BCS-based biowaivers: risks and opportunities

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LIST OF ABBREVIATIONS

API	active pharmaceutical ingredient
AUC	area under the curve
BCS	Biopharmaceutics Classification System
CI	confidence interval
C_{max}	peak plasma concentration
CV	coefficient of variation
D	dose
DD	degree of deacetylation
DMEM	Dulbecco's modified Eagle's medium
EMA	European Medicines Agency
FDA	Food and Drug Authority
FMEA	failure mode and effects analysis
GMR	geometric mean ratio
HBSS	Hank's balanced salt solution
HCl	hydrochloride
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
kDa	kiloDalton
KRB	Krebs-Ringer-Bicarbonate buffer
LC	liquid chromatography
MES	4-morpholine ethane sulfonic acid
MS	mass spectrometry
MW	molecular weight
NTI	narrow therapeutic index
P_{app}	apparent permeability
PD	potential difference
P-gp	P-glycoprotein
Ph.Eur.	European Pharmacopoeia
Rf	transsegmental electrical resistance
S	solubility
SmPC	summary of product characteristics
TEER	transepithelial electrical resistance
TIM-1	TNO gastrointestinal model 1
T_{max}	time point at which the maximum concentration is measured

1. INTRODUCTION: BIOAVAILABILITY, BIOEQUIVALENCE AND BIOWAIVERS

1.1. Regulatory context and definitions

The oral route is the most commonly applied and a preferred route of administration for medicines. The success of drug candidate selection and development of a pharmaceutical formulation for oral administration is highly dependent on the characteristics of the compound at issue. High Throughput Screening (HTS) based on Structure Activity relationship (SAR) analysis may result in pharmacological leads. The biopharmaceutic characteristics of the compound determine its intrinsic oral bioavailability and thus its potential administration via the oral route.

The US regulation 21CFR320 (1) as applied by the Food and Drug Agency (FDA) defines bioavailability as "the rate and extent to which the active ingredient or active moiety is absorbed from a drug product and becomes available at the site of action. For drug products that are not intended to be absorbed into the bloodstream, bioavailability may be assessed by measurements intended to reflect the rate and extent to which the active ingredient or active moiety becomes available at the site of action." European Directive 2001/83/EC as amended, does not provide such explicit definition of the term 'bioavailability' (2).

During development of a new product, several dosage forms may be tested and the composition of the product can change accordingly. In the post-marketing phase, a formulation may be optimized, e.g. by introducing a new excipient. The bioavailability of the active pharmaceutical ingredient of the product may change as a result of such changes and a comparative bioavailability study may be needed to demonstrate that the plasma profiles of the drug are still equivalent i.e. bioequivalent. Consideration of bioequivalence issues thus plays a role from the early development of a formulation to the marketed stage. This applies both to the innovators and to generic products. For generics, demonstration of bioequivalence to the innovator is the key issue at any time during product development.

In the late 1960s, drug product equivalence issues attracted public interest as doubts arose on the equivalence of products and their respective innovators. Explicit evaluation of bioequivalence was first introduced in the US. In the 1970s, the FDA started asking for bioavailability information. A drug bioequivalence panel was established in the US in 1971 forming the start of current regulatory guidance on bioequivalence (3).

Abbreviated application procedures were introduced in the 1980s: preclinical and clinical tests need not be repeated for generic copies of existing approved drugs. In the US this was laid down

in the Hatch-Waxman Act in 1984 (Drug Price Competition and Patent Term Restoration Act ; US drug regulation since 1962) (4).

Today, a generic medicine must fulfil the following criteria to gain FDA approval:

- *contain the same active ingredient as the originator medicine (inactive ingredients may vary)*
- *be identical in strength, dosage form, and route of administration*
- *have the same use indications*
- *be bioequivalent*
- *meet the same batch requirements for identity, strength, purity, and quality*
- *be manufactured under the same strict standards of FDA's good manufacturing practice regulations required for originator products* (5).

The FDA works according to 21CFR320.1 which states that "Bioequivalence means the *absence of a significant difference* in the rate and extent to which the active ingredient or active moiety in pharmaceutical equivalents or pharmaceutical alternatives becomes available at the site of drug action when administered at the same molar dose under similar conditions in an appropriately designed study." (1) The FDA publishes an overview of 'Approved Drug Products with Therapeutic Equivalence Evaluations' also known as the 'Orange Book' (6).

