configuration change on the A-ring. In the original study, in the absence of a synthetic standard of the C3-epimer, the stereochemistry of the new metabolite was further confirmed by ¹H-NMR. As an alternative, the identity of the C-3 epimer could be confirmed by comparison of its mass spectral characteristics – fragment ions and intensity ratios – with those of all four possible A-ring diastereomers¹⁷.

Once a synthetic standard of the C3-epimer became available, characterization by ¹H-NMR was no longer necessary. Rather, the identity of the C3-epimer was determined using GC-MS only, by comparison of both GC retention time and EI mass spectral characteristics – fragment ions and intensity ratios – of the metabolite with that of the standard. Further confirmation is also often obtained by co-elution of the metabolite with the C-3 epimer synthetic standard on both normal and reversed phase HPLC systems. Since the elucidation of the C-3 epimerization pathway, similar GC-MS interpretation served as a basis for the structure characterization of other diastereomers or the identification of various metabolites of 1α,25(OH)₂-3-epi-D₃ itself²⁰,⁵⁷-⁵⁹.
Figure 2.7. EI-MS spectra of the TMS derivatives of 1α,25(OH)2-D3 (upper panel) and 1α,25(OH)2-3-epi-D3 (lower panel).
2.5.1.2. Vitamin D Analogs Analysis

In recent years, the introduction of vitamin D analogs has shifted the emphasis from the study of vitamin D natural metabolites to the study of the new synthetic vitamin D-like compounds. Indeed, the development of those analogs has been accompanied by a new range of MS-based applications from the structure characterization and/or confirmation of synthetic products to the investigation of metabolic and degradation pathways for those new compounds. Understanding the metabolism and degradation properties of an analog is essential for the investigation of its potential pharmaceutical applications.

Synthetic Product Structure Characterization

Although $^1$H-NMR is the technique of choice for structure characterization/confirmation of newly synthesized analogs in the absence of reference standards, it is still complemented by the use of mass spectrometry for the determination/confirmation of molecular weight. Mass spectrometric data are routinely recorded along with $^1$H-NMR data in literature reporting the synthesis of new analogs. Details are not always provided regarding the chosen MS method but electron impact is again the ionization technique of choice. The relatively hard ionization technique generally yields the molecular ion along with a few characteristic fragment ions, complementing $^1$H-NMR structural data and allowing for the definitive structure characterization of the synthetic product.

Metabolism of Analogs and Comparative Metabolism Studies

While most metabolic pathways have now been elucidated for natural vitamin D, the metabolism of many vitamin D analogs still remains unknown. A generally efficient way
to elucidate metabolic pathways of analogs is to conduct comparative metabolism studies between the analog and the parent or natural compound and subject the isolated metabolites to GC-MS analysis. While some compounds will be shown to follow the same pathways as the natural hormone, when that is not the case a comparative study may still reveal informative details regarding the new pathway and yield to the isolation and characterization of novel metabolites\textsuperscript{60-66}. This will be further illustrated in Chapter 3 and Chapter 4.

\textit{2.5.1.3. Complementary Use of GC-MS and LC-MS}

Several groups reported using LC-MS as a complementary technique for the structure elucidation of vitamin D related compounds\textsuperscript{67}. Although LC-MS is a more sensitive technique that can therefore help overcome some of the drawbacks of GC-MS, the structural information gained using ESI or APCI remains quite limited. The mass spectral patterns of vitamin D related compounds observed by LC-ESI-MS or LC-APCI-MS are generally relatively simple ([M+H]\textsuperscript{+}, [M+H-H\textsubscript{2}O]\textsuperscript{+}), making it possible to readily assign molecular ions. However, ESI or APCI ionization usually fails to yield further characteristic fragment ions necessary to elucidate the chemical structure of the side-chain or vitamin D skeleton. Therefore, LC-MS cannot be used as a stand-alone technique for the structural elucidation of unknown metabolites but should be more suitable for preliminary identification with synthetic postulated metabolites. As shown by Ishigai \textit{et al.}, the complementary use of both techniques enables the rapid and detailed characterization of vitamin D related compounds\textsuperscript{67}. They investigated characteristics of GC-MS and LC-MS analyses for 22-oxa-calcitriol and its metabolites and demonstrated
that, although LC-MS is a more convenient and sensitive technique for determining the molecular masses of non-volatile and thermo-labile vitamin D-related compounds without any derivatization required, GC-MS is a superior technique to identify and characterize these metabolites. In particular, GC-MS is a more powerful technique to elucidate in detail the chemical structure of small amounts of unknown compounds.

Even though GC-MS has proved to be a gold standard in the qualitative analysis of vitamin D compounds, it also suffers from some disadvantages in that it is difficult to analyze polar, non-volatile and thermo-labile compounds with sufficient sensitivity because of poor ionization or low recovery from derivatization. Moreover, when derivatized, vitamin D compounds generally undergo thermo-isomerization in the GC injection port, yielding two pyro- and isopyroisomers as depicted in Figure 2.8. Most vitamin D compounds are therefore detected by GC-MS as a doublet. This does not affect the qualitative analysis since both isomers exhibit identical mass spectral characteristics. However, the thermo-isomerization effect may prevent quantitative analysis by GC-MS.

In view of the limitations outlined above, considerable interest has been focused on the development of sensitive LC-MS methods using ESI or APCI for the quantitative analysis of vitamin D compounds. Although LC-MS generally provides little information on chemical structures, the sensitivity of detection is well suited to quantitative applications.
2.5.2. Quantitative analysis of vitamin D compounds

The quantitative analysis of vitamin D compounds in biological fluids is often a tedious and challenging process. The reasons for this include the low concentrations of circulating metabolites, their chemical similarities, hydrophobicity, physico-chemical instability in the presence of heat or UV light and the presence of a large number of interfering substances within the biological matrix. Therefore, highly sensitive and specific methods are required. The measurement of endogenous metabolites is widely used for the assessment of vitamin D status and the detection of various related disorders. In addition, quantitative assays are being developed to investigate the therapeutic potential of new synthetic analogs and support preclinical and clinical pharmacokinetic studies.
2.5.2.1. Non-MS based methods – Assessment of vitamin D status

The prevalence of vitamin D deficiency in the general population has become a major public health problem. Vitamin D deficiency may have significant consequences not only on bone health, but accumulating evidence also suggests that it may be linked to an increase in cancer risk\textsuperscript{69-71}. This new reality has stimulated the development of accurate assays for the assessment of vitamin D status. The measurement of total 25(OH)D – sum of 25(OH)D\textsubscript{2} and 25(OH)D\textsubscript{3} – is currently the preferred test. The serum/plasma concentration of 25(OH)D, with a half-life of \textit{ca.} three weeks and systemic levels 500- to 1000-fold greater than 1\textalpha,25(OH)\textsubscript{2}D\textsubscript{3}, is considered the best indicator of cumulative effects of exposure to sunlight and dietary intake of vitamin D\textsuperscript{71}.

Currently, numerous formats exist for measuring total serum 25(OH)D concentrations. Most are based on saturation analysis such as radioimmunoassay (RIA), enzyme immunoassay (EIA), enzyme-linked immunosorbent assay (ELISA), and chemiluminescence protein-binding assay (CLPBA)\textsuperscript{72-77}. Commercial immunoassays and protein binding assays are widely used in clinical laboratories. However, simultaneous detection of 25(OH)D\textsubscript{2} and 25(OH)D\textsubscript{3} often represents a challenge and cross-reactivity of 25(OH)D assays with other vitamin D metabolites is a common issue\textsuperscript{78,79}. Although much effort has been focused on addressing these limitations, inter-laboratory variability of 25(OH)D analyses is still observed, as highlighted in several recent publications\textsuperscript{69,75,80-83}.

Likewise, bioassays such as competitive protein binding assay (CPBA) or radioreceptor assay (RRA), based on the vitamin D binding protein and vitamin D receptor, are currently used for the quantification of vitamin D analogs. However, most methods do
not provide adequate specificity or sensitivity. A few bioassays demonstrate high
sensitivity but these involve extensive purification to remove endogenous metabolites or
other cross-reactive species so that the methods are not suitable for routine analysis.

HPLC methods have also been reported for the quantification of metabolites or analogs in
biological fluids. UV detection demonstrates satisfactory sensitivity only for 25(OH)D,
circulating at relatively high levels (mid to high ng/mL), and is still considered the gold
standard for assessing vitamin D status\textsuperscript{81}. Other metabolites and analogs, occurring at
much lower concentrations (pg/mL to low ng/mL), cannot be detected by UV. Higher
sensitivity may be achieved using electrochemical or fluorometric detection but these
methods involve complicated pretreatment steps, again preventing their use for routine
analysis\textsuperscript{84,85}.

Overall, very few assays can match the accuracy, sensitivity, and specificity of mass
spectrometry. As a result, LC-MS/MS is becoming the method of choice for most vitamin
D quantitative applications, allowing for the detection and quantification of a wide range
of metabolites and analogs.

2.5.2.2. \textit{LC-MS methods – Quantification of low-level vitamin D metabolites and
analogs}

LC-MS is considered to be a rapid, specific, and convenient method for the determination
of vitamin D compounds in biological fluids and provides a powerful alternative to
traditional bioassays. The high specificity of LC-MS permits the development of assays
with minimum interferences and relatively short run times. Liquid chromatography provides the ability to separate compounds based on their relative polarities while the mass spectrometric dimension allows for further separation based on mass-to-charge ratios. The coupling of these two mature technologies therefore provides a synergistic analytical advantage that no other technique currently offers. However, the poor ionization efficiencies of most vitamin D compounds may present a challenge for their analysis. This does not represent a major limitation for the quantification of 25(OH)D since its plasma/serum levels are relatively high (10-200 ng/mL). Several LC-MS methods have been implemented for the assessment of vitamin D status with reported limits of quantitation (LOQ) as low as 5 ng/mL. However, the majority of metabolites and analogs circulate at much lower levels (picogram range). Unfortunately, due to poor ionization efficiencies, conventional LC-ESI-MS or LC-APCI-MS methods often cannot attain the required sensitivity.

Derivatization with a Cookson-type reagent (4-substituted 1,2,4-triazoline-3,5-dione) is generally used to overcome ionization efficiency problems. The triazoline dione is a powerful dienophile which rapidly and quantitatively reacts with the C10-C19:C5-C6 s-cis-diene system of vitamin D compounds to form a stable Diels-Alder adduct. Examples of Cookson-type reagents (PTAD, MBOTAD, FMTAD and DMEQTAD) are shown in Figure 2.9.
The addition of a tagging agent rich in elements with high proton-affinity, such as oxygen or nitrogen, provides suitable sites for protonation resulting in enhanced ionization efficiency and sensitivity in positive ESI- and APCI-MS. The derivatization with Cookson-type reagents has another advantage in that the molecular weight of the analyte is being shifted to a higher mass range where background noise is relatively low. The Diels-Alder derivative consists of $6R$ and $6S$ isomers because the reagent attacks at the $s$-$cis$-diene of the vitamin D compound from the $\alpha$- and $\beta$-sides (Figure 2.10). Therefore, each derivative typically exhibits characteristic twin peaks on its mass chromatogram, but only the major isomer is monitored for quantification.
Figure 2.10. Derivatization of 25(OH)D$_3$ with PTAD.
The protonated derivative \([M+H]^+\) is generally observed as the base ion in the mass spectrum. In some cases, sodium or ammonium adduct ions ([M+Na]+ or [M+NH₄]+) give a more abundant base ion. To achieve greater sensitivity, MS/MS analysis can be performed in the selected reaction monitoring mode (SRM) using the protonated molecular ion or adduct ion as a precursor. Fragmentation of the protonated molecule often results in the loss of one or several H₂O molecules; however, if sufficient collision energy is applied, it also yields a very characteristic A-ring fragment ion derived from the cleavage of the C6-C7 bond of the vitamin D backbone (Figure 2.11). The backbone cleavage is specific to derivatized compounds; it does not occur during the fragmentation of underivatized vitamin D analytes. The characteristic A-ring fragment ion is generally produced with sufficient intensity so that this specific transition may be used for quantification of the analyte.

![Figure 2.11. Fragmentation of 25(OH)D₃-PTAD.](image)
Figure 2.12 nicely illustrates the advantages of using derivatization. A 100- to 200-fold increase in sensitivity has been observed in some instances, a limit of detection (LOD) in the femtogram range has even been reported for the quantification of 1α-hydroxy-vitamin D₃ [1α(OH)D₃]⁹¹.

Figure 2.12. ESI-MS and ESI-MS/MS spectra of intact 25(OH)D₃ (panels a and b) and 25(OH)D₃-PTAD (panels c and d). (Reproduced from 93)

In summary, high specificity and enhanced sensitivity make LC-MS/MS a valuable technique for the quantification of low level vitamin D metabolites and analogs.
2.6. Conclusions

The discovery of new biological functions of 1α,25(OH)₂D₃ along with the development of vitamin D analogs has reinvigorated the vitamin D field over the past decade. Many synthetic analogs have proven to be potent drug candidates for various therapeutic applications such as psoriasis, hyperparathyroidism or even cancer. Some even entered preclinical or clinical trials, which reinforced the need for highly sensitive and specific analytical techniques. Recent advances in mass spectrometry significantly benefited the vitamin D community. Several LC-MS/MS methods have been implemented to support pharmacokinetics and toxicology studies involving vitamin D analogs. LC-MS also supersedes traditional bioassays and is becoming a reference technique for the assessment of vitamin D status. On the other hand, GC-MS remains the gold standard for the structural characterization of vitamin D metabolites and analogs.

Chapters 3 and 4 will further illustrate the essential role of mass spectrometry, particularly GC-MS, in the discovery of novel metabolic pathways – C-3 epimerization and C-1 esterification pathways – and the structural elucidation of novel vitamin D analogs.
REFERENCES


(51) Vieth, R. Bone Miner. 1990, 11, 267-72.


Chapter 3:

Vitamin D₃ C-3 Epimerization Pathway

and its Effects on Calcitriol Metabolism
3.1. Isolation and Characterization of 3-epi-Vitamin D₃: A New Metabolite of Vitamin D₃

3.1.1. Introduction

Reddy et al. first reported the metabolic conversion of 1α,25-dihydroxy-vitamin-D₃ (1α,25(OH)₂D₃) into 1α,25-dihydroxy-3-epi-vitamin-D₃ (1α,25(OH)₂-3-epi-D₃) in human neonatal keratinocytes¹. The metabolic conversion of 1α,25(OH)₂D₃ into 1α,25(OH)₂-3-epi-D₃ was then observed in several different tissues and cancer cell lines²-⁴. 1α,25(OH)₂-3-epi-D₃ has also been identified as a circulating metabolite in rats dosed with pharmacological amounts of 1α,25(OH)₂D₃⁵. More recently, the metabolism of several synthetic vitamin D analogs into their respective 3-epimers was also reported. This novel vitamin D metabolic pathway which involves the change in the stereochemistry of 3-hydroxyl group of the A-ring from β to α orientation is now well accepted in the literature as “C-3-epimerization pathway”⁶. Until recently, it was unclear whether specific structural features, such as the presence of 25-hydroxyl or 1α-hydroxyl groups, were essential to the C-3 epimerization conversion of vitamin D compounds or analogs. Recent studies reported the metabolism of 24,25(OH)₂D₃, 25(OH)D₃, and 1α(OH)D₃, which are lacking either of the aforementioned features, into their respective C-3 epimers⁷,⁸ and therefore demonstrated that neither 25- nor 1α-hydroxyl groups are required for the C-3 epimerization. These observations provided an indirect evidence that vitamin D₃ itself may be converted into its 3-epimer. However, as of today, the C-3 epimerization of vitamin D₃ has not been reported.
3.1.2. Project Goals

In view of the aforementioned considerations, we undertook the present study to investigate the metabolism of vitamin D₃ both in vitro and in vivo and demonstrate the conversion of vitamin D₃ into its 3-epimer. In vitro studies were performed with human hepatocyte cells (Hep G2) which were previously shown to express the C-3 epimerization pathway⁸⁻⁹. For in vivo studies, rats were fed with pharmacological amounts of vitamin D₃. The produced metabolites were isolated and characterized by HPLC, GC-MS and ¹H-NMR.

3.1.3. Materials and Methods

3.1.3.1. Materials

The human hepatocellular carcinoma cells (Hep G2) and MEM medium (ATCC 30-2003) were purchased from ATCC (Manassas, VA). Streptomycin, penicillin and trypsin-EDTA were purchased from Gibco (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Invitrogen (Carlsbad, CA). Power-Sil GC derivatizing agent was purchased from Alltech Associates (Deerfield, IL).

