# BIOANALYSIS OF PHARMACEUTICALS

## Sample Preparation, Separation Techniques and Mass Spectrometry

EDITED BY

Steen Honoré Hansen
Stig Pedersen-Bjergaard

WITH CONTRIBUTIONS FROM

Leon Reubsaet
Trine Grønhaug Halvorsen
Astrid Gjelstad
Martin Jørgensen
Morten A. Kall

WILEY

# Bioanalysis of Pharmaceuticals

# Bioanalysis of Pharmaceuticals

## Sample Preparation, Separation Techniques, and Mass Spectrometry

**Editors**

STEEN HONORÉ HANSEN

School of Pharmaceutical Sciences, University of Copenhagen, Denmark

STIG PEDERSEN-BJERGAARD

School of Pharmacy, University of Oslo, Norway
School of Pharmaceutical Sciences, University of Copenhagen, Denmark

WILEY

This edition first published 2015
© 2015 John Wiley & Sons, Ltd

*Registered office*
John Wiley & Sons Ltd, The Atrium, Southern Gate, Chichester, West Sussex, PO19 8SQ, United Kingdom

For details of our global editorial offices, for customer services and for information about how to apply for permission to reuse the copyright material in this book please see our website at www.wiley.com.

The right of the author to be identified as the author of this work has been asserted in accordance with the Copyright, Designs and Patents Act 1988.

All rights reserved. No part of this publication may be reproduced, stored in a retrieval system, or transmitted, in any form or by any means, electronic, mechanical, photocopying, recording or otherwise, except as permitted by the UK Copyright, Designs and Patents Act 1988, without the prior permission of the publisher.

Wiley also publishes its books in a variety of electronic formats. Some content that appears in print may not be available in electronic books.

Designations used by companies to distinguish their products are often claimed as trademarks. All brand names and product names used in this book are trade names, service marks, trademarks or registered trademarks of their respective owners. The publisher is not associated with any product or vendor mentioned in this book.

Limit of Liability/Disclaimer of Warranty: While the publisher and author have used their best efforts in preparing this book, they make no representations or warranties with respect to the accuracy or completeness of the contents of this book and specifically disclaim any implied warranties of merchantability or fitness for a particular purpose. It is sold on the understanding that the publisher is not engaged in rendering professional services and neither the publisher nor the author shall be liable for damages arising herefrom. If professional advice or other expert assistance is required, the services of a competent professional should be sought.

The advice and strategies contained herein may not be suitable for every situation. In view of ongoing research, equipment modifications, changes in governmental regulations, and the constant flow of information relating to the use of experimental reagents, equipment, and devices, the reader is urged to review and evaluate the information provided in the package insert or instructions for each chemical, piece of equipment, reagent, or device for, among other things, any changes in the instructions or indication of usage and for added warnings and precautions. The fact that an organization or Website is referred to in this work as a citation and/or a potential source of further information does not mean that the author or the publisher endorses the information the organization or Website may provide or recommendations it may make. Further, readers should be aware that Internet Websites listed in this work may have changed or disappeared between when this work was written and when it is read. No warranty may be created or extended by any promotional statements for this work. Neither the publisher nor the author shall be liable for any damages arising herefrom.

*Library of Congress Cataloging-in-Publication Data applied for.*

A catalogue record for this book is available from the British Library.

Hardback: 9781118716816
Paperback: 9781118716823

Set in 10/12pt TimesLTStd by SPi Global, Chennai, India

1 2015

# Contents


| | |
|---|---|
| ***Contributing Authors*** | **ix** |
| ***Preface*** | **xi** |
| **1. Introduction**<br>*Stig Pedersen-Bjergaard* | **1** |
| 1.1 What Is Bioanalysis? | 1 |
| 1.2 What Is the Purpose of Bioanalysis, and Where Is It Conducted? | 2 |
| 1.3 Bioanalysis Is Challenging | 6 |
| 1.4 The Different Sections of This Textbook | 7 |
| **2. Physicochemical Properties of Drug Substances**<br>*Steen Honoré Hansen and Leon Reubsaet* | **9** |
| 2.1 Bioanalysis in General | 9 |
| 2.2 Protolytic Properties of Analytes | 10 |
| 2.3 Partitioning of Substances | 12 |
| 2.4 Stereochemistry | 15 |
| 2.5 Peptides and Proteins | 18 |
| **3. Biological Samples: Their Composition and Properties, and Their Collection and Storage**<br>*Steen Honoré Hansen* | **23** |
| 3.1 Introduction | 23 |
| 3.2 Blood, or Whole Blood | 24 |
| 3.3 Plasma and Serum | 26 |
| 3.4 Urine | 26 |
| 3.5 Feces | 27 |
| 3.6 Saliva | 28 |
| 3.7 Cerebrospinal Fluid | 28 |
| 3.8 Synovial Fluid | 28 |
| 3.9 Hair and Nails | 29 |
| 3.10 Tissue (Biopsies) | 29 |

**4. General Chromatographic Theory and Principles** **31**
*Steen Honoré Hansen*

4.1 General Introduction 31
4.2 General Chromatographic Theory 32
4.3 Theory of Partition 33
4.4 Retention 34
4.5 Separation Efficiency 35
4.6 Resolution 36
4.7 Selectivity 37
4.8 The Separation Process 38
4.9 Chromatographic Principles 41
4.10 Reversed Phase Chromatography 45
4.11 Size Exclusion Chromatography (SEC) 54
4.12 Ion Exchange Chromatography 56
4.13 Chiral Separations 57

**5. Quantitative and Qualitative Chromatographic Analysis** **61**
*Steen Honoré Hansen*

5.1 Collection of Chromatographic Data 61
5.2 Quantitative Measurements 62
5.3 Calibration Methods 63
5.4 Validation 67
5.5 Qualitative Analysis 69

**6. Sample Preparation** **73**
*Stig Pedersen-Bjergaard, Astrid Gjelstad, and Trine Grønhaug Halvorsen*

6.1 Why Is Sample Preparation Required? 73
6.2 What Are the Main Strategies? 75
6.3 Protein Precipitation 75
6.4 Liquid–Liquid Extraction 80
6.5 Solid-Phase Extraction 94
6.6 Dilute and Shoot 110
6.7 What Are the Alternative Strategies? 113