In Europe, regulatory arrangements to control market access of (generic) drugs developed in parallel. Directive 65/65/EEC was the first European law on the registration of medicinal products by the national competent authorities. Its amendments by Directive 87/21/EEC described an "abridged application procedure" for a "medicinal product [that] is essentially similar to a product which has been authorised within the Community, in accordance with Community provisions in force, for not less than six years and is marketed in the Member State for which the application is made […]. In such a case, "the applicant shall not be required to provide the results of pharmacological and toxicological tests or the results of clinical trials…"

A formal definition of a generic product was introduced in the EU legislation only in 2004 (Directive 2004/27 amending 2001/83): *"generic medicinal product" shall mean a medicinal product which has the same qualitative and quantitative composition in active substances and the same pharmaceutical form as the reference medicinal product, and whose bioequivalence with the reference medicinal product has been demonstrated by appropriate bioavailability studies. The different salts, esters, ethers, isomers, mixtures of isomers, complexes or derivatives of an active substance shall be considered to be the same active substance, unless they differ significantly in properties with regard to safety and/or efficacy. In such cases, additional information providing proof of the safety and/or efficacy of the various salts, esters or derivatives of an authorised active*

substance must be supplied by the applicant. The various immediate-release oral pharmaceutical forms shall be considered to be one and the same pharmaceutical form. Bioavailability studies need not be required of the applicant if he can demonstrate that the generic medicinal product meets the relevant criteria as defined in the appropriate detailed guidelines (2).

Current European bioequivalence guidance defines "two medicinal products containing the same active substance as bioequivalent if they are pharmaceutically equivalent or pharmaceutical alternatives and their bioavailabilities (rate and extent) after administration in the same molar dose *lie within acceptable predefined limits*. These limits are set to ensure comparable *in vivo* performance, i.e. similarity in terms of safety and efficacy"(7). So, although the work definitions are not identical in the two regions, similar principles apply in practice.

Bioequivalence of oral dosage forms with systemic action

For solid oral dosage forms of an active substance with systemic action, both EU and US guidance describe how the plasma-concentration versus time curve can be used as surrogate for efficacy and safety studies. *In vivo* bioequivalence studies based on this principle are the gold standard for demonstration of bioequivalence. During such studies, healthy volunteers receive the test and reference product in a cross-over design and the blood plasma levels of the API are measured for statistical comparison of the relevant pharmacokinetic parameters. The area under the curve (AUC) and maximum plasma concentration C_{max} are the main pharmacokinetic parameters at issue. Detailed regulatory guidance on the conduct and evaluation of bioequivalence studies of oral drug products is given in documents published by the regulatory authorities (7, 8).

According to EMA and FDA guidance, *in vivo* bioequivalence studies may be waived if an assumption of equivalence in *in vivo* performance can be justified by satisfactory *in vitro* data. A so-called BCS-based biowaiver approach is meant to reduce *in vivo* bioequivalence studies and may represent a surrogate for *in vivo* bioequivalence (7).

The introduction of the concept of 'generic' medicinal products in US and EU laws led to reduction of the need for clinical and non-clinical trials involving test animals and human subjects, thereby improving drug legislation from an ethical point of view. The biowaiver approach offers even more reduction of human testing.

From an economic and social perspective these subsequent options in the regulatory system facilitated market access and consequential availability of affordable (generally cheaper than the innovator) generic medicines. Generic pharmaceutical industry, of course, confirms these

advantages e.g. on websites like http://www.egagenerics.com and http://www.gphaonline.org. However, the actual generic market is complicated by patent laws, national health systems and reimbursement policies and last but not least, by the individual patient or consumer. The patient may perhaps be convinced of the regulatory equivalence of generics with the innovator. However, he or she is generally less happy with insurance companies translating this equivalence directly to mandatory interchangeability/substitutability of generics and brand name drugs.

1.2. Scientific context

When developing a formulation that is bioequivalent to the reference product, the goal is to achieve the same systemic availability as this reference product. The systemic availability (F_{sys}) of an active compound is determined by the product of the fraction absorbed (F_a), the fraction metabolized (F_m) and the hepatic clearance (F_h). Assuming minor metabolism in the gut, and a constant hepatic clearance independent of the formulation, the main parameter to manipulate is the fraction absorbed. In cases of relevant metabolism during gastrointestinal residence, this aspect should of course also be taken into account (9). Both the API's characteristics as well as the formulation's characteristics will influence the fraction absorbed.

