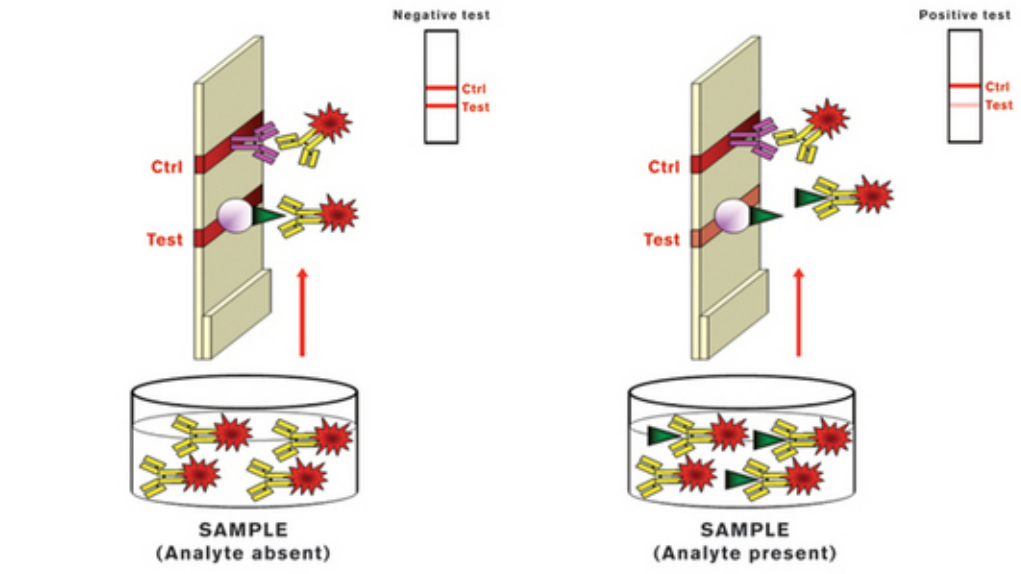
Wiley Series on Mass Spectrometry
Dominic M. Desiderio and Nico M. Nibbering, Series Editors

Chemical Analysis of Antibiotic Residues in Food

Edited by
Jian Wang, James D. MacNeil, *and* Jack F. Kay



WILEY

CHEMICAL ANALYSIS OF ANTIBIOTIC RESIDUES IN FOOD

WILEY SERIES IN MASS SPECTROMETRY

Series Editors

Dominic M. Desiderio
Departments of Neurology and Biochemistry
University of Tennessee Health Science Center

Nico M. M. Nibbering
Vrije Universiteit Amsterdam, The Netherlands

John R. de Laeter • *Applications of Inorganic Mass Spectrometry*
Michael Kinter and Nicholas E. Sherman • *Protein Sequencing and Identification Using Tandem Mass Spectrometry*
Chhabil Dass • *Principles and Practice of Biological Mass Spectrometry*
Mike S. Lee • *LC/MS Applications in Drug Development*
Jerzy Silberring and Rolf Eckman • *Mass Spectrometry and Hyphenated Techniques in Neuropeptide Research*
J. Wayne Rabalais • *Principles and Applications of Ion Scattering Spectrometry: Surface Chemical and Structural Analysis*
Mahmoud Hamdan and Pier Giorgio Righetti • *Proteomics Today: Protein Assessment and Biomarkers Using Mass Spectrometry, 2D Electrophoresis, and Microarray Technology*
Igor A. Kaltashov and Stephen J. Eyles • *Mass Spectrometry in Biophysics: Confirmation and Dynamics of Biomolecules*
Isabella Dalle-Donne, Andrea Scaloni, and D. Allan Butterfield • *Redox Proteomics: From Protein Modifications to Cellular Dysfunction and Diseases*
Silas G. Villas-Boas, Ute Roessner, Michael A.E. Hansen, Jorn Smedsgaard, and Jens Nielsen • *Metabolome Analysis: An Introduction*
Mahmoud H. Hamdan • *Cancer Biomarkers: Analytical Techniques for Discovery*
Chabbil Dass • *Fundamentals of Contemporary Mass Spectrometry*
Kevin M. Downard (Editor) • *Mass Spectrometry of Protein Interactions*
Nobuhiro Takahashi and Toshiaki Isobe • *Proteomic Biology Using LC-MS: Large Scale Analysis of Cellular Dynamics and Function*
Agnieszka Kraj and Jerzy Silberring (Editors) • *Proteomics: Introduction to Methods and Applications*
Ganesh Kumar Agrawal and Randeep Rakwal (Editors) • *Plant Proteomics: Technologies, Strategies, and Applications*
Rolf Ekman, Jerzy Silberring, Ann M. Westman-Brinkmalm, and Agnieszka Kraj (Editors) • *Mass Spectrometry: Instrumentation, Interpretation, and Applications*
Christoph A. Schalley and Andreas Springer • *Mass Spectrometry and Gas-Phase Chemistry of Non-Covalent Complexes*
Riccardo Flamini and Pietro Traldi • *Mass Spectrometry in Grape and Wine Chemistry*
Mario Thevis • *Mass Spectrometry in Sports Drug Testing: Characterization of Prohibited Substances and Doping Control Analytical Assays*
Sara Castiglioni, Ettore Zuccato, and Roberto Fanelli • *Illicit Drugs in the Environment: Occurrence, Analysis, and Fate Using Mass Spectrometry*
Ángel Garciá and Yotis A. Senis (Editors) • *Platelet Proteomics: Principles, Analysis, and Applications*
Luigi Mondello • *Comprehensive Chromatography in Combination with Mass Spectrometry*
Jian Wang, James MacNeil, and Jack F. Kay • *Chemical Analysis of Antibiotic Residues in Food*