**7. High-Performance Liquid Chromatography (HPLC) and High-Performance Liquid Chromatography–Mass Spectrometry (LC-MS)** **123**
*Steen Honoré Hansen and Leon Reubsaet*

7.1 Introduction 123
7.2 The Solvent Delivery System 125
7.3 Degassing and Filtering of Mobile Phases 127
7.4 Injection of Samples 128
7.5 Temperature Control 128
7.6 Mobile Phases 129

7.7 Stationary Phases and Columns 130
7.8 Detectors 135
7.9 Mass Spectrometric Detection 143

**8. Gas Chromatography (GC) 173**
*Stig Pedersen-Bjergaard*

8.1 Basic Principles of GC 173
8.2 GC Instrumentation 174
8.3 Carrier Gas 177
8.4 Stationary Phases 178
8.5 Separation Selectivity in GC 180
8.6 Columns 182
8.7 Injection Systems 183
8.8 Detectors 185
8.9 Derivatization 187
8.10 Gas Chromatography–Mass Spectrometry (GC-MS) 188

**9. Analysis of Small-Molecule Drugs in Biological Fluids 207**
*Steen Honoré Hansen and Stig Pedersen-Bjergaard*

9.1 Plasma and Serum Samples 207
9.2 Whole Blood Samples 234
9.3 Dried Blood Spots 241
9.4 Urine Samples 245
9.5 Saliva 253
References 259

**10. Analysis of Peptide and Protein Drugs in Biological Fluids 261**
*Leon Reubsaet and Trine Grønhaug Halvorsen*

References 282

**11. Regulated Bioanalysis and Guidelines 283**
*Martin Jørgensen and Morten A. Kall*

11.1 Introduction 283
11.2 The Evolution of Regulated Bioanalysis 284
11.3 Bioanalytical Method Validation 286
11.4 Pre-study Validation 287
11.5 In-Study Validation 299
11.6 Documentation 300
11.7 Regulatory Requirements to Bioanalysis 300
11.8 Quality Systems in Regulated Bioanalysis 301

***Index* 305**

# Contributing Authors

**Leon Reubsaet**
*School of Pharmacy, University of Oslo, Norway*
Chapters 2, 7, and 10

**Trine Grønhaug Halvorsen**
*School of Pharmacy, University of Oslo, Norway*
Chapters 6 and 10

**Astrid Gjelstad**
*School of Pharmacy, University of Oslo, Norway*
Chapter 6

**Martin Jørgensen**
*Drug ADME Research, H. Lundbeck AS, Denmark*
Chapter 11

**Morten A. Kall**
*Department of Bioanalysis, H. Lundbeck AS, Denmark*
Chapter 11

# Preface

The field of bioanalysis is very broad, complex, and challenging, and therefore writing an introductory textbook in this field is a difficult task. From our point of view, a good introductory student textbook is limited in the number of pages, discusses the different principles and concepts clearly and comprehensively, and contains many relevant and educational examples. Given these criteria, we have narrowed our focus on bioanalysis. First, we have limited our discussion to the chemical analysis of *pharmaceuticals* that are present in biological fluids. The focus is directed toward substances that are administered as human drugs, including low-molecular drug substances, peptides, and proteins. Endogenous substances are not discussed. Second, the discussion of different analytical methods has been limited to those based on *chromatography* and *mass spectrometry*. Certainly, different immunological methods are also used, but teaching all the principles and applications of chromatographic, mass spectrometric, and immunological methods was too ambitious to meet our criteria for a good introductory student textbook.

The present book is the first introductory student textbook on chromatography and mass spectrometry of pharmaceuticals present in biological fluids, highlighting an educational presentation of the principles, concepts, and applications. We discuss the chemical structures and properties of low- and high-molecular pharmaceuticals, the different types of biological samples and fluids that are used, how to prepare the samples by extraction, and how to perform the final analytical measurement by use of chromatography and mass spectrometry. Many examples illustrate the theory and applications, and the examples discuss all practical aspects, including the calculations. Thus, in this textbook, you will even learn how to convert the numbers recorded by the instrument to the concentration of the actual drug substances in the biological sample.

Bioanalysis is an applied scientific discipline, and this represents another challenge in terms of writing an introductory student textbook. University professors are well trained in teaching the basic principles. However, bioanalysis is mainly performed outside the university by researchers in the pharmaceutical industry, in contract laboratories, and in hospital laboratories. Thus, the researchers outside the university have the best overview of the most important applications and techniques in practical use. To address this, both university professors and researchers from the pharmaceutical industry have authored this textbook. Hopefully, this has resulted in a textbook that reflects bioanalysis in the year 2015. The authors have been in close contact with colleagues for advice, and we would especially like to thank Elisabeth Leere Øiestad for fruitful discussions.

The present textbook is intended for the fourth- or fifth-year university pharmacy or chemistry student. Reading the textbook requires basic knowledge in organic chemistry and biochemistry, as well as in analytical chemistry. With respect to the latter, we have

given priority to discuss the analytical techniques in a fundamental and educational frame, and detailed knowledge on instrumental analytical methods is not required prior to reading this textbook.

Good luck with the reading!

*Oslo and Copenhagen, June 2014*
*Steen Honoré Hansen, Stig Pedersen-Bjergaard, Leon Reubsaet, Astrid Gjelstad, Trine Grønhaug Halvorsen, Martin Jørgensen, and Morten A. Kall*

Readers can access PowerPoint slides of all figures at http://booksupport.wiley.com

# 1

# Introduction

**Stig Pedersen-Bjergaard**

*School of Pharmacy, University of Oslo, Norway*
*School of Pharmaceutical Sciences, University of Copenhagen, Denmark*

Welcome to the field of bioanalysis! Through reading of this textbook, we hope you get fascinated by the world of bioanalysis, and also we hope that you learn to understand that bioanalysis is a highly important scientific discipline. In this chapter, five fundamental questions are raised and briefly discussed as an introduction to the textbook: (i) What is bioanalysis? (ii) What is the purpose of bioanalysis? (iii) Where is bioanalysis conducted? (iv) Why do you need theoretical understanding and skills in bioanalysis? And (v) how do you gain the understanding and the skills from reading this textbook?