Vitamin D₃ was purchased from Sigma-Aldrich. 25(OH)D₃, 25(OH)-3-epi-D₃, 1α(OH)D₃, 1α(OH)-3-epi-D₃, 1α,25(OH)₂D₃, 1α,25(OH)₂-3-epi-D₃ were a gift from Dr. Milan Uskokovic at Hoffmann-La Roche (Nutley, NJ, USA).
3.1.3.2. Synthesis of 3-epi-vitamin D₃

3-epi-vitamin D₃ was synthesized by Dr. Milan Uskokovic (Roche, Nutley, NJ). The synthetic scheme is depicted in Figure 3.1 and was adapted from a published procedure¹⁰. Briefly, cholesta-3,5,7-trien-3-ol was first acetylated and then reduced with sodium borohydride (NaBH₄) to yield 7-dehydrocholesterol (major) and its 3-epimer (minor). The minor product of the reduction, 3-epi-7-dehydrocholesterol, was dissolved in n-hexane and irradiated with a medium pressure mercury arc lamp in an argon atmosphere to yield 3-epi-previtamin D₃. The isolated product was dissolved in toluene and heated at 65°C for 20 hours under argon, which resulted in the thermo-isomerization of 3-epi-previtamin D₃ into 3-epi-vitamin D₃. 3-epi-vitamin D₃ was purified by preparative TLC and HPLC prior to its characterization by UV (Absₘₐₓ at λ=265 nm and Absₘᵟᵦ at λ=228 nm) and ¹H-NMR.
Figure 3.1. Chemical synthesis of 3-epi-vitamin D₃
3.1.3.3. **Cells and cell culture**

Hep G2 cells were maintained in MEM medium (ATCC 30-2003) supplemented with 10% FBS and antibiotics (penicillin (100 IU/mL) and streptomycin (100 µg/mL)). Cell culture medium was replenished every 3-4 days. For the metabolism studies, 3 x 10^6 cells were seeded in T150 tissue culture bottles and grown to confluence. The incubations were carried out at 37°C in a humidified 5% CO₂ atmosphere. All metabolism studies were performed by Dr. Satya Reddy (Epimer LLC, Providence, RI).

3.1.3.4. **Metabolism studies in Hep G2 cells**

Hep G2 cells (3 x 10^6 cells/mL) were incubated with a 10 µM concentration of vitamin D₃ in 50 mL of medium containing 10% FBS. The incubations were quenched after 24, 48 or 72 hours with 50 mL of methanol and the lipids from both cells and media were extracted according to an extraction procedure developed by Bligh and Dyer. The lipid extracts were then subjected to HPLC analysis for isolation and purification of the produced metabolites. Control incubations were also performed in which vitamin D₃ was incubated in cell-free medium containing 10% FBS. HPLC analysis of the lipid extracts from control incubations indicated that vitamin D₃ did not undergo any chemical change or breakdown during the incubation period or the extraction procedure.

3.1.3.5. **In vivo metabolism of vitamin D₃ in rats**

Sprague-Dawley rats (ca. 250 g) were adapted to laboratory conditions for at least 5 days; the rats were housed two per cage in a regular environment with a 12-hour light-dark cycle and had free access to food and water. The *in vivo* metabolism studies were
performed on five rats, each of them was fed with a daily intravenous bolus dose of 500 µg of vitamin D₃. After five days, the rats were sacrificed and their blood, collected by catheterization of the aorta, was immediately centrifuged. About 25 mL of serum obtained from the five rats was subjected to lipid extraction procedure. The vitamin D₃ metabolites in the lipid extracts were analyzed by HPLC. A control experiment was performed in which a rat was fed a regular diet – no vitamin D₃ supplementation – to ensure that the serum was free of any vitamin D₃ metabolites (data not shown).

3.1.3.6. HPLC analysis

HPLC analysis of the lipid extracts from the cells and media was performed on a Waters instrument equipped with a photodiode array detector (PDA). The vitamin D₃ compounds were isolated and purified on a normal-phase Zorbax-Sil column, using two different solvent systems (system # 1: 10% isopropanol in hexane – 2 mL/min; system # 2: 0.1% isopropanol in hexane – 2 mL/min). UV absorbance was monitored at 265 nm.

3.1.3.7. GC-MS analysis

GC-MS analysis was performed on an Agilent 6890 GC-MSD 5973 system. Prior to analysis, all isolated vitamin D₃ metabolites were dried under vacuum then reconstituted in a 1:1 mixture of acetonitrile and trimethylsilyl (TMS) derivatizing reagent (Power-Sil Prep) and incubated at 70°C for 15 minutes to ensure complete derivatization. All samples were prepared to a final concentration of 10 µg/mL and 3 µL of each TMS derivative was injected onto a HP-Ultra 1 capillary column (100% dimethylpolysiloxane – 23 m x 0.2 mm I.D. x 0.11 µm film thickness). Ultra-high purity helium was used as the
carrier gas at a flow rate of 0.8 mL/min. A temperature program was run where the initial oven temperature 150°C was ramped up to 300°C at a rate of 10°C/min. The final temperature was held for an additional 5 minutes and the total run time was 20 minutes. Full-scan electron impact mass spectra were acquired across the mass range of m/z 50 to 750; the published spectra were averaged and background-subtracted.

3.1.3.8. 1H-NMR analysis

The 500-MHz 1H-NMR spectra were acquired by Dr. Gino Sasso (Roche, Nutley, NJ) on a Varian VXR-500 instrument equipped with a nano-probe. Two-dimensional COSY and NOESY spectra were obtained as described before8,12,13. Authentic standards and isolated metabolites were dissolved in CDCl3 containing tetramethylsilane as an internal zero reference.

3.1.4. Results

3.1.4.1. In vitro metabolism of vitamin D3 – Isolation of the produced metabolites by HPLC

We first examined the metabolism of vitamin D3 in Hep G2 cells, which have previously been shown to express the C-3 epimerization pathway8. Hep G2 cells were incubated with 10 μM vitamin D3 for 24, 48 and 72 hours and the lipid extracts from both medium and cells were analyzed by HPLC (Figure 3.2). The substrate eluted under peak 1. This was confirmed by co-elution with the synthetic standard of vitamin D3 and by its UV profile exhibiting a maximum absorbance at 265 nm and minimum at 228 nm. Two other
metabolites were produced by incubation of the Hep G2 cells with vitamin D₃. The less polar metabolite (P1) exhibited a UV profile characteristic of pre-vitamin D₃ (Absₓ-max at 260 nm, Absₓ-min at 228 nm). Metabolite 2 was identified as 25-hydroxy-D₃ (25(OH)D₃) based on both its UV profile (Absₓ-max at 265 nm, Absₓ-min at 228 nm) and co-elution with the synthetic standard of 25(OH)D₃. As the incubation time increased, the intensity of the substrate’s peak (1) decreased while that the polar metabolite (2) increased, which further illustrated the conversion of vitamin D₃ into its 25-hydroxylated form. This metabolic transformation was expected as Hep G2 cells have been shown to possess the enzymatic ability to hydroxylate vitamin D₃ at the C-25 position. 25(OH)D₃ was not observed in the control incubation, only vitamin D₃ and pre-vitamin D₃ were detected.

In order to investigate the production of 3-epi-vitamin D₃ in Hep G2 cells, peak 1 was reanalyzed using HPLC system # 2 (Figure 3.3). Due to extensive structural similarities, we suspected that the 3-epimer, if present, may have co-eluted with vitamin D₃ under the system # 1 conditions. As illustrated in Figure 3.3 (lower panel), the new conditions allow for the complete resolution of the vitamin D₃ synthetic standard (S1) from its 3-epimer (S1’).

Peak 1 and peak P1, observed on the 24h, 48h and 72h incubations profiles (Figure 3.3, upper three panels), were again identified as the substrate (vitamin D₃) and pre-vitamin D₃, respectively. A minor metabolite (1’), co-eluting with the synthetic 3-epi-D₃ standard (S1’), was also detected; its production increased with incubation time. Based on its UV profile (Absₓ-max at 265 nm, Absₓ-min at 228 nm) and its co-elution with S1’ (Figure 3.3,
lower panel) metabolite 1’ was proposed to be 3-epi-vitamin D₃ but further characterization by GC-MS and ¹H-NMR was required to confirm this assignment.

In order to produce an adequate amount of metabolite 1’ for its unequivocal structure identification, the incubation of Hep G2 cells with vitamin D₃ was repeated multiple times. The Hep G2 cells were grown in thirty T150 flasks. Each flask was incubated with vitamin D₃ (10 μM) for 72 h. A total of 30 μg of metabolite 1’ was isolated.
Figure 3.2. Time course study of vitamin D₃ metabolism in Hep G2 cells (24-hour, 48-hour and 72-hour incubations). HPLC profiles of lipid extracts from both cells and medium were generated using System #1. The UV spectra of the metabolite peaks (P1, 1 and 2) are shown in the insert.
Figure 3.3. HPLC resolution of peaks 1 and 1’ (isolated from 24-hour, 48-hour and 72-hour incubations) using System # 2 (upper three panels). The UV spectra of the metabolite peaks (P1, 1 and 1’) are shown in the insert. HPLC profiles of synthetic standards of vitamin D₃ (S1) and 3-epi-vitamin D₃ (S1’) are represented in the lower panel.
3.1.4.2. Identification of metabolite 1’ as 3-epi-vitamin D₃ by GC-MS

Following HPLC purification, the isolated metabolite 1’ was subjected to GC-MS analysis for further characterization (Figure 3.4). The purified metabolite was converted into its trimethylsilyl (TMS) ether by derivatization of all hydroxyl groups prior to analysis in order to enhance its volatility and yield more unique fragment ions. The GC-MS characteristics of the derivatized metabolite 1’ (Figure 3.4, panels C1-C2) were compared to those of the TMS derivatives of synthetic standards of vitamin D₃ and 3-epi-vitamin D₃ (panels A1-A2 and B1-B2, respectively) for structural identification.

One major GC peak was observed for metabolite 1’ at 13.64 min that was not detected for either synthetic standard. It was identified as the TMS derivative of cholesterol by library match of the corresponding mass spectrum with the NIST database. It would be reasonable to assume that cholesterol was present in the cell medium and co-eluted with metabolite 1’ during HPLC purification due to its structural similarities with vitamin D compounds. Even though cholesterol yields a major GC peak, it should be treated as a sample contamination and should not be taken into consideration for the structural characterization of metabolite 1’.

Beside the interfering cholesterol peak, the GC chromatogram of derivatized metabolite 1’ exhibited a doublet at 12.92 and 13.35 min (panel C1). Similarly, derivatized vitamin D₃ and 3-epi-vitamin D₃ standards each eluted as a doublet (panels A1 and B1). This is a phenomenon commonly observed for vitamin D compounds, which, upon thermal isomerization, yield two pyro- and isopyroisomers (Figure 2.8). Both isomers generally exhibit virtually identical mass spectral characteristics. The metabolite 1’ doublet was
observed at the exact same retention time as 3-epi-vitamin D₃ standard while vitamin D₃ standard eluted slightly earlier (12.59 and 13.49 min). In addition, the mass spectrum of metabolite 1’ at 12.92 min (panel C2) was virtually identical to that of the 3-epi-vitamin D₃ standard (panel B2). Similar molecular ions and fragment ions were observed for all three compounds, however slight differences in ion abundances were detected for vitamin D₃ (panel A2), especially for fragment ions at m/z 325, 351 and 366.

For each compound, the molecular ion (M⁺) was detected at m/z 456 and, upon the loss of a methyl group, yielded a fragment ion at m/z 441. The m/z 366 fragment resulted from the cleavage of a trimethylsilanol moiety (TMSOH) from the molecular ion. Subsequent loss of a methyl group yielded a fragment ion at m/z 351. The fragment ion at m/z 325 (loss of 131 Da) arose from the characteristic cleavage across the A-ring.

Based on both GC retention times and mass spectral characteristics, found virtually identical to those of 3-epi-vitamin D₃, we were able to further confirm the proposed structural identification of the metabolite 1’ as 3-epi-vitamin D₃.
Figure 3.4. GC-MS analysis of vitamin D₃ standard, 3-epi-D₃ standard and purified metabolite 1’.
The GC chromatograms of the TMS derivatives of vitamin D₃, 3-epi-vitamin D₃, and the isolated metabolite 1’ are represented in panels A1, B1 and C1, respectively. The corresponding mass spectra of the thermal isomers (peaks 1 and 2) of each compound are shown in panels A2, B2 and C2, respectively.
3.1.4.3. Confirmation of the stereochemistry of metabolite 1’ by $^1$H-NMR

$^1$H-NMR provided additional supporting evidence with regard to the $\alpha$-configuration of the 3-hydroxyl group. The $^1$H-NMR spectra of vitamin D$_3$ and 3-epi-vitamin D$_3$ standards were recorded and compared to that of metabolite 1’. $^1$H chemical shifts were assigned based on one- and two-dimensional COSY and NOESY spectra and are summarized in Table 3.1. The $^1$H-NMR spectra of metabolite 1’ and 3-epi-vitamin D$_3$ standard were superimposable, thereby confirming the identity and stereochemistry of metabolite 1’.

The most pronounced difference between vitamin D$_3$ and 3-epi-vitamin D$_3$ was the chemical shift of H-3 (vitamin D$_3$, 3.93 ppm; 3-epi-D$_3$, 3.86 ppm). The upfield shift of 3-epi-vitamin D$_3$ at H-3 position appears to be responsible. The resonances from all other protons were proof of an unchanged side chain and intact cis-triene system. These results led to the final conclusion that vitamin D$_3$ was metabolized into its C-3 epimer, namely 3-epi-vitamin D$_3$, in Hep G2 cells.
<table>
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<tr>
<th>Component</th>
<th>Vitamin D₃ standard</th>
<th>3-epi-vitamin D₃ standard</th>
<th>Metabolite 1’</th>
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<td>2.38</td>
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</tr>
<tr>
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<tr>
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</tr>
<tr>
<td>H-9</td>
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<td>0.84</td>
</tr>
<tr>
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<td>0.85</td>
<td>0.85</td>
</tr>
<tr>
<td>H-11</td>
<td>0.90</td>
<td>0.90</td>
<td>0.90</td>
</tr>
</tbody>
</table>

Table 3.1. NMR analysis of vitamin D₃ standard, 3-epi-vitamin D₃ standard and metabolite 1’. ¹H chemical shifts are in ppm and coupling constants in Hz.
3.1.4.4. In vivo metabolism of vitamin D₃ in rats

After demonstrating that vitamin D₃ undergoes C-3 epimerization in vitro, we investigated the in vivo production of 3-epi-vitamin D₃ in rats fed toxic levels of vitamin D₃. Following the metabolism study, lipids were extracted from the rat serum and analyzed by HPLC using the two solvent systems described previously. Similarly to the in vitro study, three metabolites were isolated along with the unmetabolized substrate. The metabolites were identified as pre-vitamin D₃, 3-epi-vitamin D₃ and 25-hydroxy-vitamin D₃ based on both their UV profiles and co-elution with their respective synthetic standards. The identification of 3-epi-vitamin D₃ was further confirmed by GC-MS and ¹H-NMR analysis. The metabolite isolated in vivo exhibited identical mass spectral and ¹H-NMR characteristics as metabolite 1’ isolated in vitro. We were therefore able to demonstrate that vitamin D₃ is metabolized through the C-3 epimerization pathway, both in vitro and in vivo, to yield 3-epi-vitamin D₃.
3.1.5. Discussion

In this project, we provided evidence for the metabolic conversion of vitamin D$_3$ into 3-epi-vitamin D$_3$ in Hep G2 cells. The unequivocal identity of 3-epi-vitamin D$_3$ produced in Hep G2 cells was established based on UV absorption spectrophotometry, HPLC co-elution with the synthetic 3-epi-vitamin D$_3$ standard, GC-MS and $^1$H-NMR analysis. Furthermore, we also identified 3-epi-vitamin D$_3$ as a circulating metabolite in rats treated with pharmacological amounts of vitamin D$_3$. Although the 3-epi-vitamin D$_3$ production was slightly greater in Hep G2 cells, only ca. 3% of the vitamin D$_3$ substrate was converted into its 3-epimer, both in vitro and in vivo. This confirmed previous observations that the C-3 epimerization pathway is a minor metabolic pathway.

In addition, we demonstrated that neither the 1$\alpha$-hydroxyl nor 25-hydroxyl groups are required for the C-3 epimerization to occur, unlike the C-24 oxidation pathway in which the presence of a 25-hydroxyl group is essential.