CHEMICAL ANALYSIS OF ANTIBIOTIC RESIDUES IN FOOD

Edited by

JIAN WANG
JAMES D. MacNEIL
JACK F. KAY

A JOHN WILEY & SONS, INC., PUBLICATION

Published by John Wiley & Sons, Inc., Hoboken, New Jersey
Published simultaneously in Canada

Library of Congress Cataloging-in-Publication Data:
Chemical analysis of antibiotic residues in food / edited by Jian Wang, James D. MacNeil, Jack F. Kay.
p. ; cm.
Includes bibliographical references and index.
ISBN 978-0-470-49042-6 (cloth)
1. Veterinary drug residues–Analysis. 2. Antibiotic residues–Analysis. 3. Food of animal origin–Safety measures. I. Wang, Jian, 1969– II. MacNeil, James D. III. Kay, Jack F.
[DNLM: 1. Anti-Bacterial Agents–analysis. 2. Chemistry Techniques, Analytical–methods. 3. Drug Residues. 4. Food Safety. QV 350]
RA1270.V47C44 2011
615.9′54–dc22
2010054065

Printed in the United States of America

ePDF ISBN: 978-1-118-06718-5
oBook ISBN: 978-1-118-06720-8
ePub ISBN: 978-1-118-06719-2

10 9 8 7 6 5 4 3 2 1

CONTENTS

PREFACE

Food safety is of great importance to consumers. To ensure the safety of the food supply and to facilitate international trade, government agencies and international bodies establish standards, guidelines, and regulations that food producers and trade partners need to meet, respect, and follow. A primary goal of national and international regulatory frameworks for the use of veterinary drugs, including antimicrobials, in food-producing animals is to ensure that authorized products are used in a manner that will not lead to non-compliance residues. However, analytical methods are required to rapidly and accurately detect, quantify, and confirm antibiotic residues in food to verify that regulatory standards have been met and to remove foods that do not comply with these standards from the marketplace.

The current developments in analytical methods for antibiotic residues include the use of portable rapid tests for on-site use or rapid screening methods, and mass spectrometric (MS)-based techniques for laboratory use. This book, *Chemical Analysis of Antibiotic Residues in Food*, combines disciplines that include regulatory standards setting, pharmacokinetics, advanced MS technologies, regulatory analysis, and laboratory quality management. It includes recent developments in antibiotic residue analysis, together with information to provide readers with a clear understanding of both the regulatory environment and the underlying science for regulations. Other topics include the choice of marker residues and target animal tissues for regulatory analysis, general guidance for method development and method validation, estimation of measurement uncertainty, and laboratory quality assurance and quality control. Furthermore, it also includes information on the developing area of environmental issues related to veterinary use of antimicrobials. For the bench analyst, it provides not only information on sources of methods of analysis but also an understanding of which methods are most suitable for addressing the regulatory requirements and the basis for those requirements.

The main themes in this book include antibiotic chemical properties (Chapter 1), pharmacokinetics, metabolism, and distribution (Chapter 2); food safety regulations (Chapter 3); sample preparation (Chapter 4); screening methods (Chapter 5); chemical analysis focused mainly on LC-MS (Chapters 6 and 7), method development and validation (Chapter 8), measurement uncertainty (Chapter 9), and quality assurance and quality control (Chapter 10).

The editors and authors of this book are internationally recognized experts and leading scientists with extensive firsthand experience in preparing food safety regulations and in the chemical analysis of antibiotic residues in food. This book represents the cutting-edge state of the science in this area. It has been deliberately written and organized with a balance between practical use and theory to provide readers or analytical laboratory staff with a reference book for the analysis of antibiotic residues in food.

Jian Wang
James D. MacNeil
Jack F. Kay

Canadian Food Inspection Agency, Calgary, Canada
St. Mary's University, Halifax, Canada
University of Strathclyde, Glasgow, Scotland

ACKNOWLEDGMENT

The editors are grateful to Dr. Dominic M. Desiderio, the editor of *Mass Spectrometry Reviews*, for the invitation to contribute a book on antibiotic residues analysis; to individual chapter authors, leading scientists in the field, for their great contributions as the result of their profound knowledge and many years of firsthand experience; and to the editors' dear family members for their unending support and encouragement during this book project.

EDITORS

Dr. Jian Wang received his PhD at the University of Alberta in Canada in 2000, and then worked as a Post Doctoral Fellow at the Agriculture and Agri-Food Canada in 2001. He has been working as a leading Research Scientist at the Calgary Laboratory with the Canadian Food Inspection Agency since 2002. His scientific focus is on the method development using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and UPLC/QqTOF for analyses of chemical contaminant residues, including antibiotics, pesticides, melamine, and cyanuric acid in various foods. He also develops statistical approaches to estimating the measurement uncertainty based on method validation and quality control data using the SAS program.