## 1.1 What Is Bioanalysis?

In this textbook, we define *bioanalysis* as the chemical analysis of pharmaceutical substances in biological samples. The purpose of the chemical analysis is normally both to *identify* (identification) and to *quantify* (quantification) the pharmaceutical substance of interest in a given biological sample. This is performed by a *bioanalytical chemist* (scientist) using a *bioanalytical method*. The pharmaceutical substance of interest is often termed the *analyte*, and this term will be used throughout the textbook. Identification of the analyte implies that the exact chemical identity of the analyte is established unequivocally. Quantification of the analyte implies that the concentration of the analyte in the biological sample is measured. It is important to emphasize that quantification is associated with small inaccuracies, and the result is prone to errors. Thus, the quantitative data should be considered as an estimate of the true concentration. Based on theoretical and practical skills, and based on careful optimization and testing of the bioanalytical methods, the bioanalytical chemist tries to reduce the error level, providing concentration estimates that are very close to the true values.

*Bioanalysis of Pharmaceuticals: Sample Preparation, Separation Techniques and Mass Spectrometry*, First Edition. Steen Honoré Hansen and Stig Pedersen-Bjergaard.
© 2015 John Wiley & Sons, Ltd. Published 2015 by John Wiley & Sons, Ltd.

Bioanalytical data are highly important in many aspects. As an example, a patient serum sample is analyzed for the antibiotic drug substance gentamicin, and gentamicin is measured in the sample at a concentration of 5 μg/ml. First, the identification of gentamicin in the blood serum sample confirms that the patient has taken the drug. This is important information because not all patients actually comply with the prescribed medication. Second, the exact concentration of gentamicin measured in the blood serum sample confirms that the amount of gentamicin taken is appropriate, as the recommended concentration level should be in the range of 4–10 μg/ml. For aminoglycoside antibiotics such as gentamicin, it is recommended to monitor the concentration in blood if the treatment is expected to continue for more than 72 hours as these antibiotics have the potential to cause severe adverse reactions, such as nephrotoxicity and ototoxicity.

As will be discussed in much more detail in this book, not only blood serum samples are used for bioanalysis. Bioanalysis can be performed on raw blood samples (whole blood) or on blood samples from which the blood cells have been removed (serum or plasma). Alternatively, bioanalysis can be performed from urine or saliva as examples, depending on the purpose of the bioanalysis. Bioanalysis is performed both on human samples and on samples from animal experiments.

## 1.2 What Is the Purpose of Bioanalysis, and Where Is It Conducted?

Bioanalysis is conducted in the *pharmaceutical industry*, in *contract laboratories* associated with the pharmaceutical industry, in *hospital laboratories*, in *forensic toxicology laboratories*, and in *doping control laboratories*. In the pharmaceutical industry and in the associated contract laboratories, bioanalysis is basically conducted to support the development of new drugs and new drug formulations. In hospital laboratories, bioanalysis is used to monitor existing drugs in patient samples, to check that individual patients take their drugs correctly. In forensic toxicology laboratories and doping laboratories, bioanalysis is used to check for abuse of drugs and drug-related substances.

### 1.2.1 Bioanalysis in the Pharmaceutical Industry

Bioanalytical laboratories are highly important in the development of new drugs and new drug formulations in the pharmaceutical industry. Thus, identification and quantification of drug substances and metabolites in biological samples like blood plasma, urine, and tissue play a very important role during drug development. Drug development begins with the identification of a medical need and hypotheses on how therapy can be improved. *Drug discovery* is the identification of new *drug candidates* based on combinatorial chemistry, high-throughput screening, genomics, and ADME (absorption, distribution, metabolism, and elimination). By combinatorial chemistry, a great number of new drug candidates are synthesized, and these are tested for pharmacological activity and potency in high-throughput screening (HTS) systems. The HTS systems simulate the interaction of the drug candidates with a specific biological receptor or target. Once a *lead compound* is found, a narrow range of similar drug candidates is synthesized and screened to improve the activity toward the specific target. Other studies investigate the ADME profile of drug candidates by analyzing samples collected at different time points from dosed laboratory animals (*in vivo testing*) and tissue cultures (*in vitro testing*).

Drug candidates passing the discovery phase are subjected to toxicity testing and further metabolism and pharmacological studies in the *preclinical development* phase. Both in vivo and in vitro tests are conducted, and various animal species are used to prove the pharmacokinetic profile of the candidate. The detailed information about the candidate forms the basis for further pharmaceutical research on the synthesis of raw materials, the development of dosage forms, quality control, and stability testing.

The *clinical development* phase can begin when a regulatory body has judged a drug candidate to be effective and to appear safe in healthy volunteers. In *phase I*, the goal is to establish a safe and efficient dosage regimen and to assess pharmacokinetics. Blood samples are collected and analyzed from a small group of healthy volunteers (20–80 persons). The data obtained form the basis for developing controlled *phase II* studies. The goal of phase II studies is to demonstrate a positive benefit–risk balance in a larger group of patients (200–800) and to further study pharmacokinetics. Monitoring of efficacy and monitoring of possible side effects are essential. Phase II studies can take up to two years to fulfill. At the end of phase II, a report is submitted to the regulatory body, and conditions for phase III studies are discussed. Additional information supporting the claims for a new drug is provided. *Phase III* begins when evidence for the efficacy of the drug candidate and supporting data have demonstrated a favorable outcome to the regulatory body. The phase III studies are large-scale efficacy studies with focus on the effectiveness and safety of the drug candidate in a large group of patients. In most cases, the drug candidate is compared with another drug already in use for treatment of the same condition. Phase III studies can last two to three years or more, and 3000–5000 patients can be involved. Carcinogenetic tests, toxicology tests, and metabolic studies in laboratory animals are conducted in parallel. The cumulative data form the basis for filing a new drug application to the regulatory body and for future plans for manufacturing and marketing. The regulatory body thoroughly evaluates the documentation that is provided before a market approval can be authorized and the drug product can be legally marketed. The time required from drug discovery to product launch is up to 12 years. Phase IV studies are studies that are conducted after product launch to demonstrate long-term effects and new claims, expand on approved claims, examine possible drug–drug interactions, and further assess pharmacokinetics. Several thousand patients participate in phase IV studies.