API characteristics and bioavailability

Many systems to assess and classify relevant API characteristics exist. For example, partition coefficients like the octanol-water partition coefficient (logP) have a long history of use in relation to predictions on hydrophilicity and lipophilicity (10). From the 1990s on, other systematic approaches to characterize compounds were developed. Lipinski's rule of five as published in 1997 aimed at facilitating drug candidate selection in early development by combining experimental and computational data to predict the bioavailability of a compound. It predicts that poor absorption or permeation is more likely when there are more than 5 H-bond donors, 10 H-bond acceptors, the molecular weight is greater than 500 and the calculated LogP (CLogP) is greater than 5 (11). The review as compiled by Bergström *et al.* lists additional and alternative physicochemical profiling tools that are currently used to characterize the API in preclinical development (12). The authors also identify several aspects that may be improved including development of adequate *in silico* models, miniaturized methods and optimization of the *in vivo* relevance of available tools for additional characterization of APIs.

A system well-known in current regulatory context is the Biopharmaceutics Classification System (BCS) as introduced by Amidon *et al.* in 1995 in the context of drug formulation development. The BCS classifies active substances according to their solubility and permeability, based on the applied dose. It also aims at correlating *in vitro* dissolution to *in vivo* bioavailability (13).

The BCS simplified the concepts of biopharmaceutic thinking while offering possibilities of risk-based approaches to bioequivalence testing. Formulations including APIs for which the risk of bioinequivalence is considered unlikely to depend on any other mechanism than the release of the API from the dosage form, could now be exempted from the obligation to perform a comparative bioequivalence study (BCS based biowaiver). A positive comparative dissolution test is deemed sufficient.

Wu and Benet proposed a modified BCS in 2005: the Biopharmaceutic Drug Disposition Classification System (BDDCS), which categorizes drug substances using the major route of drug elimination or the extent of drug metabolism. The cut-off value for "extensive metabolism" was originally set at 50% but it was later recommended to apply 90% as a cut-off value for regulatory waivers of *in vivo* bioequivalence studies (14, 15). In 2013, Benet described how the BCS and BDDCS co-exist each with their own function. "The purpose of BCS is to characterize drugs for which products of those drugs may be eligible for a biowaiver of *in vivo* bioequivalence studies. The purpose of BDDCS is to predict drug disposition and potential drug-drug interactions in the intestine and the liver, and potentially the kidney and brain." Both systems apply regulatory definitions of solubility to differentiate between high and low solubility drugs. The definitions of permeability differ: the BCS focuses on extent of intestinal absorption and the BDDCS uses the permeability rate and relates it to the extent of metabolism (16). Current regulatory biowaiver guidance offers the option to exempt from *in vivo* testing for immediate release products containing an API with a high fraction of dose absorbed (optionally determined taking into account the extent of metabolism). Values of 85% (EMA) or 90% (FDA) are used as cut-off value to differentiate between high and low extent of absorption.

Formulation and excipient effects

Formulation effects

The planned performance of the formulation can be summarized in a quality target product profile (QTPP). The most suitable formulation starts by the characteristics of the API. In addition to the substance-based BCS or BDDCS, aspects like particle size and polymorphic form as well as potential interactions with excipients should be taken into account.

Pharmaceutical industries have developed aids for (pre-)formulation development decisions using BCS and/or other classification strategies as starting point (17-20). The review published within the IMI financed Orbito project describes some of these current strategies (12). With the new quality paradigm (ICH Q8-9-10) early involvement of pre-formulation specialists in development of the final formulation becomes more common and explicit (21).

Here again, the research on technical formulation strategies in early development and the discussion from a regulatory viewpoint need to be distinguished. Butler and Dressman proposed the developability classification system (DCS) in 2010 (22). They presented this system in a more regulatory context with reference to Quality by Design concepts and the offer of a scientific framework for discussion with regulators as to the risk of bioinequivalence.

In parallel, discussions on the concept of a drug product Manufacturing Classification System (MCS) are ongoing. As stated in the program of an event in September 2014: The MCS is intended as a tool for pharmaceutical scientists to rank the feasibility of different processing routes for the manufacture of oral solid dosage forms, based on selected properties of the Active Pharmaceutical Ingredient (API) and the needs of the formulation. The proposed MCS could be used to develop a risk assessment for manufacturing based on "ideal" ranges for API physical properties and therefore indicate how robust a manufacturing process is likely to be in relation to those properties (23).