Dr. James D. MacNeil received his PhD from Dalhousie University, Halifax, NS, Canada in 1972 and worked as a government scientist until his retirement in 2007. During 1982–2007 he was Head, Centre for Veterinary Drug Residues, now part of the Canadian Food Inspection Agency. Dr. MacNeil has served as a member of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), cochair of the working group on methods of Analysis and Sampling, Codex Committee on Veterinary Drugs in Foods (CCRVDF), is the former scientific editor for "Drugs, Cosmetics & Forensics" of J.AOAC Int., worked on IUPAC projects, has participated in various consultations on method validation and is the author of numerous publications on veterinary drug residue analysis. He is a former General Referee for methods for veterinary drug residues for AOAC International and was appointed scientist emeritus by CFIA in 2008. Dr. MacNeil holds an appointment as an adjunct professor in the Department of Chemistry, St. Mary's University.

Dr. Jack F. Kay received his PhD from the University of Strathclyde, Glasgow, Scotland in 1980 and has been involved with veterinary drug residue analyses since 1991. He works for the UK Veterinary Medicines Directorate to provide scientific advice on residue monitoring programmes and manages the research and development (R&D) program. Dr. Kay helped draft Commission Decision 2002/657/EC and is an International Standardization Organization (ISO)-trained assessor for audits to ISO 17025. He served as cochair of the CCRVDF ad hoc Working Group on Methods of Sampling and Analysis and steered Codex Guideline CAC/GL 71–2009 to completion after Dr. MacNeil retired. Dr. Kay now cochairs work to extend this to cover multi-residue method performance criteria. He assisted JECFA in preparing an initial consideration of setting MRLs in honey, and is now developing this further for the CCRVDF. He also holds an Honorary Senior Research Fellowship at the Department of Mathematics and Statistics at the University of Strathclyde.

CONTRIBUTORS

Bjorn Berendsen, Department of Veterinary Drug Research, RIKILT—Institute of Food Safety, Unit Contaminants and Residues, Wageningen, The Netherlands

Alistair Boxall, Environment Department, University of York, Heslington, York, United Kingdom

Andrew Cannavan, Food and Environmental Protection Laboratory, FAO/IAEA Agriculture & Biotechnology Laboratories, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency, Vienna, Austria

Martin Danaher, Food Safety Department, Teagasc, Ashtown Food Research Centre, Ashtown, Dublin 15, Ireland

Leslie Dickson, Canadian Food Inspection Agency, Saskatoon Laboratory, Centre for Veterinary Drug Residues, Saskatoon, Saskatchewan, Canada

Rick Fedeniuk, Canadian Food Inspection Agency, Saskatoon Laboratory, Centre for Veterinary Drug Residues, Saskatoon, Saskatchewan, Canada

Lynn G. Friedlander, Residue Chemistry Team, Division of Human Food Safety, FDA/CVM/ONADE/HFV-151, Rockville, Maryland

Kevin J. Greenlees, Office of New Animal Drug Evaluation, HFV-100, USFDA Center for Veterinary Medicine, Rockville, Maryland

Jack F. Kay, Veterinary Medicines Directorate, New Haw, Surrey, United Kingdom; *also* Department of Mathematics and Statistics, University of Strathclyde, Glasgow, United Kingdom (honorary position)

Bruno Le Bizec, Food Safety, LABERCA (Laboratoire d'Etude des Résidus et Contaminants dans les Aliments), ONIRIS—Ecole Nationale Vétérinaire, Agroalimentaire et de l'Alimentation Nantes, Atlantique, Nantes, France

Peter Lees, Veterinary Basic Sciences, Royal Veterinary College, University of London, Hatfield, Hertfordshire, United Kingdom

James D. MacNeil, Scientist Emeritus, Canadian Food Inspection Agency, Dartmouth Laboratory, Dartmouth, Nova Scotia, Canada; *also* Department of Chemistry, St. Mary's University, Halifax, Nova Scotia, Canada

Ross A Potter, Veterinary Drug Residue Unit Supervisor, Canadian Food Inspection Agency (CFIA), Dartmouth Laboratory, Dartmouth, Nova Scotia, Canada

Philip Thomas Reeves, Australian Pesticides and Veterinary Medicines Authority, Regulatory Strategy and Compliance, Canberra, ACT (Australian Capital Territory), Australia

Jacques Stark, DSM Food Specialities, Delft, The Netherlands

Sara Stead, The Food and Environment Research Agency, York, North Yorkshire, United Kingdom

Alida A. M. (Linda) Stolker, Department of Veterinary Drug Research, RIKILT—Institute of Food Safety Unit Contaminants and Residues, Wageningen, The Netherlands

Jonathan A. Tarbin, The Food and Environment Research Agency, York, North Yorkshire, United Kingdom

Pierre-Louis Toutain, UMR181 Physiopathologie et Toxicologie Experimentales INRA, ENVT, Ecole Nationale Veterinaire de Toulouse, Toulouse, France

Sherri B. Turnipseed, Animal Drugs Research Center, US Food and Drug Administration, Denver, Colorado