Bioanalytical measurements are conducted during drug discovery, preclinical development, and clinical development, and they are intended to (among other things) generate the experimental data to establish the *pharmacokinetics*, the *toxicokinetics*, and the *exposure–response* relationships for a new drug. The pharmacokinetics of a certain drug substance describes how the body affects the drug after administration (ADME): how the drug is absorbed (A) and distributed (D) in the body, and how the drug is metabolized (M) by metabolic enzymes and chemically changed to different types of metabolites, which in turn are excreted (E) from the body. Bioanalysis is used extensively in pharmacokinetic studies, among others, to establish blood concentration–time profiles, and to measure the rate of drug metabolism and excretion. This involves a large number of both animal and human samples.

Toxicokinetics studies, in contrast, are intended to investigate the relationship between the exposure of a new drug candidate in experimental animals and its toxicity. This type of information is used to establish a relationship between the possible toxic properties of a drug in animals and those in humans. Toxicokinetic studies involve bioanalysis in both animal and human samples.

Exposure–response studies investigate the link between pharmacokinetics and pharmacodynamics. *Pharmacodynamics* is the study of the biochemical and physiological effects of a drug substance on the body. Exposure–response studies thus establish the link between the dose, the blood concentration, and the effect. Also for exposure–response studies, bioanalysis of a large number of blood samples has to be conducted. Frequently, such bioanalytical measurements are outsourced by the pharmaceutical company to a contract laboratory that is highly specialized in bioanalysis. High quality of the bioanalytical data is mandatory, because these data from drug discovery, preclinical, and clinical studies are used to support the regulatory filings.

### 1.2.2 Bioanalysis in Hospital Laboratories

Bioanalysis is also very important in many hospital laboratories. Here, the focus is on measuring the drug concentration in blood samples of patients to check that they are properly medicated. This is called *therapeutic drug monitoring* (TDM), and it refers to the individualization of dosage by maintaining serum or plasma drug concentrations within a target range to optimize efficacy and to reduce the risk of adverse side effects. The target range of a drug is also called the *therapeutic range* or the *therapeutic window*; it is the concentration range between the lowest drug concentration that has a positive effect and the concentration that gives more adverse effects than positive effects. Variability in the dose–response relationship between individual patients is due to *pharmacokinetic variability* and *pharmacodynamic variability*, as shown in Figure 1.1.

Pharmacodynamic variability arises from variations in drug concentrations at the receptor and from variations in the drug–receptor interaction. Pharmacokinetic variability is due to variations in the dose to plasma concentration relationship. Major sources of pharmacokinetic variability are age, physiology, disease, compliance, and genetic polymorphism of drug metabolism. Indications for including a drug in a therapeutic drug-monitoring program are:

- There is an experimentally determined relationship between the plasma drug concentration and the pharmacological effect.
- There is a narrow therapeutic window.
- The toxicity or lack of effectiveness of the drug puts the patient at risk.
- There are potential patient compliance problems.
- The dose cannot be optimized by clinical observations alone.

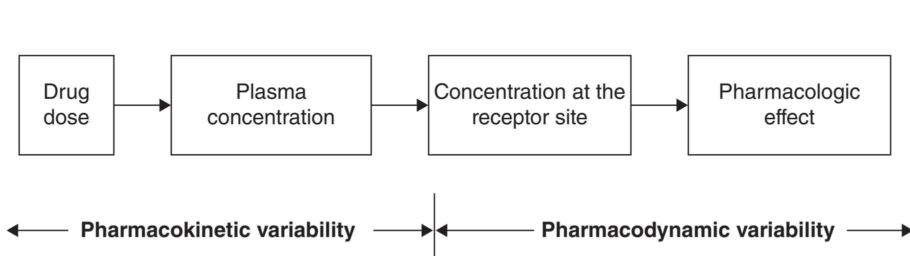



***Figure 1.1*** *Effects of pharmacokinetics and pharmacodynamics on the dose–response relationship*

***Table 1.1*** *Therapeutic range of common drugs subjected to therapeutic drug monitoring*

| Drug | Therapeutic range | Drug | Therapeutic range |
|---|---|---|---|
| Amitriptyline | 120–150 ng/ml | Nortriptyline | 50–150 ng/ml |
| Carbamazepine | 4–12 μg/ml | Phenobarbital | 10–40 μg/ml |
| Desipramine | 150–300 ng/ml | Phenytoin | 10–20 μg/ml |
| Digoxine | 0.8–2.0 ng/ml | Primidone | 5–12 μg/ml |
| Disopyramide | 2–5 μg/ml | Theophylline | 10–20 μg/ml |
| Ethosuximide | 40–100 μg/ml | Valproic acid | 50–100 μg/ml |
| Lithium | 4–8 μg/ml | | |

Many drugs do not meet the criteria to be included in a TDM program. They are safely taken without determining drug concentrations in plasma because the therapeutic effect can be evaluated by other means. For example, the coagulation time effectively measures the efficacy of an anticoagulant drug, and the blood pressure indicates the efficacy of a drug used in the treatment of hypertension. In these situations, it is preferred to adjust the dosage on the basis of medical response.

The two major situations when TDM is advised are (1) for drugs used prophylactically to maintain the absence of a condition (e.g., depressive or manic episodes, seizures, cardiac arrhythmias, organ rejection, and asthma relapses) and (2) to avoid serious toxicity for drugs with a narrow therapeutic window (e.g., antiepileptic drugs, antidepressant drugs, digoxin, phenytoin, theophylline, cyclosporine and HIV protease inhibitors, and aminoglycoside antibiotics). The therapeutic range of some drugs subjected to TDM is shown in Table 1.1.

### 1.2.3 Bioanalysis in Forensic Toxicology Laboratories

Bioanalysis is a core discipline also in forensic toxicology laboratories, where a large number of blood, urine, and saliva samples are analyzed to identify abuse of drugs and narcotics. The focus is on drugs and their metabolites, narcotics, and other substances that are toxicologically relevant. Serious cases that are of criminal relevance may include:

- Analysis of pharmaceuticals and addictive drugs that may impair human behavior.
- Detection of poisons and evaluation of their relevance in determining causes of death.

In forensic toxicology, the analyte is essentially unknown. Therefore, samples are first screened for the presence of drugs or drugs of abuse. In case of a positive sample, the drug or the drug of abuse is confirmed with a second bioanalytical method. Due to the serious legal consequences of forensic cases, particular emphasis is placed on the quality and reliability of bioanalytical results. The work always involves the application of at least two different analytical methods (screening and confirmation) based on different physical or chemical principles.