Through the present work, we are able to complete the elucidation of the C-3 epimerization pathway as it relates to vitamin D$_3$ and its two hydroxylated metabolites. As illustrated in Figure 3.5, 3-epi-vitamin D$_3$ may, like vitamin D$_3$, undergo metabolism via successive C-1 and C-25 hydroxylations to yield the biologically active metabolite, 1$\alpha$,25(OH)$_2$-3-epi-D$_3$. Just like vitamin D$_3$, 3-epi-vitamin D$_3$ itself is not expected to exert significant biological activity.
Figure 3.5. C-3 epimerization and activation pathways of vitamin D₃.
The C-3 epimerization pathway had already been shown to have a significant influence on the biological activity and metabolism of vitamin D₃ compounds. For instance, Brown and Reddy recently reported that 1α-OH-3-epi-D₃ is a potent suppressor of PTH secretion but without raising serum calcium. The mechanism for the unique biological actions of 1α-OH-3-epi-D₃ is still unclear. 1α,25-dihydroxy-3-epi-vitamin D₃, C-3 epimer of the vitamin D₃’s active metabolite, was also reported to exert interesting biological activities. More importantly, 3-epi compounds generally exhibit a lower calcemic potential relative to their 3β-counterpart, which significantly reduces their risk of inducing hypercalcemia, a major side-effect of vitamin D₃ compounds. Finally, the C-3 epimerization conversion may also affect the subsequent metabolism of epimerized vitamin D₃ compounds, again leading to enhanced or unique activities. An example of such effect will be illustrated in the following section (3.2).
3.2. Comparative Metabolism Study between 1α,25-dihydroxy-vitamin D₃ and 1α,25-dihydroxy-3-epi-vitamin D₃: New Evidence for the Metabolic Stability of 3-epi-Calcitriol

3.2.1. Introduction

The discovery of the metabolic conversion of 1α,25(OH)₂D₃ (calcitriol) into 1α,25(OH)₂-3-epi-D₃ (3-epi-calcitriol) was first reported more than a decade ago in human keratinocytes¹. Since then, it became obvious that the C-3 epimerization of various natural and synthetic vitamin D compounds including vitamin D₃ itself is a natural phenomenon²,³,⁵,⁶,¹⁴. Most C-3 epimers of vitamin D compounds are found to be relatively less calcemic, therefore reducing their potential toxicity. Moreover, they appear to possess interesting biological actions such as potent suppression of parathyroid hormone secretion and anti-proliferative effects on both normal and malignant cells³,¹⁴. However, the mechanisms responsible for the unique biological actions of C-3 epimers remain unknown. Such activity cannot be explained based on their stronger binding to the vitamin D receptor (VDR) as C-3 epimers are reported to bind to VDR with less affinity³. In a recent study, Reddy et al. proposed the metabolic stability of C-3 epimers as one of the possible mechanisms³.
3.2.2 Project Goals

We undertook the present study in collaboration with Epimer LLC and Teijin Pharma Ltd. to investigate the metabolic stability of the C-3 epimers of vitamin D compounds, more specifically 1α,25(OH)₂-3-epi-D₃, and provide further supporting evidence for Dr. Reddy’s hypothesis. To that effect, we compared the metabolism of 1α,25(OH)₂D₃ and its 3-epimer, 1α,25(OH)₂-3-epi-D₃, through the C-24 oxidation pathway in the perfused rat kidney. The metabolites produced from 1α,25(OH)₂-3-epi-D₃ were first isolated and purified by HPLC before further structural characterization by GC-MS. The amount of each 3-epi metabolite was then estimated and compared to that of their respective 3β-counterpart produced from 1α,25(OH)₂D₃, providing further evidence for the metabolic stability of 1α,25(OH)₂-3-epi-D₃.

3.2.3. Methods

3.2.3.1. Animals

Male Sprague-Dawley rats (ca. 350-375 g) were fed a regular rodent diet, sufficient in calcium, phosphorus, and vitamin D₃. Rats were pre-treated intraperitoneally with 2µg of 1α,25(OH)₂D₃ 16 hours and 4 hours prior to isolation of their right kidney in order to increase the activity of the 24-hydroxylase enzyme involved in the further metabolism of 1α,25(OH)₂D₃. The rats were then anesthetized with an intraperitoneal injection of Ketamine (100 mg/kg) and their right kidneys were surgically removed and attached to individual kidney perfusion systems.
3.2.3.2. Study of 1α,25(OH)₂-3-epi-D₃ metabolism using the kidney perfusion technique

The metabolism of 1α,25(OH)₂D₃ was compared to that of 1α,25(OH)₂-3-epi-D₃ in rat kidney using the isolated perfused kidney system developed by Reddy et al. (Figure 3.6).

![Figure 3.6. Rat kidney perfusion apparatus]({})

Kidney perfusions were performed by Dr. Reddy as described before in detail. The comparative metabolism studies were initiated after an equilibration period of 2 hours and were performed using 1 µM substrate concentrations. The kidney perfusions were performed for a period of 2 hours with 100 mL of perfusate containing 41.6 µg of either 1α,25(OH)₂D₃ or 1α,25(OH)₂-3-epi-D₃. Lipid extraction of the kidney perfusate was then performed according to the procedure developed by Bligh and Dyer except that...
dichloromethane was substituted for chloroform. The lipid extracts from 10 mL of final perfusate were then subjected to HPLC analysis for isolation and purification of the produced metabolites.

3.2.3.4. HPLC analysis

HPLC analysis of the lipid extracts from the kidney perfusate was performed on a Waters instrument equipped with a photodiode array (PDA) detector. The vitamin D compounds were isolated and purified on a normal-phase Zorbax-Sil column, using two different solvent systems (system # 1: 10% isopropanol in hexane – 2 mL/min; system # 2: 6% isopropanol in dichloromethane – 2 mL/min). UV absorbance was monitored at 265 nm.

3.2.3.5. GC-MS Analysis

GC-MS analysis was performed on an Agilent 6890 GC-MSD 5973 system. Prior to analysis, all isolated vitamin D₃ metabolites were dried under vacuum then reconstituted in a 1:1 (v/v) mixture of acetonitrile and trimethylsilyl (TMS) derivatizing reagent (Power-Sil Prep) and incubated at 70 °C for 15 minutes to ensure complete derivatization. All samples were prepared to a final concentration of 10 µg/mL and 3 µL of each TMS derivative was injected onto a HP-Ultra 1 capillary column (100% dimethylpolysiloxane – 23 m x 0.2 mm I.D. x 0.11 µm film thickness). Ultra-high purity helium was used as the carrier gas at a flow rate of 0.8 mL/min. A temperature program was run where the initial oven temperature 150 °C was ramped up to 300 °C at a rate of 10 °C/min. The final temperature was held for an additional 5 minutes and the total run time was 20 minutes. Full-scan electron impact mass spectra were acquired across the mass range of m/z 50 to 750; the published spectra were averaged and background-subtracted.
3.2.4. Results

3.2.4.1. Metabolism of $1\alpha,25(OH)_2D_3$ in the perfused rat kidney

The HPLC profile of $1\alpha,25(OH)_2D_3$ (calcitriol) and its metabolites extracted from 10 mL of final perfusate is shown in Figure 3.7.

![HPLC profile of the lipid extracts from rat kidneys perfused with 1 μM $1\alpha,25(OH)_2D_3$.](image)

**Figure 3.7.** HPLC profile of the lipid extracts from rat kidneys perfused with 1 μM $1\alpha,25(OH)_2D_3$.

As expected, $1\alpha,25(OH)_2D_3$ was metabolized through the well characterized C-24 oxidation pathway, main side-chain metabolic pathway. $1\alpha,25(OH)_2D_3$ is first hydroxylated at C-24 to produce $1\alpha,24,25(OH)_3D_3$ (M$_1$), which upon oxidation yields $1\alpha,25(OH)_2$-24-oxo-D$_3$ (M$_2$). Further hydroxylation at C-23 leads to the formation of
1α,23,25(OH)₃-24-oxo-D₃ (M₃) and subsequent cleavage across the C23-C24 bond yields 1α,23(OH)₂-24,25,26,27-tetranor-D₃ (M₄). Further oxidation of M₄ results in the formation of calcitroic acid, end product of the C-24 oxidation pathway. The chemical structures of M₁, M₂, M₃ and M₄ are represented in **Figure 3.8**.

**Figure 3.8.** Chemical structures of M₁, M₂, M₃ and M₄, metabolites of 1α,25(OH)₂D₃.
3.2.4.2. Metabolism of $1\alpha,25(OH)_2$-3-epi-$D_3$ in perfused rat kidney

The HPLC profile of $1\alpha,25(OH)_2$-3-epi-$D_3$ (3-epi-calcitriol) and its metabolites extracted from the rat kidney perfusate is shown in Figure 3.9.

![HPLC profile of the lipid extracts from rat kidneys perfused with 1 μM $1\alpha,25(OH)_2$-3-epi-$D_3$.](image)

**Figure 3.9.** HPLC profile of the lipid extracts from rat kidneys perfused with 1 μM $1\alpha,25(OH)_2$-3-epi-$D_3$.

Similarly to $1\alpha,25(OH)_2$-$D_3$, four metabolites ($M_1^*$, $M_2^*$, $M_3^*$ and $M_4^*$) are being produced upon metabolism of the 3-epimerized substrate. It would be reasonable to identify the four $M^*$ metabolites as the C-3 epimers of $M_1$, $M_2$, $M_3$ and $M_4$. However, each $M^*$ metabolite must be fully characterized by GC-MS in order to confirm the proposed structural assignment.
3.2.4.3. Identification of the isolated metabolites of $1\alpha,25(OH)_2$-3-epi-D$_3$ by GC-MS

After isolation and purification by HPLC, each $M^*$ metabolite was subjected to GC-MS analysis for further structural characterization. Prior to analysis, $1\alpha,25(OH)_2$-3-epi-D$_3$ and its four metabolites ($M_1^*$, $M_2^*$, $M_3^*$ and $M_4^*$) were derivatized into their respective trimethylsilyl (TMS) ethers in order to enhance their volatility. The mass spectra of the four trimethylsilylated metabolites are represented in Figure 3.10.

Metabolite $M_1^*$ exhibited a molecular ion at $m/z$ 720 while the TMS derivative of $1\alpha,25(OH)_2$-3-epi-D$_3$ gave a molecular ion at $m/z$ 632. The 88 mass unit shift corresponds to the incorporation of a hydroxyl group (-OTMS after derivatization) through metabolism of the substrate and is therefore well in agreement with the structure proposed for $M_1^*$, namely $1\alpha,24,25(OH)_3$-3-epi-D$_3$. $M_1^*$ exhibited additional fragment ions characteristic of vitamin D$_3$ compounds at $m/z$ 131, 217 and 589. The $m/z$ 131 ion resulted from the side chain cleavage across C24-C25 while the fragments at $m/z$ 589 (loss of 131 Da) and 217 were formed upon cleavage across the A-ring as indicated in Figure 3.10.

The TMS derivative of metabolite $M_2^*$ exhibited a molecular ion at $m/z$ 646. The 14 mass unit shift from the substrate is consistent with the proposed structure for $M_2^*$, $1\alpha,25(OH)_2$-24-oxo-3-epi-D$_3$. The TMS derivatization being specific to hydroxyl groups, the keto functionality remained intact upon reaction with the TMS agent. This explains why we only observed a 14 mass unit shift from the substrate and not 88 Da (-OTMS). Similarly to $M_1^*$, the mass spectrum of trimethylsilylated $M_2^*$ also exhibited characteristic fragment ions at $m/z$ 131, 515 (loss of 131 Da) and 217.
The TMS derivative of M$_3^*$ yielded a molecular ion at $m/z$ 734. However, its intensity was so low that it was not detectable on the mass spectrum. Instead, we observed a fragment at $m/z$ 644 resulting from the elimination of a TMSOH group from M$^+$. The fragment ion at $m/z$ 603 corresponding to the loss of 131 mass units upon cleavage across the A-ring further confirmed that the molecular ion, had it been intense enough, would have been detected at $m/z$ 734. The 88 mass unit shift from the TMS derivative of metabolite M$_2^*$ is well in agreement with the incorporation of an additional hydroxyl group. Metabolite M$_3^*$ was therefore identified as 1α,23,25(OH)$_3$-24-oxo-3-epi-D$_3$.

Lastly, with a molecular ion at $m/z$ 576, the identity of M$_4^*$ was confirmed as 1α,23(OH)$_2$-24,25,26,27-tetranor-3-epi-D$_3$.

The mass spectra of the four M$^*$ metabolites were also compared to those of the known metabolites of 1α,25(OH)$_2$D$_3$ (data not shown). The mass spectrum of each M$^*$ metabolite was found virtually identical to that of the corresponding metabolite of 1α,25(OH)$_2$D$_3$; only the retention times were slightly shifted relative to the 1α,25(OH)$_2$D$_3$ metabolites, which had also been observed on the HPLC profiles. This additional observations further confirmed that the four M$^*$ metabolites are the respective C-3 epimers of M1, M2, M3 and M4.

Thus, the comparative metabolism study between 1α,25(OH)$_2$D$_3$ and 1α,25(OH)$_2$-3-epi-D$_3$ in the isolated perfused rat kidney indicates that, like the natural hormone, 1α,25(OH)$_2$-3-epi-D$_3$ is metabolized through the C-24 oxidation pathway.
Figure 3.10. Mass spectra (GC-MS) of the M* metabolites produced from 1α,25(OH)₂-3-epi-D₃.
3.2.4.4. Relative quantification of the various metabolites of $1\alpha,25(OH)_2D_3$ and $1\alpha,25(OH)_2$-3-epi-D$_3$ isolated from the kidney perfusions

Following their structural characterization, we determined the relative amounts of the various metabolites produced from both $1\alpha,25(OH)_2D_3$ and $1\alpha,25(OH)_2$-3-epi-D$_3$. The relative amounts were estimated based on the HPLC peak areas of the metabolites isolated from three kidney perfusions and are reported in Table 3.2. Each data point represents the mean of three experiments.

<table>
<thead>
<tr>
<th>Unmetabolized Substrate</th>
<th>$1\alpha,25(OH)_2D_3$</th>
<th>$1\alpha,25(OH)_2$-3-epi-D$_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8.20 µg</td>
<td>7.40 µg</td>
</tr>
<tr>
<td>$1\alpha,24,25(OH)_3$D$_3$</td>
<td>0.37 µg (M$_1$)</td>
<td>2.13 µg (M$_1$*)</td>
</tr>
<tr>
<td>$1\alpha,25(OH)_2$-24-oxo-D$_3$</td>
<td>0.29 µg (M$_2$)</td>
<td>0.64 µg (M$_2$*)</td>
</tr>
<tr>
<td>$1\alpha,23,25(OH)_3$-24-oxo-D$_3$</td>
<td>0.68 µg (M$_3$)</td>
<td>1.56 µg (M$_3$*)</td>
</tr>
<tr>
<td>$1\alpha,23(OH)_2$-24,25,26,27-tetranor-D$_3$</td>
<td>0.96 µg (M$_4$)</td>
<td>0.27 µg (M$_4$*)</td>
</tr>
</tbody>
</table>

Table 3.2. Relative amounts of various metabolites of $1\alpha,25(OH)_2D_3$ and $1\alpha,25(OH)_2$-3-epi-D$_3$ isolated from three kidney perfusions.

The results shown in Table 3.2 indicate that the concentration of unmetabolized $1\alpha,25(OH)_2D_3$ was almost similar to that of $1\alpha,25(OH)_2$-3-epi-D$_3$. However, it is significant to note that the amounts of $1\alpha,24,25(OH)_3$-3-epi-D$_3$, $1\alpha,25(OH)_2$-24-oxo-3-epi-D$_3$ and $1\alpha,23,25(OH)_3$-24-oxo-3-epi-D$_3$ present in the perfusate were much higher than the corresponding intermediary metabolites of $1\alpha,25(OH)_2D_3$. In contrast, the amount of $1\alpha,23(OH)_2$-24,25,26,27-tetranor-3-epi-D$_3$ was much lower than the corresponding metabolite of $1\alpha,25(OH)_2D_3$. This difference in the metabolism of
1α,25(OH)\textsubscript{2}-3-epi-D\textsubscript{3}, leading to the accumulation of intermediary metabolites, is better illustrated in Figure 3.11. A partial block in the conversion of 1α,23,25(OH)\textsubscript{3}-24-oxo-3-epi-D\textsubscript{3} (M\textsubscript{3\*}) into 1α,23(OH)\textsubscript{2}-24,25,26,27-tetranor-3-epi-D\textsubscript{3} (M\textsubscript{4\*}) was observed, leading to the accumulation of the intermediary metabolites, 1α,24,25(OH)\textsubscript{3}-3-epi-D\textsubscript{3} (M\textsubscript{1\*}) and 1α,23,25(OH)\textsubscript{3}-24-oxo-3-epi-D\textsubscript{3} (M\textsubscript{3\*}).