Jian Wang, Canadian Food Inspection Agency, Calgary Laboratory, Calgary, Alberta, Canada

1

ANTIBIOTICS: GROUPS AND PROPERTIES

PHILIP THOMAS REEVES

1.1 INTRODUCTION

The introduction of the sulfonamides in the 1930s and benzylpenicillin in the 1940s completely revolutionized medicine by reducing the morbidity and mortality of many infectious diseases. Today, antimicrobial drugs are used in food-producing animals to treat and prevent diseases and to enhance growth rate and feed efficiency. Such use is fundamental to animal health and well-being and to the economics of the livestock industry, and has seen the development of antimicrobials such as ceftiofur, florfenicol, tiamulin, tilmicosin, tulathromycin, and tylosin specifically for use in food-producing animals.[1,2] However, these uses may result in residues in foods and have been linked to the emergence of antibiotic-resistant strains of disease-causing bacteria with potential human health ramifications.[3] Antimicrobial drug resistance is not addressed in detail in this text, and the interested reader is referred to an excellent overview by Martinez and Silley.[4]

Many factors influence the residue profiles of antibiotics in animal-derived edible tissues (meat and offal) and products (milk and eggs), and in fish and honey. Among these factors are the approved uses, which vary markedly between antibiotic classes and to a lesser degree within classes. For instance, in some countries, residues of quinolones in animal tissues, milk, honey, shrimp, and fish are legally permitted (maximum residue limits [MRLs] have been established). By comparison, the approved uses of the macrolides are confined to the treatment of respiratory disease and for growth promotion (in some countries) in meat-producing animals (excluding fish), and to the treatment of American foulbrood disease in honeybees. As a consequence, residues of macrolides are legally permitted only in edible tissues derived from these food-producing species, and in honey in some countries. Although a MRL for tylosin in honey has not been established, some countries apply a safe working residue level, thereby permitting the presence of trace concentrations of tylosin to allow for its use. Substantial differences in the approved uses of antimicrobial agents also occur between countries. A second factor that influences residue profiles of antimicrobial drugs is their chemical nature and physicochemical properties, which impact pharmacokinetic behavior. Pharmacokinetics (PK), which describes the timecourse of drug concentration in the body, is introduced in this chapter and discussed further in Chapter 2.

Analytical chemists take numerous parameters into account when determining antibiotic residues in food of animal origin, some of which are discussed here.

1.1.1 Identification

A substance needs to be identified by a combination of the appropriate identification parameters including the name or other identifier of the substance, information related to molecular and structural formula, and composition of the substance.

International nonproprietary names (INNs) are used to identify pharmaceutical substances or active pharmaceutical ingredients. Each INN is a unique name that is internationally consistent and is recognized globally. As of October 2009, approximately 8100 INNs had been designated, and this number is growing every year by some 120–150 new INNs.[5] An example of an INN is tylosin, a macrolide antibiotic.

Chemical Analysis of Antibiotic Residues in Food, First Edition. Edited by Jian Wang, James D. MacNeil, and Jack F. Kay.

International Union of Pure and Applied Chemistry (IUPAC) names are based on a method that involves selecting the longest continuous chain of carbon atoms, and then identifying the groups attached to that chain and systematically indicating where they are attached. Continuing with tylosin as an example, the IUPAC name is [(2*R*,3*R*,4*E*,6*E*,9*R*,11*R*,12*S*,13*S*,14*R*)-12-{[3,6-dideoxy-4-*O*-(2,6-dideoxy-3-*C*-methyl-α-L-ribohexopyranosyl)-3-(dimethylamino)-β-D-glucopyranosyl]oxy}-2-ethyl-14-hydroxy-5,9,13-trimethyl-8,16-dioxo-11-(2-oxoethyl)oxacyclohexadeca-4,6-dien-3-yl]methyl 6-deoxy-2,3-di-*O*-methyl-β-D-allopyranoside.

The Chemical Abstract Service (CAS) Registry Number is the universally recognized unique identifier of chemical substances. The CAS Registry Number for tylosin is 1401-69-0.

Synonyms are used for establishing a molecule's unique identity. For the tylosin example, there are numerous synonyms, one of which is Tylan.

1.1.2 Chemical Structure

For the great majority of drugs, action on the body is dependent on chemical structure, so that a very small change can markedly alter the potency of the drug, even to the point of loss of activity.[6] In the case of antimicrobial drugs, it was the work of Ehrlich in the early 1900s that led to the introduction of molecules selectively toxic for microbes and relatively safe for the animal host. In addition, the presence of different sidechains confers different pharmacokinetic behavior on a molecule. Chemical structures also provide the context to some of the extraction, separation, and detection strategies used in the development of analytical methods. Certain antibiotics consist of several components with distinct chemical structures. Tylosin, for example, is a mixture of four derivatives produced by a strain of *Streptomyces fradiae*. The chemical structures of the antimicrobial agents described in this chapter are presented in Tables 1.2–1.15.