### 1.2.4 Bioanalysis in Doping Control Laboratories

Bioanalysis also is very important in doping control laboratories, where blood and urine samples are tested for doping agents. Only laboratories accredited by the World

Anti-Doping Agency (WADA) take part in the testing. WADA was established in 1999 as an international agency to promote, coordinate, and monitor the fight against doping in sport. One of WADA's most significant achievements was the acceptance and implementation of the World Anti-Doping Code (the Code). The Code is the core document that provides the framework for antidoping policies, rules, and regulations within sport organizations and among public authorities. The Code works in conjunction with five international standards aimed at bringing harmonization among antidoping organization in various areas. The standards are:

- List of Prohibited Substances and Methods
- International Standard for Testing
- International Standard for Laboratories
- International Standard for Therapeutic Use Exemptions
- International Standard for the Protection of Privacy and Personal Information.

The prohibited list is the standard that defines substances and methods that are prohibited to athletes at all times (both in competition and out of competition), substances prohibited in competition, and substances prohibited in particular sports. The prohibited list is updated annually.

The purpose of the International Standard for Testing is to plan for effective in-competition and out-of-competition testing and to maintain the integrity and identity of the samples collected. The International Standard for Therapeutic Use Exemptions and the International Standard for the Protection of Privacy and Personal Information ensure that the process of granting an athlete therapeutic-use exemptions is harmonized and that all relevant parties adhere to the same set of privacy protections.

The purpose of the International Standard for Laboratories is to ensure that laboratories produce valid test results. The standard further ensures that uniform and harmonized results are reported from all accredited laboratories. In addition, the document specifies the criteria that must be fulfilled by antidoping laboratories to achieve and maintain their WADA accreditation.

## 1.3 Bioanalysis Is Challenging

Bioanalysis is highly challenging because most target pharmaceutical substances are present in blood, urine, and saliva samples at very low concentrations. Typically, the concentration level is at the low ng/ml level, but in some cases, target pharmaceuticals have to be detected even down to the pg/ml level. This relies on very sensitive instrumentation and high operator skills. In addition, the target pharmaceuticals coexist with a broad range of endogenous compounds that are naturally present in biological samples. There can be thousands of different components, and many of them can be present at high concentration levels. Therefore, in most cases, a successful bioanalysis procedure requires the isolation of target pharmaceuticals from the biological matrix, before the final measurement with a sensitive instrument. Thus, experience and skills on how to prepare samples are extremely important in bioanalysis. The intention of the current textbook is to provide the reader with the required theoretical understanding and skills related to the understanding, development, and application of bioanalytical methods and procedures.

## 1.4 The Different Sections of This Textbook

The first part of this book is focused on the chemical properties of drug substances (Chapter 2) and the properties of the different biological fluids in use (Chapter 3). Careful reading of Chapter 2 is important for readers who are not familiar with pharmaceutical substances or the chemical properties of these substances. Understanding the chemical properties of the target pharmaceuticals is highly important in order to understand the bioanalytical procedures. Chapter 3 discusses the properties of different biological fluids, and if you are unfamiliar with biological fluids, you should read this chapter carefully. Understanding the properties of the biological fluids is mandatory in order to understand bioanalytical procedures.

Chapters 4–8 teach the different techniques and their principles, with foci on sample preparation, separation, and detection. These are chapters that are similar to the content of general textbooks in analytical chemistry. So, if you have been through general courses in analytical chemistry, this part of the textbook will be repetition. The discussion about sample preparation gives the reader an understanding of how to isolate target pharmaceuticals from the bulk biological matrix. The discussion about separation is focused on chromatographic separation, in which target pharmaceuticals are separated from any other substances in the sample, and the discussion about detection is focused on the final measurement of the substance, in most cases by mass spectrometry.

Chapters 9 and 10 are a collection of examples of bioanalytical procedures that are typically not found in general textbooks in analytical chemistry. In this part of the textbook, we make use of all previous knowledge and try to give you the full understanding. In these chapters, we discuss practical examples, and all discussions are related to the theory presented in Chapters 4–8. Remember when you read Chapters 9 and 10 that you should understand rather than remember all the details. Hopefully, the combination of Chapters 4–10 should give the reader a very good understanding of bioanalytical procedures. Finally, regulatory aspects related to bioanalytical procedures are discussed in Chapter 11. This is important to make sure that we can rely on the data generated from the application of bioanalytical methods.

Good luck with your journey into the world of bioanalysis!

# 2

# Physicochemical Properties of Drug Substances

**Steen Honoré Hansen[1] and Leon Reubsaet[2]**

[1]*School of Pharmaceutical Sciences, University of Copenhagen, Denmark*
[2]*School of Pharmacy, University of Oslo, Norway*

The development of both sample preparation strategies and chromatographic methods is based on the physicochemical properties of the substances to be analyzed as well as the principles of the analytical technique used. Why is it that in some cases mass spectrometric detection is needed to determine a substance, whereas in other cases UV detection is sufficient? Can chromatographic behavior be predicted from simply looking at the chemical structure of the analyte? In this chapter, the most important physicochemical properties of small-molecule drug substances as well as those of peptide and protein biopharmaceuticals are discussed. The discussions are short and comprehensive, as most of this information should already be known from learning general chemistry. The properties discussed here will be used in other chapters in this book in relation to sample preparation and subsequent analysis.

## 2.1 Bioanalysis in General

In bioanalysis, the task often is to perform *qualitative or quantitative measurements* of analytes in complex matrices consisting of thousands of other chemical entities. Therefore, a high degree of selectivity is needed to be able to “pick the needle out of the haystack” and in this way increase the *reliability* of the data obtained. In many bioanalytical methods, the *selectivity* is incorporated at several stages: in the sample preparation, in the following chromatographic separation, and in the detection step. To be able to optimize the selectivity, a basic knowledge of some fundamental chemical and *physicochemical properties* is needed.

*Bioanalysis of Pharmaceuticals: Sample Preparation, Separation Techniques and Mass Spectrometry*,
First Edition. Steen Honoré Hansen and Stig Pedersen-Bjergaard.
© 2015 John Wiley & Sons, Ltd. Published 2015 by John Wiley & Sons, Ltd.

## 2.2 Protolytic Properties of Analytes

Many small molecules show *protolytic properties*, which cause them to be present in an ionic state as well as a neutral form. The degree of ionization controlled by the surrounding aqueous solvent very much influences the properties of the molecules and thus their behavior in each step in the bioanalytical method.