Figure 3.11. Relative amounts of various metabolites of 1α,25(OH)\textsubscript{2}D\textsubscript{3} and 1α25(OH)\textsubscript{2}-3-epi-D\textsubscript{3}.
3.2.5. Discussion

In the present study, we demonstrated that, like $1\alpha,25$(OH)$_2$D$_3$, $1\alpha,25$(OH)$_2$-3-epi-D$_3$ is further metabolized in rat kidney into several polar metabolites. The various metabolites were unequivocally identified as $1\alpha,24,25$(OH)$_3$-3-epi-D$_3$, $1\alpha,25$(OH)$_2$-24-oxo-3-epi-D$_3$, $1\alpha,23,25$(OH)$_3$-24-oxo-3-epi-D$_3$ and $1\alpha,23$(OH)$_2$-24,25,26,27-tetranor-3-epi-D$_3$. Thus, both $1\alpha,25$(OH)$_2$D$_3$ and its C-3 epimer are metabolized through the same C-24 oxidation pathway as illustrated in Figure 3.12.
Figure 3.12. C-24 oxidation pathway of 1α,25(OH)₂D₃ (calcitriol) and 1α,25(OH)₂-3-epi-D₃ (3-epi-calcitriol) in perfused rat kidney.
The rate of disappearance of $1\alpha,25(\text{OH})_2$-3-epi-D$_3$ from the perfusate is comparable to that of $1\alpha,25(\text{OH})_2$D$_3$. However, the amounts of various metabolites of both compounds accumulated over time differ significantly. Our findings indicate that the side chain cleavage of $1\alpha,23,25(\text{OH})_2$-24-oxo-3-epi-D$_3$ to yield $1\alpha,23(\text{OH})_2$-24,25,26,27-tetranor-3-epi-D$_3$ is hindered. This partial metabolic block results in the accumulation of both $1\alpha,24,25(\text{OH})_3$-3-epi-D$_3$ and $1\alpha,23,25(\text{OH})_3$-24-oxo-3-epi-D$_3$ in the kidney perfusate. These observations provide supporting evidence with regard to the metabolic stability of 3-epi-calcitriol, proposed as one of the possible mechanisms responsible for its enhanced biological actions. The metabolic stability of $1\alpha,25(\text{OH})_2$-3-epi-D$_3$ is not at substrate level but rather occurs through its conversion into stable intermediary metabolites.

Further work was conducted in collaboration with Dr. Alex Brown (Washington School of Medicine, St Louis, MO) to study the biological activity of these stable metabolites. However, neither $1\alpha,24,25(\text{OH})_3$-3-epi-D$_3$ nor $1\alpha,23,25(\text{OH})_3$-24-oxo-3-epi-D$_3$ was reported to exert significant activity. These observations confirm that $1\alpha,25(\text{OH})_2$-3-epi-D$_3$ is indeed the active form directly responsible for its unique biological actions. Its enhanced potency may rather be the result of its slower metabolism, and therefore slower inactivation, relative to $1\alpha,25(\text{OH})_2$D$_3$. Further studies will be undertaken to further understand what prevents the side chain from being cleaved in the C-24 oxidation pathway of $1\alpha,25(\text{OH})_2$-3-epi-D$_3$.

ACKNOWLEDGEMENTS

This study was conducted in collaboration with Epimer LLC (Providence, RI) and Teijin Ltd. (Japan).
REFERENCES


Chapter 4:

Elucidation of a Novel Metabolic Pathway for 20-epi Analogs of Vitamin D₃:

C-1 Esterification with Stearic and Oleic Acids
4.1. Introduction

More than two decades ago, it was discovered that structural modifications of the secosteroid hormone \( \alpha{25(OH)}_2\text{D}_3 \) can result in the dissociation of its calcemic actions from its non-calcemic functions such as the regulation of cell growth and differentiation. This discovery led to the synthesis of numerous vitamin D analogs with a wide array of biological actions\(^1\). The 20-epi vitamin D analogs, in which the methyl group at C-20 is in its unnatural orientation, have received a great deal of interest\(^2\). Among such compounds, 1\(\alpha\),25-dihydroxy-20-epi-vitamin D\(_3\) (1\(\alpha\),25(OH)\(_2\)-20-epi-D\(_3\)) has become prominent because of its unique biological activity. The chemical structures of 1\(\alpha\),25(OH)\(_2\)-20-epi-D\(_3\) and other 20-epi vitamin D analogs are shown in Figure 4.1. It has been reported that 1\(\alpha\),25(OH)\(_2\)-20-epi-D\(_3\) decreases cell proliferation and promotes cell differentiation with a potency several orders of magnitude greater than that of 1\(\alpha\),25(OH)\(_2\)\text{D}_3\(^3\text{-}^6\). The mechanisms responsible for this enhanced potency of 1\(\alpha\),25(OH)\(_2\)-20-epi-D\(_3\) are not fully understood. In an earlier study, it was reported that 1\(\alpha\),25(OH)\(_2\)-20-epi-D\(_3\) is metabolized in its target tissues at a slower rate when compared to 1\(\alpha\),25(OH)\(_2\)\text{D}_3 and this was proposed as one possible mechanism\(^7\). Since then, our understanding of the target tissue metabolism of the natural hormone, 1\(\alpha\),25(OH)\(_2\)\text{D}_3, has advanced and we have re-examined the metabolism of the 20-epi vitamin D analogs in light of these new advances. It is now well established that 1\(\alpha\),25(OH)\(_2\)\text{D}_3 is metabolized in its target tissues through modifications of both the side chain and the A-ring. The C-24 oxidation pathway is the major pathway through which the side chain of 1\(\alpha\),25(OH)\(_2\)\text{D}_3 undergoes a series of oxidations at C-24 and C-23 followed by cleavage
between C-23 and C-24 for its final conversion into calcitroic acid\textsuperscript{8,9}. The C-3 epimerization pathway is the minor pathway through which the A-ring of 1α,25(OH)\textsubscript{2}D\textsubscript{3} undergoes a single modification of epimerization of the hydroxyl group at C-3 for its final conversion into 1α,25(OH)\textsubscript{2}-3-epi-D\textsubscript{3}\textsuperscript{10-12}. During the past decade, in collaboration with Dr. Satya Reddy (Epimer LLC, Providence, RI), we performed a series of studies to identify the differences between 1α,25(OH)\textsubscript{2}D\textsubscript{3} and 1α,25(OH)\textsubscript{2}-20-epi-D\textsubscript{3} in their target tissue metabolism through both the C-24 oxidation and C-3 epimerization pathways. Metabolism studies were performed both in the isolated perfused rat kidney which expresses only the C-24 oxidation pathway and in rat osteoblastic osteosarcoma cells (UMR-106) which express both C-24 oxidation and C-3 epimerization pathways. During these studies, it was observed that 1α,25(OH)\textsubscript{2}-20-epi-D\textsubscript{3} – but not 1α,25(OH)\textsubscript{2}D\textsubscript{3} – is metabolized in UMR-106 cells into novel less polar metabolites (LPMs). These LPMs were reported to possess significant biological activity and their production was found to be tissue specific as they are formed only in UMR-106 cells but not in the isolated perfused rat kidney\textsuperscript{13}.

Another 20-epi vitamin D analog, 1α,25(OH)\textsubscript{2}-16-ene-20-epi-D\textsubscript{3}, was also observed to be metabolized into LPMs in UMR-106 cells. The addition of the 16-ene modification to 1α,25(OH)\textsubscript{2}-20-epi-D\textsubscript{3} did not have any effect on its metabolism into LPMs. However, further addition of a 23-yne modification to yield 1α,25(OH)\textsubscript{2}-16-ene-23-yne-20-epi-D\textsubscript{3} completely blocked the new metabolic pathway, suggesting that not all 20-epi vitamin D analogs get metabolized into LPMs. Despite these valuable observations, the identity of the novel less polar metabolites remained to be elucidated.
4.2. Project Goals

We undertook the present study in an attempt to elucidate the structure of the aforementioned novel LPMs produced by metabolism of selected 20-epi analogs in rat UMR-106 osteoblastic osteosarcoma cells. At the time of the study, $1\alpha,25(\text{OH})_2$-16-ene-20-epi-D$_3$ was available in milligram quantities (gift from Dr. Milan Uskokovic) while only a very limited amount of $1\alpha,25(\text{OH})_2$-20-epi-D$_3$ was available. We therefore performed the metabolism study with $1\alpha,25(\text{OH})_2$-16-ene-20-epi-D$_3$ as we could not produce the LPMs of $1\alpha,25(\text{OH})_2$-20-epi-D$_3$ in sufficient quantity for their unequivocal identification. The LPMs produced via metabolism of $1\alpha,25(\text{OH})_2$-16-ene-20-epi-D$_3$
were first isolated and purified by HPLC before being subjected to GC-MS, ESI-MS and $^1$H-NMR analysis for structural characterization. We later obtained milligram quantities of $1\alpha,25$(OH)$_2$-20-epi-D$_3$ and were also able to confirm the identity of the LPMs produced with that substrate.

4.3. Materials and Methods

4.3.1. Materials

UMR-106 osteoblastic osteosarcoma cells were purchased from ATCC (Rockville, MD). Streptomycin, penicillin, McCoy’s media were obtained from Life Technologies (Gaithersburg, MD). Fetal calf serum (FCS) was purchased from Hyclone (Logan, UT). $1\alpha,25$-dihydroxy-vitamin D$_3$ ($1\alpha,25$(OH)$_2$D$_3$), $1\alpha,25$-dihydroxy-16-ene-vitamin D$_3$ ($1\alpha,25$(OH)$_2$-16-ene-D$_3$), $1\alpha,25$-dihydroxy-16-ene-20-epi-vitamin D$_3$ ($1\alpha,25$(OH)$_2$-16-ene-20-epi-D$_3$), and $1\alpha,25$-dihydroxy-16-ene-20-epi-3-epi-vitamin D$_3$ ($1\alpha,25$(OH)$_2$-16-ene-20-epi-3-epi-D$_3$) were synthesized at Hoffmann-La Roche (Nutley, NJ).

4.3.2. Cells and cell culture

Rat UMR-106 osteoblast-like osteosarcoma cells were maintained in McCoy’s culture media supplemented with 10% FCS and antibiotics (penicillin (100 IU/mL) and streptomycin (100 μg/mL)). Cell culture medium was replenished every 3-4 days. For the metabolism studies, $3 \times 10^6$ cells were seeded in T150 tissue culture bottles and grown to confluence. The incubations were carried out at 37 °C in a humidified 5% CO$_2$ atmosphere.
4.3.3. Metabolism studies in UMR-106 cells

All metabolism studies were performed by Dr. Satya Reddy (Epimer LLC, Providence, RI). UMR-106 cells (3 x 10^6 cells/mL) were incubated with various concentrations of 1α,25(OH)2-16-ene-20-epi-D₃ or its 3-epimer, namely 1α,25(OH)₂-16-ene-20-epi-3-epi-D₃, in 50 mL of medium containing 10% FCS. The incubations were quenched after 24 hours by addition of 50 mL of methanol and the lipids from both cells and media were extracted according to the extraction procedure described by Bligh and Dyer¹⁴ except that dichloromethane was substituted for chloroform. The lipid extracts were then subjected to HPLC analysis for isolation and purification of the generated metabolites. Control incubations were also performed in which the substrate was incubated in cell-free medium to ensure that the substrate did not undergo any chemical change or breakdown during the incubation period or the extraction procedure (data not shown).

4.3.4. HPLC analysis

HPLC analysis of the lipid extracts from the cells and media was performed on a Waters instrument equipped with a photodiode array detector (PDA). The vitamin D₃ compounds were isolated and purified using three different chromatographic methods. The conditions for the three HPLC methods are summarized in Table 4.1. The lipid extracts were first subjected to HPLC Method-I. The metabolites of interest were further resolved and purified using Method-II and Method-III prior to GC-MS analysis. UV absorbance was monitored at 265 nm.
<table>
<thead>
<tr>
<th>Method</th>
<th>HPLC Column</th>
<th>Mobile phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method I</td>
<td>Zorbax-SIL (9.4 x 250 mm) (Normal phase)</td>
<td>90:10 hexane:isopropanol (2 mL/min – isocratic)</td>
</tr>
<tr>
<td>Method II</td>
<td>Zorbax-SIL (9.4 x 250 mm) (Normal phase)</td>
<td>98:2 hexane:isopropanol (2 mL/min – isocratic)</td>
</tr>
<tr>
<td>Method III</td>
<td>Zorbax-ODS (4.6 x 250 mm) (Reversed phase)</td>
<td>90:10 acetonitrile:dichloromethane (2 mL/min – isocratic)</td>
</tr>
</tbody>
</table>

Table 4.1. HPLC Conditions for the analysis of lipid extracts from cells.

4.3.5. GC-MS analysis

GC-MS analysis was performed on an Agilent 6890 GC-MSD 5973 system. Prior to analysis, all isolated vitamin D$_3$ metabolites were dried under vacuum then reconstituted in a 1:1 (v/v) mixture of acetonitrile and trimethylsilyl (TMS) derivatizing reagent (Power-Sil Prep) and incubated at 70 °C for 15 minutes to ensure complete derivatization. All samples were reconstituted to a final concentration of 10 µg/mL and 3-5 µL of each TMS derivative was injected onto a HP-Ultra 1 capillary column (100% dimethylpolysiloxane – 23 m x 0.2 mm I.D. x 0.11 µm film thickness). Ultra-high purity helium was used as the carrier gas at a flow rate of 0.8 mL/min. A temperature program was run where the initial oven temperature 150 °C was ramped up to 300 °C at a rate of 10 °C/min. The final temperature was held for an additional 5 minutes, resulting in a total run time of 20 minutes. Full-scan electron impact mass spectra were acquired across the mass range of m/z 50 to 750; the published spectra were averaged and background-subtracted.
4.3.6. ESI-MS analysis (infusion)

Further mass spectrometric analysis was performed on a Thermo-Finnigan LCQ Classic ion trap equipped with a nanoelectrospray (nano-ESI) source developed in our laboratory. Prior to infusion, the underivatized vitamin D3 compounds were dried and reconstituted in a 60:40 acetonitrile:water solution containing 0.1% formic acid. Full scan MS and MS/MS mass spectra were acquired. MS/MS experiments were performed in the ion trap in the presence of helium with the relative collision energy set at 32%.

4.3.7. ¹H-NMR analysis

The ¹H-NMR spectra were acquired by Dr. Gino Sasso at Roche (Nutley, NJ) on a Varian UNITYplus® 400-MHz spectrometer. The samples were dissolved in deuterochloroform containing tetramethylsilane as an internal zero reference.

4.4. Results

4.4.1. Metabolism of 1α,25(OH)₂-16-ene-D₃ and its C-20 epimer in UMR-106 cells

We compared the metabolism of the natural hormone, 1α,25(OH)₂-D₃ (S1), in UMR-106 cells to that of 1α,25(OH)₂-16-ene-D₃ (S2) and its C-20 epimer 1α,25(OH)₂-16-ene-20-epi-D₃ (S3). Cells were incubated with each substrate (1 µM) for 24h. 

Figure 4.2 shows the normal-phase profiles (HPLC Method-I) of the metabolites produced from each substrate. As expected, all three substrates were metabolized through the C-3 epimerization and the C-24 oxidation pathways to yield their respective C-3 epimers as well as 24-hydroxy- and 24-oxo-derivatives. A small amount of substrate was
converted back to the pre-vitamin form (Peak P). Based on these HPLC profiles, the incorporation of the 16-ene unsaturation did not appear to affect the metabolism of the hormone. However, further modification around C-20 to yield 1α,25(OH)₂-16-ene-20-epi-D₃ (S3) led to the production of additional metabolites (Peaks X and Y). These novel metabolites, eluting very early, are significantly less polar than the metabolites produced through the C-3 epimerization or C-24 oxidation pathways. When the incubation was performed using the C-3 epimerized substrate, namely 1α,25(OH)₂-16-ene-20-epi-3-epi-D₃ (S4), it was noted that only metabolite Y was being produced (Figure 4.2). This observation suggested that metabolite Y may be produced after C-3 epimerization of 1α,25(OH)₂-16-ene-20-epi-D₃ (S3).
Figure 4.2. Normal-phase profiles (HPLC Method-I) of the metabolites produced from 1α,25(OH)₂D₃ (S1), 1α,25(OH)₂-16-ene-D₃ (S2), 1α,25(OH)₂-16-ene-20-epi-D₃ (S3) and 1α,25(OH)₂-16-ene-20-epi-3-epi-D₃ (S4) in UMR 106 cells.
Moreover, as depicted in **Figure 4.3**, the formation of the novel LPMs, particularly metabolite X, noticeably increased when the substrate concentration was increased. At 10 μM, the LPMs production became so prominent that the C-24 oxidation metabolites were no longer detected.