1.1.3 Molecular Formula

By identifying the functional groups present in a molecule, a molecular formula provides insight into numerous properties. These include the molecule's water and lipid solubility, the presence of fracture points for gas chromatography (GC) determinations, sources of potential markers such as chromophores, an indication as to the molecule's UV absorbance, whether derivatization is likely to be required when quantifying residues of the compound, and the form of ionization such as protonated ions or adduct ions when using electrospray ionization. The molecular formulas of the antimicrobial agents described in this chapter are shown in Tables 1.2–1.15.

1.1.4 Composition of the Substance

Regulatory authorities conduct risk assessments on the chemistry and manufacture of new and generic antimicrobial medicines (formulated products) prior to granting marketing approvals. Typically, a compositional standard is developed for a new chemical entity or will already exist for a generic drug. A compositional standard specifies the minimum purity of the active ingredient, the ratio of isomers to diastereoisomers (if relevant), and the maximum permitted concentration of impurities, including those of toxicological concern. The risk assessment considers the manufacturing process (the toxicological profiles of impurities resulting from the synthesis are of particular interest), purity, and composition to ensure compliance with the relevant standard. The relevant test procedures described in pharmacopoeia and similar texts apply to the active ingredient and excipients present in the formulation. The overall risk assessment conducted by regulatory authorities ensures that antimicrobial drugs originating from different manufacturing sources, and for different batches from the same manufacturing source, have profiles that are consistently acceptable in terms of efficacy and safety to target animals, public health, and environmental health.

1.1.5 pK_a

The symbol pK_a is used to represent the negative logarithm of the acid dissociation constant K_a, which is defined as $[H^+][B]/[HB]$, where B is the conjugate base of the acid HB. By convention, the acid dissociation constant (pK_a) is used for weak bases (rather than the pK_b) as well as weak organic acids. Therefore, a weak acid with a high pK_a will be poorly ionized, and a weak base with a high pK_a will be highly ionized at blood pH. The pK_a value is the principal property of an electrolyte that defines its biological and chemical behavior. Because the majority of drugs are weak acids or bases, they exist in both ionized and un-ionized forms, depending on pH. The proportion of ionized and un-ionized species at a particular pH is calculated using the Henderson–Hasselbalch equation. In biological terms, pK_a is important in determining whether a molecule will be taken up by aqueous tissue components or lipid membranes and is related to the partition coefficient log P. The pK_a of an antimicrobial drug has implications for both the fate of the drug in the body and the action of the drug on microorganisms. From a chemical perspective, ionization will increase the likelihood of a species being taken up into aqueous solution (because water is a very polar solvent). By contrast, an organic molecule that does not readily ionize will often tend to stay in a non-polar solvent. This partitioning behavior affects the efficiency of extraction and clean-up of analytes and is an important consideration when developing enrichment methods. The pK_a values for many

of the antimicrobial agents described in this chapter are presented in Tables 1.2–1.15. The consequences of pK_a for the biological and chemical properties of antimicrobial agents are discussed later in this text.

1.1.6 UV Absorbance

The electrons of unsaturated bonds in many organic drug molecules undergo energy transitions when UV light is absorbed. The intensity of absorption may be quantitatively expressed as an extinction coefficient ε, which has significance in analytical application of spectrophotometric methods.

1.1.7 Solubility

From an *in vitro* perspective, solubility in water and in organic solvents determines the choice of solvent, which, in turn, influences the choice of extraction procedure and analytical method. Solubility can also indirectly impact the timeframe of an assay for compounds that are unstable in solution. From an *in vivo* perspective, the solubility of a compound influences its absorption, distribution, metabolism, and excretion. Both water solubility and lipid solubility are necessary for the absorption of orally administered antimicrobial drugs from the gastrointestinal tract. This is an important consideration when selecting a pharmaceutical salt during formulation development. Lipid solubility is necessary for passive diffusion of drugs in the distributive phase, whereas water solubility is critical for the excretion of antimicrobial drugs and/or their metabolites by the kidneys.

1.1.8 Stability

In terms of residues in food, stability is an important parameter as it relates to (1) residues in biological matrices during storage, (2) analytical reference standards, (3) analytes in specified solvents, (4) samples prepared for residue analysis in an interrupted assay run such as might occur with the breakdown of an analytical instrument, and (5) residues being degraded during chromatography as a result of an incompatible stationary phase.

Stability is also an important property of formulated drug products since all formulations decompose with time.[7] Because instabilities are often detectable only after considerable storage periods under normal conditions, stability testing utilizes high-stress conditions (conditions of temperature, humidity, and light intensity, which are known to be likely causes of breakdown). Adoption of this approach reduces the amount of time required when determining shelf life. Accelerated stability studies involving the storage of products at elevated temperatures are commonly conducted to allow unsatisfactory formulations to be eliminated early in development and for a successful product to reach market sooner. The concept of accelerated stability is based on the Arrhenius equation:

$$k = Ae^{(-E_a/RT)}$$

where k is the rate constant of the chemical reaction; A, a pre-exponential factor; E_a, activation energy; R, gas constant; and T, absolute temperature.

In practical terms, the Arrhenius equation supports the generalization that, for many common chemical reactions at room temperature, the reaction rate doubles for every 10°C increase in temperature. Regulatory authorities generally accept accelerated stability data as an interim measure while real-time stability data are being generated.