*pH* is defined in dilute aqueous solution and is an expression for the acidity or alkalinity of an aqueous solution. The pH concept is extremely important and has great influence on living organisms as well as in analytical chemistry. Water can react with itself to form a hydronium ion and a hydroxide ion:

$$2H_2O \rightleftarrows H_3O^+ + OH^- \tag{2.1}$$

This is called *autoprotolysis*, as the water in this case acts as an acid and a base. The autoprotolysis constant is:

$$K_w = \frac{[H_3O^+][OH^-]}{[H_2O]^2} = 10^{-14} \tag{2.2}$$

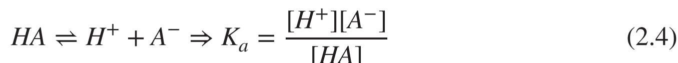
and it indicates that only a very small amount of water is ionized. The concentration of the two ions, $H_3O^+$ and $OH^-$, in pure water is therefore $10^{-7}$ M of each ion.

pH is defined as the negative logarithm to the activity, $a_H{}^+$, or the concentration of the hydrogen ion, $[H^+]$ (being equivalent to the hydronium ions):

$$pH = -\log(a_{H^+}) \approx -\log([H^+]) \tag{2.3}$$

Strong acids and strong bases are fully ionized in dilute aqueous solution, and the activity and concentration of $[H^+]$ therefore can be considered to be identical.

Weak acids and weak bases are not completely ionized in aqueous solution and are therefore in equilibrium with the unionized acid or base. When we ignore the weak autoprotolysis of water, we get the following general equation for a weak acid:



$$HA \rightleftharpoons H^+ + A^- \Rightarrow K_a = \frac{[H^+][A^-]}{[HA]} \tag{2.4}$$

When an acid ($H^+$) is added to such a system, the $H^+$ will partly be removed by association with $A^-$ to form HA, and if a base ($OH^-$) is added it will be partly neutralized by $H^+$ and more HA will dissociate. pH will thus be maintained in the solution. A system like this is called a *buffer system*, and the purpose of a buffer is to maintain the pH in the solution. *$pK_a$* is defined as the negative logarithm to $K_a$:

$$pK_a = -\log K_a \tag{2.5}$$

and it is obvious that the highest *buffer capacity* is achieved at a pH value equivalent to the $pK_a$ value of the buffer substance. Combining Equations 2.4 and 2.5 results in a most useful equation called the *Henderson–Hasselbalch equation*:

$$pH = pK_a + \log\frac{[A^-]}{[HA]} \tag{2.6}$$

At $pH = pK_a$, equal concentrations of the acid and corresponding base are present. If the ratio between HA and $A^-$ becomes 9/1 (only 10% base) pH will decrease one unit, and if the ratio becomes 99/1 (1% base) the pH value will decrease by two units. This is illustrated

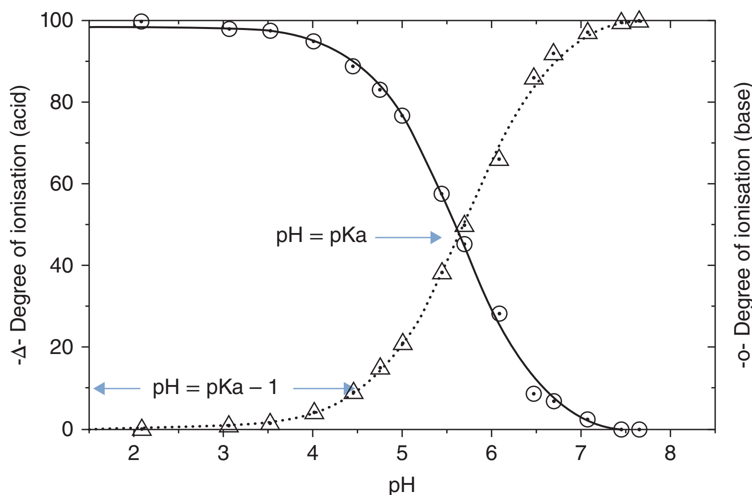



***Figure 2.1*** *Ionization of acids and bases as a function of pH*

in Figure 2.1. Equivalent estimations can be performed when increasing the base content. One example of a buffer system is arterial plasma, and this is featured in Box 2.1.

## Box 2.1 Arterial Plasma Is a Buffer System

pH in arterial plasma is buffered by a special bicarbonate system. When acid is added $CO_2$ is formed, which is actively controlled by the lungs, and if base is added the

***Table 2.1*** *Typical $pK_a$ values of important functional groups*

| Functional group | $pK_a$ | Comments (depending on chemical structure) |
|---|---|---|
| R-COOH, carboxylic acid | 4–5 | Can be lower (more acidic)[a] |
| R-$NH_2$; R1, R2, NH; and R1, R2, R3, N, aliphatic amines | 8–11 | Can be lower (less basic)[a] |
| Aromatic amines | About 5 | — |
| Quaternary ammonium ions | — | Ions with no protolytic properties; are always positively charged |
| Ar-OH, phenols | 8–10 | Can be lower (more acidic)[a] |
| R-OH, alcohols | >12 | Can for practical purposes be considered as neutral substances |
| R-$SO_2$OH, sulfonic acid | About 1 | Are for all practical purposes always negatively charged |
| R-CO—NH—CO—R and R—$SO_2$NH—R | 7–11 | Weak to very weak acids |

[a] This depends on other chemical groups in the molecule.

increase in bicarbonate is actively controlled by the kidneys. In this way it is possible to maintain a pH of 7.4, although the $pK_a$ value of the bicarbonate is 6.1. Plasma collected for bioanalysis no longer has the contact to the lungs and the kidneys, but the plasma still has some buffer capacity, mainly due to the content of about 8% of proteins.

It is convenient to have general knowledge of the $pK_a$ values of a number of functional groups, as presented in Table 2.1.