**Figure 4.3.** Metabolism of 1α,25(OH)₂-16-ene-20-epi-D₃ in UMR 106 cells at different substrate concentrations.
4.4.2. **HPLC isolation and purification of the less polar metabolites (LPMs) produced from 1α,25(OH)2-16-ene-20-epi-D3 and 1α,25(OH)2-16-ene-20-epi-3-epi-D3**

The LPMs isolated and collected using HPLC Method-I were further resolved by switching to HPLC Method-II (conditions described in Section 4.3.4.). As shown in **Figure 4.4**, 1α,25(OH)2-16-ene-20-epi-D3 is actually metabolized into four LPMs (X1, X2, Y1 and Y2), two of which (Y1 and Y2) being produced after C-3 epimerization of the substrate. The four LPMs were completely resolved in a reversed-phase system (HPLC Method-III); their LC profiles are shown in **Figure 4.4** (inserts). Each purified metabolite was collected manually for further structural characterization. In order to obtain sufficient amounts of each LPM for GC-MS, ESI-MS and 1H-NMR analysis, we incubated 55 culture bottles with 1α,25(OH)2-16-ene-20-epi-D3 (10 μM) and 35 culture bottles with 1α,25(OH)2-16-ene-20-epi-3-epi-D3 (10 μM). After final purification, we isolated 523 μg of X1, 316 μg of X2, 859 μg of Y1 and 448 μg of Y2.
Figure 4.4. Further resolution of LPMs produced from 1α,25(OH)_{2}-16-ene-20-epi-D_{3} and 1α,25(OH)_{2}-16-ene-20-epi-3-epi-D_{3} using HPLC Method-II. Complete resolution was obtained with HPLC Method-III (inserts).
4.4.3. Structure elucidation of the less polar metabolites X1, X2, Y1 and Y2

4.4.3.1. GC-MS analysis

The GC chromatogram of the TMS derivative of 1α,25(OH)2-16-ene-20-epi-D3 is represented in Figure 4.5 (Panel A). Two peaks were observed at 14.04 and 14.77 min corresponding to the pyro- and isopyroisomers of the analyte. Thermal isomerization is a phenomenon commonly observed in the GC-MS analysis of vitamin D compounds. The formation of the pyro- and isopyroisomers is described in more detail in Chapter 2 (Figure 2.8). The thermal isomerization generally does not affect the analysis as both isomers exhibit virtually identical mass spectra. The molecular ion of trimethylsilylated 1α,25(OH)2-16-ene-20-epi-D3 was detected at m/z 630 and, upon elimination of trimethylsilanol groups (TMSOH), yielded fragment ions at m/z 540 and m/z 450 (Figure 4.5 Panel B). Each TMSOH accounts for 90 mass units. The mass spectrum of the derivatized substrate exhibited additional fragments characteristic of vitamin D compounds at m/z 131, 217, and 499. The m/z 131 ion resulted from a side-chain cleavage across C-24/C-25 whereas the two fragments at m/z 499 (loss of 131 Da) and 217 are characteristic of a cleavage across the A-ring as depicted in Figure 4.5 (Panel B).
Figure 4.5. GC-MS analysis of trimethylsilylated $1\alpha,25$(OH)$_2$-16-ene-20-epi-D$_3$.

The GC chromatograms of trimethylsilylated metabolites X1 and X2 (Figure 4.6 Panels A1 and A2) yielded a doublet at 14.46 and 15.07 min (thermal isomers). The mass spectra under the GC peaks are virtually identical (Figure 4.6 Panel B). For both X1 and X2, the molecular ion of the TMS derivative was detected at $m/z$ 540 which is 90 mass units less than that of the parent, $1\alpha,25$(OH)$_2$-16-ene-20-epi-D$_3$ ($m/z$ 630) (Figure 4.5 Panel B). This observation indicates the loss or substitution of a hydroxyl group relative to the parent molecule. The presence of the fragment ion at $m/z$ 131 – characteristic of a side chain cleavage across the C-24/C-25 bond – in both the substrate’s and the
metabolites’ mass spectra suggests that the side chain was not altered during metabolism. Therefore, the structural alteration must have occurred at either C-1 or C-3. The absence of fragment ions at \( m/z \, 409 ([M^+\cdot-131]) \) or at \( m/z \, 217 \), both resulting from cleavage across the A-ring, provides further evidence for the hypothesized A-ring modification. Based on these observations and the 90 mass unit shift previously mentioned, we reasoned that the GC peaks represent A-ring dehydrated species resulting from the loss of a hydroxyl group from the substrate at either C-1 or C-3. Therefore, we initially proposed that X1 and X2 were dehydration products of the parent analog.

However, further examination of the GC chromatogram of trimethylsilylated X1 (Figure 4.6 Panel A1) revealed the presence of an additional peak at 7.72 min that was not present in the GC chromatogram of the parent (Figure 4.5 Panel A). Upon searching of the NIST library, the corresponding mass spectrum was found to be virtually identical to that of trimethylsilylated stearic acid (Figure 4.6 Panel C1). Similarly, the GC chromatogram of trimethylsilylated X2 exhibited an extra peak at 7.53 min. The mass spectrum (Figure 4.6 Panel C2) corresponded in this case to the TMS derivative of a different fatty acid, namely oleic acid, therefore ruling out the possibility of the fatty acid being an impurity in the samples. Instead, it could be suspected that the fatty acid moiety was cleaved off the metabolite’s A-ring upon injection on the GC system.
Figure 4.6. GC-MS analysis of trimethylsilylated metabolites X1 and X2. Panels A1 and A2 represent the GC chromatograms of TMS-X1 and TMS-X2, respectively. Panel B represents the mass spectrum at 15.07 min (X1 and X2). Panels C1 and C2 represent the mass spectra of the fatty acid moieties of X1 (stearic acid – 7.72 min) and X2 (oleic acid – 7.53 min), respectively.
Similarly, the GC-MS analysis of metabolites Y1 and Y2 produced from the C-3 epimerized substrate, 1α,25(OH)₂-16-ene-20-epi-3-epi-D₃, yielded an “A-ring dehydrated substrate” species along with a fatty acid – stearic acid for Y1 and oleic acid for Y2 (Figure 4.7). The slight shift in retention time for the “A-ring dehydrated substrate” species relative to X1 and X2 confirms that Y1 and Y2 are products of the C-3 epimerized substrate – the retention time of the 1α,25(OH)₂-16-ene-20-epi-3-epi-D₃ substrate itself was slightly shifted relatively to 1α,25(OH)₂-16-ene-20-epi-D₃.

The mass spectral findings thus indicated that metabolites X1, X2, Y1, and Y2 are formed as a result of metabolic alterations occurring on the A-ring of their parent. The data suggested that X1 and X2 may be fatty acid esters of the parent compound, 1α,25(OH)₂-16-ene-20-epi-D₃, and Y1 and Y2 fatty acid esters of 1α,25(OH)₂-16-ene-20-epi-3-epi-D₃ (Figure 4.8). Following GC-MS analysis, all four metabolites were subjected to ESI-MS analysis in an attempt to obtain a mass spectrum of the intact compounds and further confirm the proposed structural assignment.
Figure 4.7. GC-MS analysis of trimethylsilylated metabolites Y1 and Y2. Panels A1 and A2 represent the GC chromatograms of TMS-Y1 and TMS-Y2, respectively. Panel B represents the mass spectrum at 15.00 min (Y1 and Y2). Panels C1 and C2 represent the mass spectra of the fatty acid moieties of Y1 (stearic acid – 7.72 min) and Y2 (oleic acid – 7.53 min), respectively.
Figure 4.8. Proposed structures for metabolites X1, X2, Y1 and Y2.
4.4.3.2. ESI-MS analysis (infusion)

As expected, very little fragmentation was observed using electrospray ionization but, unlike GC-MS, ESI-MS allowed us to detect the intact molecules (Figure 4.9). Derivatization of the vitamin D compounds was not necessary for the analysis since the nanospray mode provided sufficient sensitivity. The full scan mass spectrum acquired for 1α,25(OH)2-16-ene-20-epi-D3 (MW 414) exhibited a sodium adduct ion \([\text{M+Na}]^+\) at \(m/z\) 437. Similarly, metabolites X1 and X2 were detected as [M+Na]\(^+\) ions at \(m/z\) 703 and 701 respectively, which would correspond to molecular weights of 680 for X1 and 678 for X2. This is well in agreement with the proposed structure assignment for X1 and X2, namely the stearic and oleic acid esters of their parent.

MS/MS experiments were also performed by isolating each [M+Na]\(^+\) ion and subjecting it to further fragmentation. This resulted in the loss of a H\(_2\)O molecule for the substrate (loss of 18 Da) and the cleavage of the fatty acid moiety for X1 and X2 (loss of 284 and 282 Da respectively). As a result, all three compounds yielded the same \(m/z\) 419 ion upon fragmentation, which correlates well with the dehydrated species detected by GC-MS. ESI-MS analysis of Y1 and Y2 yielded identical observations.

The ESI-MS observations therefore confirmed that metabolites X1 and X2 were stearic and oleic acid esters of their parent, respectively, and Y1 and Y2 stearic and oleic acid esters of 1α,25(OH)\(_2\)-16-ene-20-epi-3-epi-D3 but the position of the esterification site (C-1 or C-3) on the A-ring had yet to be determined. Following mass spectrometric analysis, all four metabolites were thus subjected to \(^1\)H-NMR analysis to determine whether the esterification occurred at C-1 or C-3.
Figure 4.9. ESI-MS of 1α,25(OH)2-16-ene-20-epi-D3 and metabolites X1 and X2. The left panels represent the full scan mass spectra acquired for each compound in the full scan MS mode. The right panels represent the MS/MS mass spectra acquired upon fragmentation of the base ions.
4.4.3.3. $^1$H-NMR analysis

The $^1$H-NMR spectra acquired for metabolites X1, X2, Y1 and Y2 were compared to the spectra of their parent compounds (Figure 4.10). In order to determine whether the esterification occurred at C-1 or C-3, we looked specifically at the chemical shifts of the hydrogen atoms attached to these two carbons, labeled H-1 and H-3. Upon metabolism, H-1 was shifted from 4.45 ppm to 5.51 ppm while H-3 remained unchanged, which was indicative of a structural alteration around C-1. The shifting of H-1 by 1.06 ppm downfield can be best explained by an acylation effect which would be well in agreement with the esterification process (–CH–O–CO–R). Moreover, the presence of a 2-proton triplet at 2.26 ppm, characteristic of a methylene attached to a carboxyl (–CH$_2$–COOR), further validated our proposed structure assignment for the metabolites as fatty acid esters of their parent compounds.
Figure 4.10. $^1$H-NMR spectra of 1α,25(OH)$_2$-16-ene-20-epi-D$_3$ (A), X1 (B), X2 (C), 1α,25(OH)$_2$-16-ene-20-epi-3-epi-D$_3$ (D), Y1 (E) and Y2 (F).
Based on both mass spectral and $^1$H-NMR findings, we were therefore able to identify X1, X2, Y1 and Y2 as 25(OH)-16-ene-20-epi-1-stearate-D$_3$, 25(OH)-16-ene-20-epi-1-oleate-D$_3$, 25(OH)-16-ene-20-epi-3-epi-1-stearate-D$_3$, and 25(OH)-16-ene-20-epi-3-epi-1-oleate-D$_3$, respectively. For all four metabolites, the fatty acid ester bond was most likely hydrolyzed in the GC injection port. The released fatty acid was subsequently derivatized *in situ* by residual vapors of the trimethylsilylating agent, yielding two distinct sets of GC peaks, an apparent dehydrated substrate and a fatty acid moiety.

4.5. Discussion

We have demonstrated that 1$\alpha$,25(OH)$_2$-16-ene-20-epi-D$_3$ undergoes C-1 esterification in UMR-106 cells. The newly elucidated metabolic pathway of the 20-epi analog is illustrated in Figure 4.11. We also confirmed the C-1 esterification of 1$\alpha$,25(OH)$_2$-20-epi-D$_3$ into stearic and oleic acid esters (data not shown).
Figure 4.11. C-1 esterification pathway of 1α,25(OH)₂-16-ene-20-epi-D₃.
Biosynthetic esterification of steroids is a well established phenomenon in steroid metabolism\textsuperscript{16}. It has been shown that many of the steroid hormones undergo \textit{in vitro} acylation in different tissues, where the hydroxyl group at C-3, C-17 or C-21 gets enzymatically esterified\textsuperscript{17-30}. However, to date, we do not have adequate information regarding the nature of the enzyme(s) responsible for the formation of C-1 fatty acid esters of vitamin D compounds. We noted that, like the C-3 epimerization pathway, the new C-1 esterification pathway is tissue-specific as the production of less polar metabolites was observed only in rat osteoblastic osteosarcoma cells but not in rat kidney. The newly elucidated pathway also appears to be substrate-specific. As summarized in Figure \textbf{4.12}, the production of LPMs was observed with 1α,25(OH)\textsubscript{2}-20-epi-D\textsubscript{3} and 1α,25(OH)\textsubscript{2}-16-ene-20-epi-D\textsubscript{3} but further addition of a triple bond on the side chain to yield 1α,25(OH)\textsubscript{2}-16-ene-23-yne-20-epi-D\textsubscript{3} completely blocked the C-1 esterification pathway. Further studies are currently in progress to better understand the characteristics of the enzyme(s) responsible for the production of fatty acid esters of vitamin D compounds.
Figure 4.12. Substrate-specificity of the C-1 esterification pathway.

The significance of biological esterification of steroids with fatty acids is not fully understood. It is hypothesized that the fatty acid may protect the steroid nucleus from metabolism and thereby prolong the life of the parent steroid. For example, the fatty acid esters of estradiol, naturally occurring metabolites of estradiol, possess a valuable therapeutic potential due to their enhanced estrogenic potency and duration of action.
when compared to estradiol⁰. The enhanced action of estradiol esters likely results from an increased resistance to metabolism. A role for the naturally occurring steroid fatty acid esters as a tissue storage form of steroids has also been considered²². Further work is ongoing to better understand the biological significance of the esterification of vitamin D compounds.

ACKNOWLEDGEMENTS

This work was supported by funds from Epimer, LLC Providence, RI and a grant from Hoffmann-LaRoche Inc, Nutley, NJ.
REFERENCES


Chapter 5:

Synthesis, Characterization and LC-MS/MS Quantification of Benzo[a]pyrene Diol Epoxide – Deoxyguanosine Adducts in TK6 cells Treated with Benzo[a]pyrene
5.1. Introduction

5.1.1. DNA Adducts

All living organisms are constantly exposed to toxic agents (endogenous or exogenous). In humans, such exposure may originate from a variety of sources including car exhaust, tobacco smoke or charbroiled food. These agents, more specifically their electrophilic or radical intermediates, can bind covalently to DNA to yield addition products, commonly referred to as DNA adducts. Covalent binding to DNA leads to structural modification of nucleic acid constituents. Normally, chemical damage to genetic material is promptly repaired by cellular defense systems; however, a small fraction of primary damage events leads to permanent genetic mutations, which in turn can trigger the development of degenerative processes such as cancer. A strong relationship between DNA adduct formation and carcinogenicity has indeed been suggested by many over the past three decades. Thus, it is generally accepted that DNA adducts represent an early, critical and detectable step in carcinogenesis, and may serve as useful biomarkers for the risk assessment of exposure to harmful environmental carcinogens.