1.2 ANTIBIOTIC GROUPS AND PROPERTIES

1.2.1 Terminology

Traditionally, the term *antibiotic* refers to substances produced by microorganisms that at low concentration kill or inhibit the growth of other microorganisms but cause little or no host damage. The term *antimicrobial agent* refers to any substance of natural, synthetic, or semisynthetic origin that at low concentration kills or inhibits the growth of microorganisms but causes little or no host damage. Neither antibiotics nor antimicrobial agents have activity against viruses. Today, the terms *antibiotic* and *antimicrobial agent* are often used interchangeably.

The term *microorganism* or *microbe* refers to (for the purpose of this chapter) prokaryotes, which, by definition, are single-cell organisms that do not possess a true nucleus. Both typical bacteria and atypical bacteria (rickettsiae, chlamydiae, mycoplasmas, and actinomycetes) are included. Bacteria range in size from 0.75 to 5 μm and most commonly are found in the shape of a sphere (coccus) or a rod (bacillus). Bacteria are unique in that they possess peptidoglycan in their cell walls, which is the site of action of antibiotics such as penicillin, bacitracin, and vancomycin. Differences in the composition of bacterial cell walls allow bacteria to be broadly classified using differential staining procedures. In this respect, the Gram stain developed by Christian Gram in 1884 (and later modified) is by far the most important differential stain used in microbiology.[8] Bacteria can be divided into two broad groups—Gram-positive and Gram-negative—using the Gram staining procedure. This classification is based on the ability of cells to retain the dye methyl violet after washing with a decolorizing agent such as absolute alcohol or acetone. Gram-positive cells retain the stain, whereas Gram-negative cells do not. Examples of Gram-positive bacteria are *Bacillus, Clostridium, Corynebacterium, Enterococcus,*

Erysipelothrix, Pneumococcus, Staphylococcus, and *Streptococcus*. Examples of Gram-negative bacteria are *Bordetella, Brucella, Escherichia coli, Haemophilus, Leptospira, Neisseria, Pasteurella, Proteus, Pseudomonas, Salmonella, Serpulina hyodysenteriae, Shigella*, and *Vibrio*. Differential sensitivity of Gram-positive and Gram-negative bacteria to antimicrobial drugs is discussed later in this chapter.

1.2.2 Fundamental Concepts

From the definitions above, it is apparent that a critically important element of antimicrobial therapy is the selective toxicity of a drug for invading organisms rather than mammalian cells. The effectiveness of antimicrobial therapy depends on a triad of bacterial susceptibility, the drug's disposition in the body, and the dosage regimen. An additional factor that influences therapeutic outcomes is the competence of host defence mechanisms. This property is most relevant when clinical improvement relies on the inhibition of bacterial cell growth rather than bacterial cell death. Irrespective of the mechanism of action, the use of antimicrobial drugs in food-producing species may result in residues.

The importance of antibacterial drug pharmacokinetics (PK) and pharmacodynamics (PD) in determining clinical efficacy and safety was appreciated many years ago when the relationship between the magnitude of drug response and drug concentration in the fluids bathing the infection site(s) was recognized. PK describes the timecourse of drug absorption, distribution, metabolism, and excretion (what the *body does to the drug*) and therefore the relationship between the dose of drug administered and the concentration of non-protein-bound drug at the site of action. PD describes the relationship between the concentration of non-protein-bound drug at the site of action and the drug response (ultimately the therapeutic effect) (what the *drug does to the body*).[9]

In conceptualizing the relationships between the host animal, drug, and target pathogens, the chemotherapeutic triangle (Fig. 1.1) alludes to antimicrobial drug PK and PD. The relationship between the host animal and the drug reflects the PK properties of the drug, whereas drug action against the target pathogens reflects the PD properties of the drug. The clinical efficacy of antimicrobial therapy is depicted by the relationship between the host animal and target pathogens.

1.2.3 Pharmacokinetics of Antimicrobial Drugs

The pharmacokinetics of antimicrobial drugs is discussed in Chapter 2. The purpose of the following discussion, then, is to introduce the concept of pharmacokinetics and, in particular, to address the consequences of an antimicrobial drug's pK_a value for both action on the target pathogen and fate in the body.

The absorption, distribution, metabolism, and excretion of an antimicrobial drug are governed largely by the drug's chemical nature and physicochemical properties. Molecular size and shape, lipid solubility, and the degree of ionization are of particular importance, although the degree of ionization is not an important consideration for amphoteric compounds such as fluoroquinolones, tetracyclines, and rifampin.[10] The majority of antimicrobial agents are weak acids and bases for which the degree of ionization depends