The *$pK_a$ value for bases* refers to the protonated form of the bases. However, the basicity of bases may also be expressed equivalent to the $pK_a$ of acids. In that case, the term $pK_b$ is used and

$$pK_a + pK_b = 14. \tag{2.7}$$

## 2.3 Partitioning of Substances

A prerequisite in chromatography as well as in many sample preparation techniques is the partitioning of molecules between more or less immiscible phases (gas–liquid, gas–solid, liquid–liquid, or liquid–solid). When molecules are in solution, they will be exposed to a number of *intermolecular interactions*. These include, among other things, diffusion, collisions, dipole–dipole interactions, hydrogen bonding, and electrostatic interactions, as illustrated in Table 2.2. The nature of the interactions taking place is dependent on the physical and chemical nature of the analytes, and these interactions will determine how the molecules are distributed between different phases.

*Ionic interactions* can be as strong as a covalent bond but are often limited to one interaction per molecule. In contrast, *van der Waal interactions* are relatively weak but have many interactions per molecule and therefore are also very important.

The partition or distribution between phases (see Figure 2.2) is also influenced by pH, and thus a thorough knowledge of the pH concept, including $pK_a$, as well as of distribution constants will ease the development of bioanalytical methods (e.g., the chromatographic separation). The distribution is dependent on the nature of the two phases as well as the temperature. If we want to alter the partition between the two phases, we must change one

**Table 2.2** *Energy in bonds or of intermolecular forces*

| Type of bond or intermolecular force | Example of interacting molecules | Energy in kJ/mol (kcal/mol) |
|---|---|---|
| Covalent | $RH_2C–CH_2R$ | 400–1200 (100–300) |
| Ionic | $R_4N^+$ • • • $^-OOC$-R | 200–800 (50–200) |
| Hydrogen bond | $H_3CO_H$ • • • $HO_H$ | 20–50 (5–12) |
| Dipole–dipole | $H_3CC{\equiv}N$ • • • $C_6H_5Cl$ | 12–40 (3–10) |
| Dipole-induced dipole | $H_3CC{\equiv}N$ • • • $C_6H_6$ | 10–25 (2–6) |
| Dispersion or van der Waals | $C_6H_6$ • • • $C_6H_{14}$ | 5–20 (1–5) |

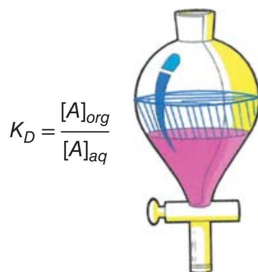



***Figure 2.2*** *Distribution of an analyte A between an upper organic phase and a lower aqueous phase*

of these variables. The equilibrium distribution for a substance A is given by the *partition ratio*, which is also called the *distribution constant*:

$$K_D = \frac{[A]_{org}}{[A]_{aq}} \tag{2.8}$$

where $[A]_{org}$ is the concentration of compound A in the organic phase and $[A]_{aq}$ is the concentration of compound A in the water phase.

The distribution constant is a constant relating to a specific molecular species, but often the molecules of a compound can be present as different species, for example by dissociation in the aqueous phase:

$$HA + H_2O \leftrightarrow A^- + H_3O^+ \tag{2.9}$$

or by dimerization in the organic phase:

$$2HA \leftrightarrow (HA)_2 \tag{2.10}$$

These equilibria are normally very fast, and it is therefore appropriate to look at the total distribution of all the species of a compound between the two phases:

$$D_C = \frac{[HA]_{org} + [A^-]_{org} + [(HA)_2]_{org}}{[HA]_{aq} + [A^-]_{aq} + [(HA)_2]_{aq}} = \frac{[HA_{total}]_{org}}{[HA_{total}]_{aq}} \tag{2.11}$$

The *concentration distribution ratio*, $D_C$, between the two phases can also be converted to the *mass distribution ratio*, $D_m$, by multiplying the concentrations with the matching phase volumes:

$$D_m = \frac{[HA_{total}]_{org} \cdot V_{org}}{[HA_{total}]_{aq} \cdot V_{aq}} = \frac{((amount\ of\ HA)_{total})_{org}}{((amount\ of\ HA)_{total})_{aq}} \approx \frac{((amount\ of\ HA)_{total})_{stat}}{((amount\ of\ HA)_{total})_{mob}} \tag{2.12}$$

where $V_{org}$ and $V_{aq}$ refer to the volumes of the organic and water phases, respectively. The terms *stat* and *mob* refer, respectively, to the stationary phase and the mobile phase used in chromatography, and this is discussed in more detail in Chapter 4.

The fundamentals of partition are also further outlined in Chapter 6 in relation to sample preparation. The greater the partition coefficient, the higher the affinity toward an organic

phase will be. In case of distribution to a solid phase, as in solid phase extraction, the partition can be governed by characteristics other than partition ratios. The partitioning of analytes in a system where one phase is a gas, as in gas chromatography, necessitates that the analytes can enter the gas phase. Discussions on the extraction and partition of compounds therefore most often refer to liquid–liquid systems. Partition ratios are estimated using distribution between n-octanol and water. If the compound can be ionized, the ionized form will have a much stronger affinity toward the aqueous phase as water molecules will solvate the ions. The distribution of an ionizable compound will therefore very much depend on the pH of the aqueous phase. From the Henderson–Hasselbalch equation given above, the following equations can be derived:

$$\text{For acids:} \qquad D_{app} = \frac{D_C}{1 + 10^{pH-pKa}} \tag{2.13}$$

$$\text{For bases:} \qquad D_{app} = \frac{D_C}{1 + 10^{pKa-pH}} \tag{2.14}$$

If the distribution ratio, $D_C$, and the $pK_a$ value are known for a compound, the *apparent distribution ratio*, $D_{app}$, at a given pH can be calculated.

Parameters such as the partition ratios in octanol–water are available as the so-called *log P values*, and the distribution ratio of compounds between octanol and water at different pH values in the water phase is tabulated as *log D values*. Computer programs can also be used for estimation of pKa values, log P values, log D values, and the water solubility of compounds (see Figure 2.3). The actual values of each parameter can vary when consulting different literature references, and this is most often due to differences in the methods used for analysis. This is particularly true for log P, log D, and water solubility data. In a bioanalytical chemical context, such parameters should primarily be used as a guide.