More than twenty classes of environmental carcinogens and mutagens have been identified over the past thirty years. The DNA adduct program implemented in the Vouros laboratory has mainly focused on DNA damage caused by aromatic amines (AAs) (e.g., 4-aminobiphenyl (4-ABP)), heterocyclic aromatic amines (HAAs) (e.g., 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)), and polycyclic aromatic hydrocarbons (PAHs) (e.g., benzo[a]pyrene (B[a]P)) (Figure 5.1).
5.1.2. Polycyclic Aromatic Hydrocarbons (PAHs) and Benzo[a]pyrene (B[a]P)

The focus of this chapter will be on polycyclic aromatic hydrocarbons, more specifically benzo[a]pyrene (B[a]P). Polycyclic aromatic hydrocarbons (PAHs) are a family of planar aromatic compounds that are formed by a variety of combustion processes. The simplest PAHs, as defined by the IUPAC, are phenanthrene and anthracene. The chemical structures of selected PAHs are represented in Figure 5.2. These ubiquitous environmental pollutants are found in car exhausts, charbroiled food, and tobacco smoke. Substantial evidence has accumulated showing that PAHs are causative agents in lung, skin, and bladder cancer.
5.1.3. Metabolic activation of PAHs

PAHs are not carcinogenic in their own right but require metabolic activation. Three pathways for the activation of PAHs to their ultimate carcinogens have been proposed\textsuperscript{17-21}. These pathways are represented in Figure 5.3 for B[a]P. The most widely accepted pathway of activation involves cytochrome-P450 (CYP)-mediated formation of B[a]P-7,8-oxide, which subsequently undergoes epoxide hydrolase-mediated hydrolysis to yield the proximate carcinogen B[a]P-7,8-dihydro-7,8-diol\textsuperscript{17,21}. CYP1A1 and CYP1B1 are
considered the major CYPs involved in the activation of B[a]P\textsuperscript{22,23}, although other isoforms including CYP1A2 are able to metabolize B[a]P\textsuperscript{23,24}.

Further activation of B[a]P-7,8-dihydrodiol (B[a]P-7,8-diol) can occur through CYP1A1- or CYP1B1-mediated metabolism to form the ultimate carcinogen, 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (B[a]PDE)\textsuperscript{24,25}. The B[a]PDE is detoxified by hydrolysis to the corresponding tetrols\textsuperscript{9,20,26} or by glutathione-S-transferase-mediated conversion into GSH-adducts\textsuperscript{9,27}. Any B[a]PDE that escapes these detoxification reactions can enter the nucleus and react with genomic DNA to form a number of diastereomeric 2′-deoxyguanosine (dG)- or 2′-deoxyadenosine (dA)-adducts\textsuperscript{9,17,21-23} as represented in Figure 5.3.

A second metabolic activation pathway involves aldo-keto reductase (AKR)-mediated oxidation of B[a]P-7,8-diol to a ketol, which rearranges to an air-sensitive catechol (B[a]P-7,8-catechol)\textsuperscript{18,20,28}. B[a]P-7,8-catechol undergoes two sequential one-electron autoxidation reactions to the ortho-quinone, B[a]P-7,8-dione, which can potentially form DNA adducts\textsuperscript{29}. In the presence of cellular reducing equivalents, redox cycling occurs to produce reactive oxygen species (ROS), such as hydroxyl radicals, that can cause oxidative DNA damage\textsuperscript{30}.

A third proposed pathway of B[a]P activation involves the intermediate formation of a radical cation through the action of CYP peroxidases\textsuperscript{31}. It has been suggested that the B[a]P-radical cation can enter the nucleus, react at C-8 of dG or N-7 of dA residues in DNA, and finally undergo a second one-electron oxidation to form depurinating DNA-adducts\textsuperscript{19}.
5.1.4. Stereochemistry of B[a]PDE and its derived DNA adducts

Two *anti*- and two *syn*-forms of B[a]PDE can arise from the P450-mediated activation pathway through the formation of (+)- and (-)-B[a]P-7,8-dihydrodiol. All four B[a]PDE diastereomers are mutagenic in bacterial and mammalian cell assays. However, (+)-*anti*-B[a]PDE is the most tumorigenic. Upon further activation, the four B[a]PDE isomers yield a total of eight B[a]PDE-dN stereoisomers represented in Figure 5.4. The present study will use the two *anti*-forms of B[a]PDE therefore yielding four B[a]PDE-dG diastereoisomers.
Figure 5.4. Stereochemistry of B[a]PDE-\(N^2\)-dG and B[a]PDE-\(N^6\)-dA adducts formed from (±)-anti-B[a]PDE and (±)-syn-B[a]PDE.
5.1.5. Interaction with DNA and toxicity

Upon metabolic activation, B[a]PDE intercalates with DNA, covalently bonding to the nucleophilic guanine or adenine nucleobases at the $N_2$- or $N_6$-position, respectively. B[a]PDE-dN adducts, more specifically B[a]PDE-$N_2$-dG, represent a strong block to DNA replication, with translesion synthesis resulting primarily in $G \rightarrow T$ transversions and a smaller percentage of $G \rightarrow A$ transition mutations. Several reports indicate that B[a]PDE targets, among other genes, the $p53$ tumor suppressor gene. By inducing $G \rightarrow T$ transversions within $p53$, there is a probability that B[a]PDE inactivates the tumor suppression ability in certain cells, potentially leading to cancer.

5.1.6. Characterization and measurement of DNA adducts

Historically, cancer risk assessment strategies have relied on measuring external exposure to carcinogens. Direct analysis of DNA adducts has proven to be more accurate and reliable in determining the carcinogenicity of xenobiotic compounds as well as allowing investigation of endogenous carcinogens. This approach allows direct quantitation of the primary damage to genetic material that may result from particular exposures. Considerable emphasis has therefore been placed on the development of sensitive and reliable methods for the detection and quantification of DNA adducts over the past three decades. DNA adducts have been examined by a variety of analytical techniques including fluorescence spectroscopy, laser induced fluorescence (LIF), immunoassays, electrochemical detection or the combination of affinity capillary electrophoresis (CE) with LIF. For many years, the method of choice has been $^{32}$P-postlabeling, a very sensitive method but which entails the use of radioactive materials.
In recent years, mass spectrometry has gained tremendous popularity for the qualitative and quantitative analysis of DNA adducts as it offers superior sensitivity, specificity as well as detailed structural information. The status of LC-MS for the analysis of DNA adducts has been critically reviewed by several authors. The Vouros research group was among the first to demonstrate the significance of capillary separation methods coupled to ESI/MS/MS for the analysis of bulky DNA adducts. Since the mid 1990s, continuous efforts have been focused on the optimization of such methods; the recent advent of chip-based technologies has contributed to significant detection improvements as will be described in this chapter.
5.2. Project Goals

This project was undertaken in collaboration with Helmut Zarbl as part of the DNA adduct program to gain a better understanding of the proposed relationship between DNA adducts and carcinogenesis. DNA adducts provide the most direct evidence of exposure and genetic damage in cells; we therefore investigated the possible correlation between the formation of B[a]PDE-dG adducts, carcinogen dose and changes in gene expression. An essential step toward achieving this goal was the development of a highly sensitive and reliable LC-MS/MS methodology for the characterization and quantification of B[a]PDE-dG adducts in cells dosed with B[a]PDE, active metabolite of B[a]P. In order to develop a quantitation method, B[a]PDE-dG authentic standards were required. Unfortunately such reference standards are not commercially available. Therefore, extensive efforts were dedicated to the synthesis and characterization of authentic B[a]PDE-dG and B[a]PDE-[15N2]-dG adduct standards as a preliminary step. The isotopically labeled adduct was used as an internal standard. The synthesis and characterization of these reference compounds will be described in detail in section 5.3. The fully validated LC-MS/MS method was subsequently applied to the detection and quantification of B[a]PDE-dG adducts in human lymphoblastoid TK6 cells treated with different levels of B[a]PDE (section 5.4.).

5.3.1. Materials and methods

5.3.1.1. Chemicals and materials

Caution: B[a]PDE and its derivatives are carcinogenic and mutagenic and must be handled with extreme care.

(±)-anti-B[a]PDE was purchased from the National Cancer Institute Chemical Carcinogen Reference Standard Repository (Midwest Research Institute, Kansas City, MO). Deoxyguanosine (dG), dimethyl sulfoxide (DMSO), triethylamine (TEA), ethyl acetate (EA), Tris-HCl, sodium phosphate monobasic, and sodium phosphate dibasic were obtained from Sigma (St. Louis, MO).

5.3.1.2. Synthesis of B[a]PDE-$N^2$-dG adducts

A solution of (±)-anti-B[a]PDE (100 μL; 5 mg/mL in DMSO) was added into a solution of dG (500 μL; 10 mg/mL of 0.1 M Tris-HCl, pH 7.5). The reaction mixture was incubated for 48 h in the dark at 37 °C with continuous stirring. After the reaction was completed, the reaction mixture was extracted three times with one volume of 1-butanol. The butanol phases were washed with water and combined before evaporation under vacuum in a Speedvac concentrator. The dried mixture was reconstituted in ca. 100 μL of methanol and subjected to thin layer chromatography (TLC) analysis.
5.3.1.3. Separation and purification of B[a]PDE-\(N^2\)-dG adducts

Isolation of B[a]PDE-\(N^2\)-dG adducts by preparative TLC

Preliminary purification of the synthesized adducts was conducted on a preparative TLC plate allowing for the resolution of the B[a]PDE-\(N^2\)-dG adducts from any unreacted material or other reaction byproducts. The TLC migration conditions were optimized and complete separation was achieved with a 4:1:0.5 EA:CH\(_3\)OH:H\(_2\)O solvent system containing 0.2\% TEA. After migration of the reaction mixture components for ca. 1.5 h, the band of interest (B[a]PDE-\(N^2\)-dG adducts) was scraped off the preparative TLC plate and the isolated adducts were redissolved in about 20 mL of migration solvent. The mixture was stirred vigorously for 30 minutes before filtration to remove the silica particles. After complete evaporation of the solvent, the isolated fraction was reconstituted in 45\% methanol and subjected to HPLC separation for further resolution of the four B[a]PDE-\(N^2\)-dG diastereomers. Prior to HPLC analysis, the reconstituted fraction was infused by mass spectrometry to confirm the identity of the collected B[a]PDE-\(N^2\)-dG.

Further separation and resolution of B[a]PDE-\(N^2\)-dG diastereomers by HPLC

Complete resolution of all four B[a]PDE-\(N^2\)-dG diastereomers was achieved in two steps. The isolated adducts were first separated on an analytical C18 column (Hyperclone ODS, 250 x 4.6 mm, 5\(\mu\)m, Phenomenex) using 50 mM phosphate buffer (pH 7.0) as mobile phase A and methanol as mobile phase B at a flow rate of 1 mL/min. The phosphate buffer was prepared fresh daily and filtered prior to use. The separation conditions were
optimized, the linear gradient was 48% B to 51% B over 50 min and UV absorbance was monitored at 260 nm and 335 nm. The separation yielded three peaks; all three peaks were collected and the homogeneity of the second and third peaks was confirmed by UV. The first peak was further resolved on a chiral column (OD-R, 250 x 4.6 mm, Chiralcel) using water as mobile phase A and acetonitrile as mobile phase B at a flow rate of 1 mL/min. The gradient was 5% B to 10% B in 30 min, followed by 20% B in 10 min, and 20% B was held for another 10 min. UV absorbance was monitored at 260 and 335 nm. Each peak was collected and the solvent was evaporated under vacuum. Desalting was conducted as a third and final purification step on the Hyperclone C18 column described above using water as mobile phase A and acetonitrile as mobile phase B. The mobile phase composition was held at 10% B for 3 min, followed by a linear gradient 10% B to 50% B in 25 min. The purity of each isolated adduct was confirmed by examination of its UV spectrum.

5.3.1.4. Synthesis, separation and purification of B[a]PDE-N2-[15N5]-dG adducts
A solution of (±)-anti-B[a]PDE (100 μL; 5 mg/mL in DMSO) was added into a solution of [15N5]-dG (500 μL; 10 mg/mL of 0.1 M Tris-HCl, pH 7.5). The reaction conditions, separation and purification procedures were the same as described above for the B[a]PDE-N2-dG adducts.

5.3.1.5. Characterization and structure identification of the synthesized standards
The UV absorbance (220-400 nm) of each purified adduct was measured on a Spectronic Genesys 5 UV/Vis spectrophotometer. MS and MS/MS spectra were acquired on an
Agilent MSD Trap XCT Ultra ion trap mass spectrometer. Each isolated adduct was dissolved in a 70:30 methanol:water infusion solvent containing 0.1% acetic acid and infused at a flow rate of 300 nL/min. The mass spectrometer was operated in the positive nanoelectrospray mode with a 1.7 kV spray voltage. Full scan MS and MS/MS spectra were acquired.

5.3.2. Results

5.3.2.1. Synthesis, separation and purification of B[a]PDE-N²-dG adducts

(±)-anti-B[a]PDE-N²-dG adduct standards were chemically synthesized. The reaction yield was fairly low (~10%) but well in agreement with data published by other groups. After synthesis, the adducts were first separated by preparative TLC from unreacted dG and hydrolyzed B[a]PDE. The adduct fraction was further purified by analytical reversed-phase HPLC (Figure 5.5 Panel A) and separated into three different elution fractions, X (retention time 26.6 min), Y (retention time 28.7 min) and Z (retention time 31.4 min). Fraction X was further resolved on a chiral column (Figure 5.5 Panel B) to yield fractions X₁ (retention time 48.9 min) and X₂ (retention time 55.1 min). After complete resolution of all four stereoisomers, each fraction was subjected to desalting on a reversed-phase column to remove any residual phosphate buffer.
Figure 5.5. LC-UV analysis of the stereoisomers of B[a]PDE-$N^2$-dG adduct standards. Panel A: Reversed-phase separation (C18 column). Panel B: Further resolution of isomers $X_1$ and $X_2$ by chiral chromatography.
5.3.2.2. Synthesis, separation and purification of B[a]PDE-N2-[15N5]-dG adducts

(±)-anti-B[a]PDE-N2-[15N5]-dG adduct internal standards were chemically synthesized and purified according to the same procedure as the dG-adducts. However, the second HPLC purification step (chiral separation) was not successful. As a result, we were only able to resolve and isolate two of the four diastereomers, Y’ and Z’, [15N5]-labeled counterparts of Y and Z respectively. This did not affect our LC-MS/MS method development, as only one internal standard was required for quantification purposes.

5.3.2.3. Characterization of B[a]PDE-N2-dG and B[a]PDE-N2-[15N5]-dG adducts

The structure and stereochemistry of the adduct stereoisomers were determined by MS and UV spectroscopy. The adducts were first subjected to MS and MS/MS analysis for structure confirmation (Figure 5.6). All of the four B[a]PDE-N2-dG adducts showed a [M+H]⁺ base ion at m/z 570. MS/MS analysis of m/z 570 yielded a unique fragment at m/z 454 resulting from the cleavage of the sugar moiety off the base (loss of 116 Da). Similarly, the full scan MS spectra of the [15N5]-labeled adducts exhibited an intense base ion at m/z 575 ([M+H]⁺). MS/MS analysis resulted in the formation of a fragment at m/z 459 (Figure 5.7).
Figure 5.6. Mass spectra of B[a]PDE-\(N^2\)-dG adduct standards. 
Upper panel: Full scan MS spectrum (\(m/z\) 400 – 800). Lower panel: MS/MS at \(m/z\) 570.2.
Figure 5.7. Mass spectra of B[a]PDE-[15N₅]-N²-dG internal standard. Upper panel: Full scan MS spectrum ($m/z$ 400 – 800). Lower panel: MS/MS at $m/z$ 575.2.
UV spectra were acquired to identify the stereochemistry of each adduct. The adducts were classified into two groups based upon their absorbance at 320-350 nm, characteristic range for the aromatic ring system of B[a]PDE. One group of adducts, B[a]PDE-dG adducts X2 and Y, exhibited a characteristic absorbance maximum at 346 nm, while the other group, adducts X1 and Z, exhibited an absorbance maximum at 345 nm. B[a]PDE-monomonucleoside adducts in the cis configuration show a stronger red-shift than those in trans configuration, therefore X2 and Y were assigned as cis stereoisomers, and X1 and Z were assigned as trans stereoisomers.

Our final stereochemistry assignments were based on published observations. It has been reported in the literature that the reaction of (±)-anti-B[a]PDE with dG significantly favors the formation of the (-)cis isomer while the (-)trans form is always a very minor product. Concentrations of authentic standard solutions were determined from UV absorbance at 345-346 nm and known extinction coefficients. The reaction of (±)-anti-B[a]PDE with dG yielded 2 μg X1, 20 μg X2, 44 μg Y, and 15 μg Z. Therefore, the minor isomer X1 was identified as (-)-anti-trans-B[a]PDE-N2-dG while Y was identified as (-)-anti-cis-B[a]PDE-N2-dG. X2 and Z, based on the previous cis/trans assignments, were identified as (+)-anti-cis-B[a]PDE-N2-dG and (+)-anti-trans-B[a]PDE-N2-dG, respectively.