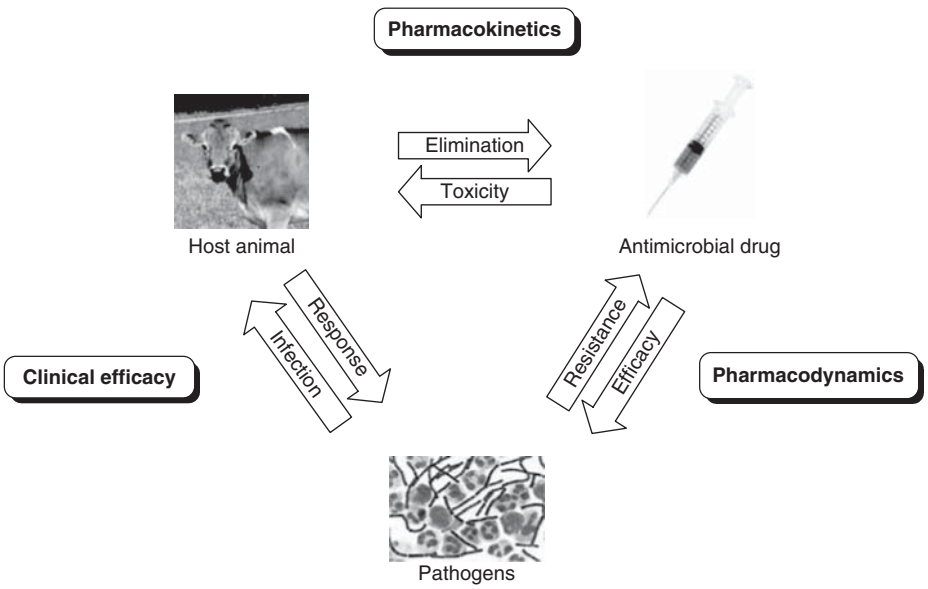


Figure 1.1 Schematic of the chemotherapeutic triangle depicting the relationships between the host animal, antimicrobial drug, and target pathogens.

on the pK_a of the drug and the pH of the biological environment. Only the un-ionized form of these drugs is lipid-soluble and able to cross cell membranes by passive diffusion. Two examples from Baggot and Brown[11] are presented here to demonstrate the implications of pK_a for the distributive phase of drug disposition. However, the same principles of passive diffusion apply to the absorption, metabolism, and excretion of drugs in the body and to the partitioning of drugs into microorganisms.

The first example relates to the sodium salt of a weak acid (with pK_a 4.4) that is infused into the mammary glands of dairy animals to treat mastitis. The pH of the normal mammary gland can be as low as 6.4, and at this pH, the Henderson–Hasselbalch equation predicts that the ratio of un-ionized to ionized drug is 1 : 100. Mastitic milk is more alkaline (with pH $\sim$ 7.4) and the ratio of un-ionized to ionized drug, as calculated by the Henderson–Hasselbalch equation, is 1 : 1000. This is identical to the ratio for plasma, which also has a pH of 7.4. This example demonstrates that, when compared to the normal mammary gland, the mastitic gland will have more drug "trapped" in the ionized form. The second example involves the injection of a lipid-soluble, organic base that diffuses from the systemic circulation (with pH 7.4) into ruminal fluid (pH 5.5–6.5) during the distributive phase of a drug. Again, the ionized form becomes trapped in the acidic fluid of the rumen; the extent of trapping will be determined by the pK_a of the organic base. In summary, weakly acidic drugs are trapped in alkaline environments and, vice versa, weakly basic drugs are trapped in acidic fluids.

A second PK issue is the concentration of antimicrobial drug at the site of infection. This value reflects the drug's distributive behavior and is critically important in terms of efficacy. Furthermore, the optimization of dosage regimens is dependent on the availability of quality information relating to drug concentration at the infection site. It raises questions regarding the choice of sampling site for measuring the concentration of antimicrobial drugs in the body and the effect, if any, that the extent of plasma protein binding has on the choice of sampling site. These matters are addressed below.

More often than not, the infection site (the biophase) is remote from the circulating blood that is commonly sampled to measure drug concentration. Several authors[12–14] have reported that plasma concentrations of free (non-protein-bound) drug are generally the best predictors of the clinical success of antimicrobial therapy. The biophase in most infections comprises extracellular fluid (plasma + interstitial fluids). Most pathogens of clinical interest are located extracellularly and as a result, plasma concentrations of free drug are generally representative of tissue concentrations; however, there are some notable exceptions:

1. Intracellular microbes such as *Lawsonia intracellularis*, the causative agent of proliferative enteropathy in pigs, are not exposed to plasma concentrations of antimicrobial drugs.
2. Anatomic barriers to the passive diffusion of antimicrobial drugs are encountered in certain tissues, including the central nervous system, the eye, and the prostate gland.
3. Pathological barriers such as abscesses impede the passive diffusion of drugs.
4. Certain antimicrobial drugs are preferentially accumulated inside cells. Macrolides, for instance, are known to accumulate within phagocytes.[15]
5. Certain antimicrobial drugs are actively transported into infection sites. The active transport of fluoroquinolones and tetracyclines by gingival fibroblasts into gingival fluid is an example.[16]

With regard to the effect of plasma protein binding on the choice of sampling site, Toutain and coworkers[14] reported that plasma drug concentrations of antimicrobial drugs that are >80% bound to plasma protein are unlikely to be representative of tissue concentrations. Those antimicrobial drugs that are highly bound to plasma protein include clindamycin, cloxacillin, doxycycline, and some sulfonamides.[17,18]

The most useful PK parameters for studying antimicrobial drugs are discussed in Chapter 2.