Liquid–liquid extraction (LLE) is often used in sample preparation, as discussed in more detail in Chapter 6. In LLE, it is of interest to determine the fraction of analytes extracted under given conditions. This is given by the general formula:

$$E_n = 1 - \left[ \frac{1}{1 + D_c\left(\frac{V_2}{V_1}\right)} \right]^n \tag{2.15}$$

where $E_n$ is the *extracted fraction*, $D_c$ is the distribution ratio between the two phases $V_2$ and $V_1$, and $n$ is the number of extractions. $V_1$ is the phase that originally contains the

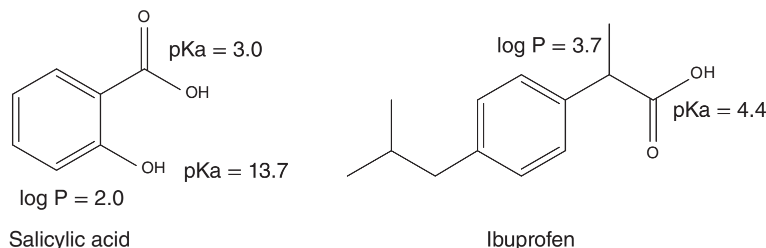



**Figure 2.3** *Chemical structures of ibuprofen and salicylic acid with log P and pKa values*

analyte, and $V_2$ is the phase to which the analyte is extracted. In bioanalysis, a high *recovery* is desired in order to minimize the loss of analyte and to improve the reliability of data obtained. Thus, a log P of at least 2 and extraction performed at favorable pH conditions are required. This is illustrated in Boxes 2.2 and 2.3.

### Box 2.2 Extraction of Ibuprofen

Ibuprofen has a log P value of 3.72 and a $pK_a$ value of 4.43. If 10 ml of a sample solution of ibuprofen at pH 6.0 is to be extracted to 30 ml of an organic solution, how much will be extracted?

A log P value of 3.72 corresponds to a $K_D$ of 5248. At pH 6.0, the apparent distribution ratio will be $5248 / 1 + 37 = 138$ (using Equation 2.13). Calculating the extracted fraction gives 0.9997, or 99.97%. Doing the same extraction at pH 7.0 will result in an extraction of 97.7%. Try to do this calculation yourself.

### Box 2.3 Extraction of Salicylic Acid

Salicylic acid has a log P of 2.0 (corresponding to a $K_D$ of 100) and pKa values of 3.0 and 13.7. Performing similar calculations as in the above example, it can be shown that only 19% of the salicylic acid is extracted into 30 ml of organic phase at pH 6.0. Lowering the pH to 5.0 will give an apparent $D_c$ of about 1 and thus an extraction of 75%.

A question could be if multiple extractions using the same total amount of organic solvent would improve the extraction yield. Consider using three extractions of only 10 ml each of organic solvent. Calculations using Equation 2.15 show that the total extraction in the combined 30 ml will be 87.5% compared to the 75% obtained in only one single 30 ml extraction. Multiple extractions are more efficient, but in the case of salicylic acid it is necessary to perform the extraction at a lower pH value if quantitative extraction is needed.

Similar extraction calculations can be performed for bases using Equations 2.14 and 2.15. It is obvious that quantitative extractions from an aqueous solution into an organic phase are more easily achieved if extraction is performed when the analytes are not ionized. Thus, extraction of carboxylic acid should take place at low pH ($pK_a - 2$–3 pH units) and extraction of bases at high pH ($pK_a + 2$–3 pH units) in the aqueous phase.

## 2.4 Stereochemistry

Many pharmacologically active molecules, including drug substances, are *chiral*. Biological systems are built from chiral building blocks, proteins and peptides are built from chiral amino acids, polysaccharides are built from chiral monosaccharides, and DNA is chiral due

to its $\alpha$-helix structure. The biological system including the human body is therefore able to distinguish between stereoisomers that differ only in their spatial configuration. This may result in very different pharmacologically responses and different ADME (adsorption, distribution, metabolism, and excretion) properties of such isomers.

To describe the stereochemistry of a drug substance is to visualize the spatial orientation of its components in space. Biological systems contain large biomolecules that are constructed from building blocks with unique stereochemistry.

Figure 2.4 shows how isomers can be divided into several groups. *Constitutional isomers* are, of course, different compounds with different chemical structures. The *diastereomers*, grouped under the stereoisomers, contain compounds in which the isomers have different physicochemical characteristics with different melting points, partition ratios, and so on. These isomers are therefore easy to separate in chromatographic systems. *Cis-trans isomers* belong to this group, and a number of drug substances can be found in this group. Two examples are given in Figure 2.5. However, *enantiomers*, which also belong to this group of isomers, require chiral systems for their separation due to their identical physicochemical properties.

*Isomerization* at the double bond is often mediated by light, and therefore the compound should be protected from light exposure.

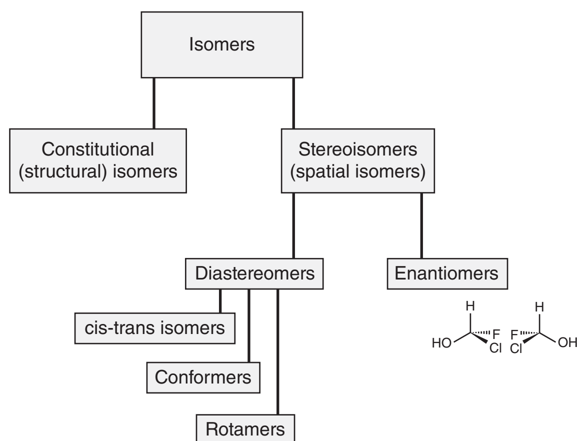



***Figure 2.4*** *Classification of isomers*

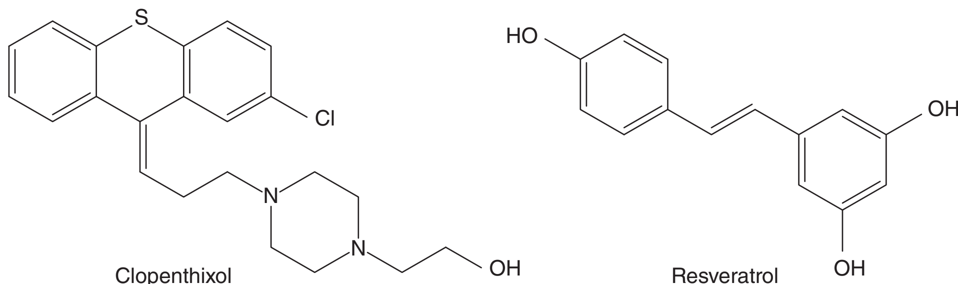



***Figure 2.5*** *The chemical structure of cis-clopenthixol and trans-resveratrol*