Likewise, B[a]PDE-N2-[15N5]-dG adducts Y’ and Z’ were identified as (-)-anti-cis-B[a]PDE-N2-[15N5]-dG and (+)-anti-trans-B[a]PDE-N2-[15N5]-dG, respectively.
5.4. Quantification of B[a]PDE-dG adducts (total adducts) in TK6 cells dosed with (±)-anti-B[a]PDE by LC-MS/MS

5.4.1. Materials and Methods

5.4.1.1. Chemicals and materials

Calf thymus DNA (ct-DNA), DNase I, and alkaline phosphatase were obtained from Sigma (St. Louis, MO). Snake venom phosphodiesterase I (SVP) was purchased from USB (Cleveland, OH). Cell culture media and reagents were obtained from Life Technologies, Inc. (Grand Island, NY). Blood and cell culture DNA extraction maxi kits were purchased from Qiagen, Inc. (Valencia, CA).

5.4.1.2. Cell culture

Human lymphoblastoid line TK6\textsuperscript{60} was derived from the parental human lymphoblastoid line HH4 and hetereozygous for thymidine kinase, an enzyme which phosphorylates thymidine and its toxic analogs in an ATP-dependent reaction. The cells were grown at MIT by our collaborator Wendy Luo in spinner flasks to allow for gentle spinning. Cells were maintained in exponential growth by daily dilution in RPMI 1640 medium supplemented with 5% donor horse serum and maintained in 37\textdegree C incubators with a 5% CO\textsubscript{2} atmosphere. Cell counts were taken daily and cultures were diluted to ~ 4-5 x 10\textsuperscript{5} cells/mL. Detailed growth records were maintained and used to ascertain any effects of the treatment on growth rate.
5.4.1.3. Chemical treatment

TK6 cells were treated with (±)-anti-B[a]PDE, the mutagenic metabolite of B[a]P, by our collaborator Wendy Luo according to the following procedure. (±)-anti-B[a]PDE stock solutions were prepared in anhydrous DMSO (99.997% purity) and used immediately. The final DMSO concentration in the culture was less than 0.1%.

Prior to mutagen treatment, the background hypoxanthine-guanine phosphoribosyl transferase (HPRT) mutant fraction was reduced. TK6 cells were first “CHAT”-treated (10^{-5} M deoxycytidine + 2 \times 10^{-4} M hypoxanthine + 2 \times 10^{-7} M aminopterin + 1.75 \times 10^{-5} M thymine) for 3 days, followed by a 2-day recovery period in “THC”\textsuperscript{61}. Cells were then treated with three concentrations (0.017 \mu M, 0.034 \mu M, and 0.12 \mu M corresponding to 5%, 15%, and 40% toxicity levels, respectively) of B[a]PDE for 1 h. Triplicate cultures were completed at each concentration. Untreated cells were used as negative controls. At 4, 12, 17, and 24 h after treatment, two 100-mL aliquots of cells (~ 5 \times 10^5 cells/mL) were removed, cells were collected by centrifugation, immediately frozen down in liquid nitrogen, and stored at –80°C.

5.4.1.4. Cellular DNA extraction

DNA was extracted from human cells in our laboratory using Qiagen Blood and Cell Culture DNA Maxi Kits according to the manufacturer’s instructions (Figure 5.8). The Qiagen genomic DNA purification procedure is designed for direct isolation of chromosomal DNA 20-150 kb in size directly from whole blood, culture cells or tissues. The procedure is based on optimized buffer systems for careful lysis of cells and/or nuclei, followed by binding of genomic DNA to Qiagen anion-exchange resin under
appropriate low-salt and pH conditions. RNA, proteins, dyes, and low-molecular-weight impurities are removed by a medium-salt wash. Genomic DNA is then eluted in high-salt buffer and then concentrated and desalted by isopropanol precipitation. Following extraction and purification, the isolated DNA was resuspended in 10 mM Tris-HCl, pH 8.5 and redissolved overnight.

**Figure 5.8.** DNA extraction procedure. (Reproduced from [http://www.qiagen.com](http://www.qiagen.com) (accessed October 2008))
The amount of isolated DNA was measured on a Qubit fluorometer using the Quant-iT dsDNA BR assay kit (Invitrogen – Molecular Probes, Eugene, OR). The assay provides accurate and sensitive DNA quantitation for samples ranging from 100 pg/μL to 1 μg/μL, using minimal sample volume (1-20 μL).

5.4.1.5. DNA digestion to mononucleosides

After isolation of DNA from the cells, a 5- to 10-μg aliquot from each sample was dissolved to a final concentration of ~ 1 mg/mL in 10 mM MgCl₂ / 5 mM Tris buffer (pH 7.2). DNA was then digested to mononucleosides by first adding 5.7 μL of DNAse I (40,000 units/mL in 10 mM MgCl₂ / 5 mM Tris). The mixture was incubated at 37 °C for 5 h before the addition of 9 μL of alkaline phosphatase (0.13 units/μL in 10 mM MgCl₂ / 5 mM Tris) and 2 μL of phosphodiesterase I (0.037 units/μL in H₂O). The digestion mixture was incubated at 37 °C for 18 h. At the end of the incubation period, 20 fmol of internal standard, B[a]PDE-N²-[¹⁵N₅]-dG, was added to each digest. The digestion was terminated by addition of 5 volumes of ice cold ethanol. The solution was immediately centrifuged at 10,000 rpm for 10 min (4°C) to remove any salt and unreacted proteins. The supernatant was transferred to a clean microcentrifuge tube and the solvent was evaporated to dryness under vacuum. Each sample was then reconstituted in 20 μL of 5% methanol for LC-MS/MS analysis. The final concentration of internal standard (IS) in each sample was 1 fmol/μL. Each sample and control was digested and run in triplicate.
5.4.1.6. Preparation of B[a]PDE-N2-dG standard curves and quality control samples

In order to mimic the real sample composition as much as possible, the calibration curve was prepared in calf-thymus DNA. 5-μg aliquots of ct-DNA were dissolved to a final concentration of ~1 mg/mL in 10 mM MgCl₂ / 5 mM Tris buffer and digested to mononucleosides according to the procedure described in Section 5.4.1.5. At the end of the second incubation period (18 h), authentic B[a]PDE-N₂-dG standards (0, 0.2, 1, 2, 5, 10, 20, 40, 100 fmol) were added to the ct-DNA digest. A 20-fmol aliquot of B[a]PDE-N₂-[¹⁵N₅]-dG internal standard was also spiked in each sample. The digestion reaction was then quenched with ice cold ethanol, and salts and proteins were removed by centrifugation as described earlier. The matrix-matched calibration standards were reconstituted in 20 μL of 5% methanol to final concentrations of 0, 0.05, 0.1, 0.25, 0.5, 1, 2 and 5 fmol/μL. The internal standard final concentration was 1 fmol/μL. Each matrix-matched standard was prepared and run in triplicate. A matrix-matched blank was also prepared, free of standard and internal standard.

Quality control (QC) samples were prepared at three different concentrations (LQC: 0.5 fmol/μL, MQC: 1.5 fmol/μL, HQC: 2.5 fmol/μL – IS: 1 fmol/μL) following the same procedure. The QC samples were prepared and run in triplicate.

5.4.1.7. LC-nESI-MS/MS instrumentation

All quantification data were acquired on an Agilent 1200 Series HPLC-Chip/MS system, a new microfluidic chip-based technology for nanospray LC-MS. The reusable HPLC-Chip comprises a reversed-phase Zorbax SB C18 column (3.5 μm, 300 Å, 4.3 cm x 360
µm OD x 75 µm ID) and a 40 nL enrichment column of the same stationary phase allowing for online two-dimensional chromatography (Figure 5.9).

![Figure 5.9. Agilent HPLC-Chip. (Reproduced from http://www.agilent.com (accessed October 2008))](image)

We took advantage of the chip’s 2D feature (Figure 5.10) to perform final sample clean-up online, thereby eliminating the need for an offline solid phase extraction (SPE) step and reducing sample loss. The HPLC-Chip integrates the two columns with a nanospray emitter tip for direct interfacing of the nanoflow LC system to an Agilent Trap XCT Ultra mass spectrometer.
**Figure 5.10.** Flow path diagram of the HPLC-Chip in a 2D configuration. (Reproduced from [http://www.agilent.com](http://www.agilent.com) (accessed October 2008))
5.4.1.8. **LC-MS/MS conditions for B[a]PDE-dG adduct quantification**

The LC-MS/MS conditions for the quantification of B[a]PDE-dG are summarized in **Table 5.1**. As mentioned earlier, a final clean-up step was performed online to remove any residual salts, proteins and unadducted nucleosides. The sample was first loaded onto the enrichment column at 7% organic and trapped for 4 min, to allow for interfering species to be washed off. After 4 min, the valve was switched and the analyte was eluted through the analytical column using a gradient at 300 nL/min (**Table 5.1**). MS and MS/MS analyses were conducted in the positive ESI mode using the following mass transitions: \( m/z \ 570.2 \rightarrow m/z \ 454.2 \) for B[a]PDE-dG and \( m/z \ 575.2 \rightarrow m/z \ 459.2 \) for B[a]PDE-[\(^{15}\)N\(_5\)]-dG.

<table>
<thead>
<tr>
<th><strong>Capillary HPLC pump (enrichment)</strong></th>
<th><strong>MS Conditions</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile phase A: 3% ACN, 0.1% formic acid</td>
<td></td>
</tr>
<tr>
<td>Mobile phase B: Methanol, 0.1% formic acid</td>
<td></td>
</tr>
<tr>
<td>Flow rate: 4 µL/min</td>
<td></td>
</tr>
<tr>
<td>Gradient:</td>
<td></td>
</tr>
<tr>
<td>7% B at 4 min (sample trapping)</td>
<td></td>
</tr>
<tr>
<td>90% B at 6 min (post valve-switching wash)</td>
<td></td>
</tr>
<tr>
<td>7% B at 8.5 min (re-equilibration)</td>
<td></td>
</tr>
<tr>
<td><strong>Nanoflow HPLC pump (separation)</strong></td>
<td></td>
</tr>
<tr>
<td>Mobile phase A: 0.1% formic acid</td>
<td></td>
</tr>
<tr>
<td>Mobile phase B: Methanol, 0.1% formic acid</td>
<td></td>
</tr>
<tr>
<td>Flow rate: 300 nL/min</td>
<td></td>
</tr>
<tr>
<td>Gradient:</td>
<td></td>
</tr>
<tr>
<td>10% B at 4 min (before column switching)</td>
<td></td>
</tr>
<tr>
<td>90% B at 8 min (analyte elution)</td>
<td></td>
</tr>
<tr>
<td>60% B at 9 min</td>
<td></td>
</tr>
<tr>
<td>30% B at 10 min</td>
<td></td>
</tr>
<tr>
<td>10% B at 10.01 min</td>
<td></td>
</tr>
<tr>
<td>Hold at 30% B for another 4 min</td>
<td></td>
</tr>
<tr>
<td><strong>Total run time: 14 min</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Wellplate sampler conditions</strong></td>
<td></td>
</tr>
<tr>
<td>384-well plate</td>
<td></td>
</tr>
<tr>
<td>Injection volume: 5 µL</td>
<td></td>
</tr>
<tr>
<td><strong>Ionization mode</strong>: positive ESI</td>
<td></td>
</tr>
<tr>
<td><strong>Drying gas flow</strong>: 4.0 L/min</td>
<td></td>
</tr>
<tr>
<td><strong>Drying gas temperature</strong>: 325°C</td>
<td></td>
</tr>
<tr>
<td><strong>Capillary voltage</strong>: – 1725 V</td>
<td></td>
</tr>
<tr>
<td><strong>Skimmer 1</strong>: 40 V</td>
<td></td>
</tr>
<tr>
<td><strong>Capillary exit</strong>: 108 V</td>
<td></td>
</tr>
<tr>
<td><strong>Oct 1 DC</strong>: 12.00 V</td>
<td></td>
</tr>
<tr>
<td><strong>Oct 2 DC</strong>: 1.70 V</td>
<td></td>
</tr>
<tr>
<td><strong>Oct Rf</strong>: 47 V</td>
<td></td>
</tr>
<tr>
<td><strong>Trap drive</strong>: 180</td>
<td></td>
</tr>
<tr>
<td><strong>Lens 1</strong>: –5</td>
<td></td>
</tr>
<tr>
<td><strong>Lens 2</strong>: –60</td>
<td></td>
</tr>
<tr>
<td><strong>Automatic MS/MS</strong>:</td>
<td></td>
</tr>
<tr>
<td>MS/MS 1: ( m/z \ 570.2 \rightarrow m/z \ 454.2 )</td>
<td></td>
</tr>
<tr>
<td>MS/MS 2: ( m/z \ 575.2 \rightarrow m/z \ 459.2 )</td>
<td></td>
</tr>
<tr>
<td><strong>Scan</strong>: 420-600 m/z</td>
<td></td>
</tr>
</tbody>
</table>

**Table 5.1.** HPLC-Chip/MS conditions for the detection and quantification of B[a]PDE-dG adducts.
5.4.2. Results and Discussion

The objective of this project was to develop and validate a highly sensitive and robust assay for the quantification of B[a]PDE-N²-dG adducts. On the basis of previous LC-MS/MS methods developed for the analysis of DNA adducts in our group, we found that significant efforts had to be devoted to the optimization of the sample clean-up steps. Major improvements have been obtained by using the 2D chromatographic setup offered by the Agilent HPLC-Chip/MS instrument. The nanospray feature also contributed to the enhanced sensitivity of the method.

Typical extracted ion chromatograms for the B[a]PDE-N²-dG adduct standard (blue trace) and the internal standard (red trace) are presented in Figure 5.11 along with the corresponding MS/MS spectra. For each standard, we monitored the MS/MS transition corresponding to the fragmentation of the sugar moiety from the dG base (loss of 116 Da) as depicted in the insert.
Figure 5.11. LC-MS/MS analysis of B[a]PDE-dG adducts.
Panel A: Extracted ion chromatograms of the B[a]PDE-dG standard (blue trace) and B[a]PDE-[15N5]-dG internal standard (red trace).
Panel B: MS/MS spectrum of the B[a]PDE-dG standard.
Panel C: MS/MS spectrum of the B[a]PDE-[15N5]-dG internal standard.
5.4.2.1. Online clean-up recovery

It has been our experience that removal of extraneous materials (salts, proteins, unadducted nucleosides, etc.) prior to LC-MS/MS analysis is critical for optimal sensitivity. Therefore, the first step in the method development for the quantification of B[a]PDE-N²-dG adducts was the optimization of the online clean-up conditions. The trapping time and mobile phase composition were adjusted to 4 min and 7% methanol, 0.1% formic acid to ensure optimal removal of unadducted nucleosides. In order to determine the % recovery of the online clean-up step, 2.5 fmol of B[a]PDE-dG was mixed with 2.5 μg of free nucleosides (dN) and injected onto the enrichment column for a 4 min trapping followed by elution through the separation column under the conditions described in Section 5.4.1.8. The online clean-up % recovery was determined by comparing the response of a matrix-matched standard (B[a]PDE-dG + dN) to that of a neat B[a]PDE-dG standard. As depicted in Figure 5.12, we achieved 83% recovery. This compares with only 20-30% recovery achieved for the same sample using traditional SPE clean-up method and illustrates the value of online approach in reducing sample loss.
Figure 5.12. LC-MS/MS of 2.5 fmol B[a]PDE-dG on column (recovery experiment). Blue trace: neat solution – Red trace: B[a]PDE-dG dissolved in nucleoside matrix.
5.4.2.2. Calibration curve and linearity

A calibration curve was constructed by plotting the peak area ratio (analyte / IS) against the analyte concentration. The isotopically labeled internal standard was introduced to correct for any sample preparation or instrument operation variability. Its concentration was kept constant at 1 fmol/μL throughout the experiment. Each sample was run in triplicate and the relative standard deviation (% RSD = SD/mean x 100%) was calculated for each standard (Table 5.2). A linear response was observed from 0.05 to 5 fmol/μL, corresponding to 250 amol (1 amol = 10^{-18} mol) to 25 fmol (1 fmol = 10^{-15} mol) on column (Figure 5.13). The limit of detection was 50 amol on column.

<table>
<thead>
<tr>
<th>B[a]PDE-dG concentration (fmol/μL)</th>
<th>fmoles on column</th>
<th>Peak area ratio (std/IS)</th>
<th>Average Std/IS</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.050</td>
<td>0.25</td>
<td>0.0572 0.0512 0.0538</td>
<td>0.541</td>
<td>5.6%</td>
</tr>
<tr>
<td>0.10</td>
<td>0.50</td>
<td>0.102 0.104 0.099</td>
<td>0.101</td>
<td>3.8%</td>
</tr>
<tr>
<td>0.25</td>
<td>1.25</td>
<td>0.181 0.160 0.129</td>
<td>0.157</td>
<td>16.8%</td>
</tr>
<tr>
<td>0.50</td>
<td>2.5</td>
<td>0.376 0.288 0.339</td>
<td>0.334</td>
<td>13.2%</td>
</tr>
<tr>
<td>1.0</td>
<td>5.0</td>
<td>0.716 0.737 0.619</td>
<td>0.691</td>
<td>9.2%</td>
</tr>
<tr>
<td>2.0</td>
<td>10</td>
<td>1.23 1.17 1.33</td>
<td>1.25</td>
<td>6.4%</td>
</tr>
<tr>
<td>5.0</td>
<td>25</td>
<td>3.20 2.49 2.94</td>
<td>2.88</td>
<td>12.4%</td>
</tr>
</tbody>
</table>

Table 5.2. B[a]PDE-dG calibration standards concentrations and corresponding standardized peak area (standard/internal standard).