1.2.4 Pharmacodynamics of Antimicrobial Drugs

The PD of antimicrobial drugs against microorganisms comprises three main aspects: spectrum of activity, bactericidal and bacteriostatic activity, and the type of killing action (i.e., concentration-dependent, time-dependent, or co-dependent). Each of these is discussed below. Also described are the PD indices—minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)—and the mechanisms of action of antimicrobial drugs.

1.2.4.1 Spectrum of Activity

Antibacterial agents may be classified according to the class of target microorganism. Accordingly, antibacterial agents that inhibit only bacteria are described as narrow- or medium-spectrum, whereas those that also inhibit mycoplasma, rickettsia, and chlamydia (so-called atypical bacteria) are described as broad-spectrum. The spectrum of activity of common antibacterial drugs is shown in Table 1.1.

A different classification describes those antimicrobial agents that inhibit only Gram-positive or Gram-negative bacteria as narrow-spectrum, and those that are active against a range of both Gram-positive and Gram-negative bacteria as broad-spectrum. However, this distinction is not always absolute.

TABLE 1.1 Spectrum of Activity of Common Antibacterial Drugs

	Class of Microorganism				
Antibacterial Drug	Bacteria	Mycoplasma	Rickettsia	Chlamydia	Protozoa
Aminoglycosides	+	+	−	−	−
β-Lactams	+	−	−	−	−
Chloramphenicol	+	+	+	+	−
Fluoroquinolones	+	+	+	+	−
Lincosamides	+	+	−	−	+/−
Macrolides	+	+	−	+	+/−
Oxazolidinones	+	+	−	−	−
Pleuromutilins	+	+	−	+	−
Tetracyclines	+	+	+	+	−
Streptogramins	+	+	−	+	+/−
Sulfonamides	+	+	−	+	+
Trimethoprim	+	−	−	−	+

Notation: Presence or absence of activity against certain protozoa is indicated by plus or minus sign (+/−).

Source: Reference 2. Reprinted with permission of John Wiley & Sons, Inc. Copyright 2006, Blackwell Publishing.

The differential sensitivity of Gram-positive and Gram-negative bacteria to many antimicrobials is due to differences in cell wall composition. Gram-positive bacteria have a thicker outer wall composed of a number of layers of peptidoglycan, while Gram-negative bacteria have a lipophilic outer membrane that protects a thin peptidoglycan layer. Antibiotics that interfere with peptidoglycan syntheses more easily reach their site of action in Gram-positive bacteria. Gram-negative bacteria have protein channels (porins) in their outer membranes that allow the passage of small hydrophilic molecules. The outer membrane contains a lipopolysaccharide component that can be shed from the wall on cell death. It contains a highly heat-resistant molecule known as *endotoxin*, which has a number of toxic effects on the host animal, including fever and shock.

Antibiotic sensitivity also differs between aerobic and anaerobic organisms. Anaerobic organisms are further classified as facultative and obligate. Facultative anaerobic bacteria derive energy by aerobic respiration if oxygen is present but are also capable of switching to fermentation. Examples of facultative anaerobic bacteria are *Staphylococcus* (Gram-positive), *Escherichia coli* (Gram-negative), and *Listeria* (Gram-positive). In contrast, obligate anaerobes die in the presence of oxygen. Anaerobic organisms are resistant to antimicrobials that require oxygen-dependent mechanisms to enter bacterial cells. Anaerobic organisms may elaborate a variety of toxins and enzymes that can cause extensive tissue necrosis, limiting the penetration of antimicrobials into the site of infection, or inactivating them once they are present.

1.2.4.2 Bactericidal and Bacteriostatic Activity

The activity of antimicrobial drugs has also been described as being bacteriostatic or bactericidal, although this distinction depends on both the drug concentration at the site of infection and the microorganism involved. Bacteriostatic drugs (tetracyclines, phenicols, sulfonamides, lincosamides, macrolides) inhibit the growth of organisms at the MIC but require a significantly higher concentration, the MBC, to kill the organisms (MIC and MBC are discussed further below). By comparison, bactericidal drugs (penicillins, cephalosporins, aminoglycosides, fluoroquinolones) cause death of the organism at a concentration near the same drug concentration that inhibits its growth. Bactericidal drugs are required for effectively treating infections in immunocompromised patients and in immunoincompetent environments in the body.

1.2.4.3 Type of Killing Action

A further classification of antimicrobial drugs is based on their killing action, which may be time-dependent, concentration-dependent, or co-dependent. For time-dependent drugs, it is the duration of exposure (as reflected in time exceeding MIC for plasma concentration) that best correlates with bacteriological cure. For drugs characterized by concentration-dependent killing, it is the maximum plasma concentration and/or area under the plasma concentration–time curve that correlates with outcome. For drugs with a co-dependent killing effect, both the concentration achieved and the duration of exposure determine outcome (see Chapter 2 for further discussion).

Growth inhibition–time curves are used to define the type of killing action and steepness of the concentration–effect curve. Typically, reduction of the initial bacterial count (response) is plotted against antimicrobial drug concentration. The killing action (time-, concentration-, or co-dependent) of an antibacterial drug is determined largely by the slope of the curve. Antibacterial drugs that demonstrate time-dependent killing activity include the β-lactams, macrolides, tetracyclines, trimethoprim–sulfonamide