The internal standard concentration was kept constant at 1 fmol/μL in all samples. All standards were run in triplicates.
Figure 5.13. Matrix-matched calibration curve (linear range 250 amol to 25 fmol B[a]PDE-dG on column).
5.4.2.3. Precision and accuracy

Quality control triplicates were prepared at three different levels (LQC, MQC, HQC) covering the analytical range and used to evaluate the precision and accuracy of the quantification method. The precision was calculated as % RSD and the accuracy was calculated as the percentage of nominal concentration (measured concentration / nominal concentration x 100%). The precision and accuracy parameters are summarized in Table 5.3. The precision was within less than 12% for all QC samples and the accuracy was within 96 to 102% at all levels.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Pk area ratio (Std/IS)</th>
<th>Calculated fmol/uL</th>
<th>Average fmol/uL</th>
<th>% RSD (Precision)</th>
<th>Actual fmol/uL</th>
<th>Accuracy</th>
<th>Average (Accuracy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LQC</td>
<td>0.37</td>
<td>0.54</td>
<td>0.54</td>
<td>9.2%</td>
<td>0.5</td>
<td>109%</td>
<td>107%</td>
</tr>
<tr>
<td>LQC</td>
<td>0.33</td>
<td>0.48</td>
<td>0.58</td>
<td></td>
<td>0.5</td>
<td>97%</td>
<td></td>
</tr>
<tr>
<td>LQC</td>
<td>0.39</td>
<td>0.58</td>
<td>0.54</td>
<td>9.2%</td>
<td>0.5</td>
<td>116%</td>
<td></td>
</tr>
<tr>
<td>MQC</td>
<td>0.93</td>
<td>1.53</td>
<td>1.44</td>
<td>5.6%</td>
<td>1.5</td>
<td>102%</td>
<td>96%</td>
</tr>
<tr>
<td>MQC</td>
<td>0.83</td>
<td>1.37</td>
<td>1.44</td>
<td></td>
<td>1.5</td>
<td>91%</td>
<td></td>
</tr>
<tr>
<td>MQC</td>
<td>0.87</td>
<td>1.43</td>
<td>1.44</td>
<td>5.6%</td>
<td>1.5</td>
<td>95%</td>
<td></td>
</tr>
<tr>
<td>HQC</td>
<td>1.41</td>
<td>2.37</td>
<td>2.56</td>
<td>11.9%</td>
<td>2.5</td>
<td>95%</td>
<td>102%</td>
</tr>
<tr>
<td>HQC</td>
<td>1.42</td>
<td>2.39</td>
<td>2.56</td>
<td></td>
<td>2.5</td>
<td>96%</td>
<td></td>
</tr>
<tr>
<td>HQC</td>
<td>1.71</td>
<td>2.91</td>
<td>2.56</td>
<td>11.9%</td>
<td>2.5</td>
<td>116%</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.3. Precision and accuracy for the determination of B[a]PDE-N²-dG adducts.

5.4.2.4. Quantification of B[a]PDE-N²-dG adducts in TK6 cells treated with B[a]PDE

TK6 cells were treated with three levels of (±)-anti-B[a]PDE (0.017 μM, 0.034 μM, and 0.12 μM) for 1 h and harvested at different time points ranging from 4 to 24 h. Following DNA extraction from the cell cultures, the isolated DNA was subjected to enzymatic hydrolysis, followed by LC-MS/MS quantification of B[a]PDE-N²-dG adducts. The number of adducts formed per number of DNA bases can be calculated from the following relationship:
Number of adducts / DNA base = \( \frac{\text{moles of adducts} \times 6.02 \times 10^{23} \text{ molecules}}{1 \text{ mole}} \) \( \times \frac{1.8 \times 10^{15} \text{ nucleotides}}{1 \mu g \text{ DNA}} \) \( \times \text{ digested} \mu g \text{ DNA} \)

The number of moles of adducts is back-calculated from the measured analyte/IS peak area ratio using the calibration curve linear regression equation and must be corrected by a factor of 4 (the digested samples were reconstituted in 20 \( \mu L \) but only 5 \( \mu L \) were injected for analysis). The digested \( \mu g \) DNA represents the exact amount of digested DNA (eventually reconstituted in 20 \( \mu L \)), measured by fluorometry before digestion.

The numbers of B[a]PDE-dG adducts were determined for each sample and are summarized in Table 5.4. As indicated in the table, we also confirmed that the control samples were free of any interferences as no adduct was detected.

The quantification data show that adducts were produced in all samples treated with B[a]PDE, with adduction levels ranging from 91 to 800 adducts per billion normal bases.

<table>
<thead>
<tr>
<th>B[a]PDE Dose (( \mu M ))</th>
<th>Time Point</th>
<th>average fmol/( \mu g ) DNA</th>
<th>% RSD</th>
<th>adducts/10^9 bases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>not detected</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.017</td>
<td>4 hrs</td>
<td>0.273</td>
<td>5.0</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>12 hrs</td>
<td>0.319</td>
<td>5.4</td>
<td>107</td>
</tr>
<tr>
<td></td>
<td>17 hrs</td>
<td>0.440</td>
<td>2.4</td>
<td>147</td>
</tr>
<tr>
<td>0.034</td>
<td>9 hrs</td>
<td>0.669</td>
<td>4.0</td>
<td>224</td>
</tr>
<tr>
<td></td>
<td>12 hrs</td>
<td>0.924</td>
<td>3.3</td>
<td>309</td>
</tr>
<tr>
<td></td>
<td>17 hrs</td>
<td>1.085</td>
<td>7.0</td>
<td>363</td>
</tr>
<tr>
<td>0.12</td>
<td>4 hrs</td>
<td>1.784</td>
<td>4.6</td>
<td>597</td>
</tr>
<tr>
<td></td>
<td>17 hrs</td>
<td>2.397</td>
<td>3.9</td>
<td>802</td>
</tr>
<tr>
<td></td>
<td>24 hrs</td>
<td>2.209</td>
<td>1.3</td>
<td>739</td>
</tr>
</tbody>
</table>

Table 5.4. Amounts of B[a]PDE-dG adducts detected in TK6 cells treated with B[a]PDE at three different concentrations. Each sample was run in triplicate.
5.4.2.5. Relationship between B[a]PDE-N\textsuperscript{2}-dG adducts, toxicity and gene expression

Correlation between number of adducts formed and cellular toxicity

As indicated in the Methods section, the three (\pm)-anti-B[a]PDE dose levels corresponded to 5\%, 15\%, and 40\% cellular toxicity levels, respectively. Toxicity levels, directly related to the survival of treated cultures, were calculated from back-extrapolation of growth curves by comparison of the growth of treated cultures to control cultures\textsuperscript{62}. A positive correlation was observed between the carcinogen levels and the number of adducts formed and is illustrated in Figure 5.14. As the dose – and therefore cellular toxicity – increased, so did the number of adducts detected.

![Correlation between BPDE dose and DNA Adducts](image)

**Figure 5.14.** Correlation between B[a]PDE dose and number of B[a]PDE-dG adducts formed in TK6 after exposure to the mutagenic metabolite of B[a]P.
Gene expression profiling

The gene expression profiles represented in Figure 5.15 were generated by our collaborator Helmut Zarbl by the microarray analysis of the mRNA isolated from TK6 cells dosed with three different doses of B[a]PDE (0.017 μM, 0.034 μM, and 0.12 μM). Each horizontal bar represents a different gene and columns represent experiments performed at different doses. After subtraction of local median background and normalization to the median signal for a microarray, the relative fold change in expression for each feature was calculated as the mean log2 ratio of fluorescence intensities relative to untreated controls. The log2 ratio expression for each gene under each treatment is represented by a red and green scale. Using this analytical approach, 1865 genes were identified with significant changes in expression after treatment with B[a]PDE. Among the genes that showed decreased expression were genes functioning in cell survival and cell growth. Their decreased expression could be indicative of toxicity. Induced genes included stress response genes, inflammatory response genes, genes involved in RNA transcription as well several genes encoding DNA damage recognition proteins involved in excision repair. Together, the subsets of upregulated genes were indicative of a general cellular response to toxicity.
**Figure 5.15.** Gene expression profiles representing the gene expression changes induced in TK6 cells as a function of carcinogen-induced toxicity. Experimental conditions are on the horizontal axis and are specified at the top of the diagram. Individual genes are represented along the vertical axis. The log2 ratio expression for each of these genes under each treatment condition is represented by a red and green scale. Positive log2 values (red scale) indicate up-regulation while genes with negative log2 values (green scale) are down-regulated. Genes expressed at constant level have a log2 of 0 and are represented in black.
5.4.3. Conclusions and Future Directions

The primary objective of this project was to develop a validated analytical assay for the characterization and quantification of dG-adducts of B[a]PDE, the mutagenic metabolite of B[a]P. The analysis of DNA adducts at levels compatible with human exposure is a highly challenging task and is therefore strongly dependent on the use of LC separation methods coupled to mass spectrometry. The development of a quantitative assay for the analysis of B[a]PDE-dG adducts first required the synthesis of authentic standards and isotopically labeled internal standards. The first part of this project was therefore devoted to the synthesis and characterization of B[a]PDE-N2-dG and B[a]PDE-N2-[15N5]-dG adducts. The reaction of (±)-anti-B[a]PDE with dG (or [15N5]-dG) yields four diastereomers, therefore extensive efforts had to be dedicated to the isolation and resolution of all isomers. This was achieved by a combination of preparative TLC, reversed-phase LC and chiral LC methodologies.

The authentic standards and internal standards were subsequently used to develop a validated LC-MS/MS assay for the quantification of B[a]PDE-dG adducts. High sensitivity was achieved by using the new Agilent HPLC-Chip/MS platform, operating at nanoflow and allowing for online sample clean-up prior to separation. These various features significantly contributed to the enhanced sensitivity of our LC-MS/MS methodology by reducing ion suppression effects as well as reducing sample handling and thereby minimizing sample loss. The LC-MS/MS assay was reproducible and highly sensitive with a limit of detection of 50 amol on column and a linear range of 250 amol to 25 fmol. The only limitation, however, was the fact that the stationary phase of the LC
chip did not permit the resolution of all four B[a]PDE-N^{2}-dG diastereomers. To date, Zorbax C18 is the only commercially available stationary phase for the Agilent LC chip. The assay was therefore developed and validated for the total B[a]PDE-N^{2}-dG adducts. Further work will be conducted to refine the assay using a custom-made chip towards the quantification of individual diastereomers. The added specificity would allow for the comparison of the relative and absolute amounts of each adduct formed after exposure to (±)-anti-B[a]PDE.

After validation, we applied the LC-MS/MS assay to the quantification of B[a]PDE-N^{2}-dG adducts formed in human lymphoblastoid TK6 cells exposed to B[a]PDE. We used human cell lines to integrate data from DNA adduct formation, toxicity and gene expression. By anchoring the observed biological outcomes to DNA adduct levels, we hoped to gain further insight into how DNA adduct formation and repair affect cellular responses to mutagenic insults. This section of the project was conducted in collaboration with Wendy Luo and Helmut Zarbl, formerly at the Fred Hutchinson Cancer Research Center, Seattle, WA. We successfully detected DNA adducts in the treated TK6 cells and observed a positive correlation between the number of adducts and the carcinogen dose, therefore linking adduct formation to toxicity. Moreover, gene expression profiling experiments conducted by Helmut Zarbl revealed significant changes in expression for genes involved in cell growth, cell death, stress responses or repair processes upon exposure of TK6 cells to B[a]PDE.

Although the present study was limited to a small number of doses and exposure time, the results provide convincing evidence for the added value of anchoring gene expression patterns to phenotypic markers, such as DNA adduct levels, toxicity and mutagenicity.
Further toxicogenomics studies are currently in progress to investigate DNA adduct formation over a broader range of carcinogen level ($10^{-12}$ to $10^{-6}$ M B[a]PDE) and their relationship to mutation, toxicity and gene expression profiling. We expect that the results of this work will help establish more definitely the degree of involvement of DNA adducts in the etiology of human cancer.

ACKNOWLEDGEMENTS

We would like to thank Wendy Luo and Helmut Zarbl for their major contributions to this project. This research was supported by the National Institute of Health (RO1CA112231).
REFERENCES


Chapter 6:

Suggestions for Future Research
The research projects presented in this dissertation have demonstrated the widespread application of mass spectrometry to the qualitative and quantitative analysis of small molecules.

6.1. Vitamin D

Chapter 2 highlights the superseding role of mass spectrometry in the characterization and quantification of vitamin D, its metabolites and other emerging analogs. The superiority of mass spectrometric methods is illustrated in Chapters 3 and 4 reporting the elucidation of two novel metabolic pathways of vitamin D: the C-1 epimerization of vitamin D (Chapter 3) and the C-1 esterification of 20-epi-vitamin D analogs with fatty acids (Chapter 4). The two newly discovered pathways are of particular importance as they may be responsible for the unique or enhanced biological actions of the derived metabolites.

GC-MS is expected to continue to play a central role in the elucidation of metabolic pathways and the discovery of novel vitamin D analogs and metabolites. C-3 epimers are of particular interest since they often exert unique biological activity while exhibiting a low calcemic potential, therefore minimizing hypercalcemic side-effects often observed with vitamin D at pharmacological doses. Further work is therefore in progress to study the metabolism and biological significance of various 3-epi-vitamin D analogs.
6.2. DNA Adducts

In Chapter 5, we developed and validated a highly sensitive LC-MS/MS assay for the quantification of DNA adducts derived from B[a]PDE, carcinogenic and mutagenic metabolite of benzo[a]pyrene. The method was developed on a HPLC-Chip-nESI/MS platform which significantly improved the assay sensitivity (LOD: 50 amol on column) by minimizing sample handling and reducing ion suppression effects. The validated assay was applied to the quantification of DNA adducts in TK6 cells treated with BPDE which allowed us to correlate DNA adduct formation, toxicity, and changes in gene expression. The observed positive dose-response correlation along with changes observed in the gene expression profiles provide further evidence for the suggested involvement of DNA adducts in carcinogenesis.

The presented study was limited to three carcinogen doses (5%, 15% and 40% toxicity levels). Further work is currently in progress to significantly extend the dose range to lower and higher toxicity levels, evaluate the DNA adduct formation and determine whether a positive dose-response correlation is still observed over a broader dose range. In the future, the study should also be repeated by treating cells with benzo[a]pyrene itself as supposed to its carcinogenic metabolite, B[a]PDE. As highlighted in Section 5.1.3., the epoxide pathway represents only one of the three proposed activation pathways of B[a]P. A new study using B[a]P treatment would therefore complement the data generated in Chapter 5 for the isolated epoxide pathway. We expect that this extended study will help provide a more comprehensive understanding of the relationship between DNA adducts and carcinogenesis.
BIOGRAPHICAL DATA

NAME          Caroline Ceailles Flarakos

PLACE OF BIRTH  Lourdes, France

EDUCATION

Graduate Teaching Assistant, 2002-2004
Department of Chemistry and Chemical Biology,
Northeastern University, Boston, MA

Research Scientist, 2001-2002
Praecis Pharmaceuticals
Waltham, MA

American Chemical Society
American Society of Mass Spectrometry
Greater Boston Mass Spectrometry Discussion Group

PROFESSIONAL
EXPERIENCE

Graduate Teaching Assistant, 2002-2004
Department of Chemistry and Chemical Biology,
Northeastern University, Boston, MA

Research Scientist, 2001-2002
Praecis Pharmaceuticals
Waltham, MA

American Chemical Society
American Society of Mass Spectrometry
Greater Boston Mass Spectrometry Discussion Group

ACTIVITIES & AWARDS

President, Graduate Students’ Association, 1999-2001
ENSCM, Montpellier, France

Barnett Institute Academic Excellence Award, 2002
Lourdes Rotary Academic Excellence Award, 1997
LIST OF PUBLICATIONS


LIST OF POSTERS AND ORAL PRESENTATIONS