Excipients

Excipients are relevant for the performance of the drug product in many ways. Technically, their use should lead to the foreseen product characteristics. Chemically, incompatibilities of selected excipients with the active ingredient are to be avoided (24). From a biopharmaceutic viewpoint, excipients may play a relevant role in the absorption of the active ingredient from the formulation (25, 26) and excipients may also exert physiological effects that change the pharmacokinetics of the API.

First, drug release from the dosage form may be affected. A physical effect on the stability of the polymorphic form or the effective surface are of the API may influence solubility of the API (27-29), while the dissolution rate of the active substance can be affected by disintegrants or wetting agents (30). An effect on stomach physiology can then lead to increased residence time using floating agents or bioadhesion or to delayed gastric emptying (31-34).

Modulation of GI fluids is possible by affecting pH of the gastrointestinal tract (35) or a change in the composition of the matrix in which the drug is transported e.g. through an effect on bile or mucus production. Examples of this last option have not been identified so far.

Enzymatic metabolism and physical stability determine the fraction of the dose that is available for absorption. It can be affected by excipients like nonionic surfactants and polymers (36-43) while intestinal enzymatic or chemical degradation processes could be prevented by protecting agents (44-46). Changes to GI transit and motility affect the available time for absorption: increased intestinal motility by osmotic agents like mannitol and xylitol as well as lactulose (47-51) was shown to reduce the transit times, while oleic acids and lipids have the potential to delay the transit times (52-54).

Several physical and physiological mechanisms have been described in relation to modulation of membrane transport through transcellular and paracellular transport routes. Agents like mucolytica or surfactants may non-specifically damage the intestinal mucosa. (55-57) Physically reduced microparticles or nanoparticles have shown to enhance bioavailability of heparin while lipid complex formation may also improve heparin's membrane transport; the paracellular or transcellular route depended on the applied technique (58). The paracellular route can be modulated through opening of the tight junctions; numerous potential modulators have been investigated with this purpose (59, 60). The influence of excipients on transporter-mediated absorption has lately been reviewed by Grube and Langguth and by Goole et al. (61, 62). Finally, Guan et al. described complexation of bergenin with phospholipid which was postulated to be transported across enterocytes by both passive diffusion and active transport by receptor-mediated endocytosis (63).

The interplay of individual effects of the API itself and excipients, their potential interactions and the characteristics of the final formulation resulting from the manufacturing process, will define the final bioavailability of the API. Consequently, many mechanisms by which an excipient may affect the bioavailability of an API are conceivable. The clinical relevance of each of these possibilities depends on the specific substances, their combinations and physiological context at issue. Generally, there is a lack of human pharmacokinetic data to evaluate this relevance. A public, systematic approach to classify excipient effects is also not available. In this thesis, possibilities to systematically improve our excipient understanding with regard to their potential effect on the absorption of the active pharmaceutical ingredient will be further discussed.

Models as a surrogate for in vivo bioavailability and bioequivalence testing

Excipient effects on bioavailability are best shown by *in vivo* bioavailability studies in humans. Knowledge of these excipient effects is especially relevant in the context of comparative studies: formulation A versus formulation B containing different excipients. In many situations, comparative bioavailability studies are needed for new formulations to demonstrate equivalence. Understanding of intended or unintended effects of excipients on the pharmacokinetics of a drug substance is particularly interesting from the perspective of waiving *in vivo* bioequivalence (BE) studies for oral immediate release dosage forms with systemic action.

Current EU Guidance states that BE studies for oral solutions of multisource drug products may be waived. However, a BE study should be conducted if the excipients in the dosage forms involved affect gastrointestinal transit (e.g. sorbitol, mannitol, etc.), absorption (e.g. surfactants or excipients that may affect transport proteins), *in vivo* solubility (e.g. co-solvents) or *in vivo* stability of the active substance, unless the differences in the amounts of these excipients can be adequately justified by reference to other data (7).

A BCS-based biowaiver may be considered for BCS class I and III (in the EU) drug compounds. As a general rule, for both BCS class I and III drug substances, well-established excipients in usual amounts should be employed and possible interactions affecting drug bioavailability and/or solubility characteristics should be considered and discussed. A description of the function of the excipients is required with a justification whether the amount of each excipient is within the normal range.

To study the effects of excipients on the bioavailability the pharmaceutical industry and academia apply different techniques, each with their own limitations. *In vitro* dissolution testing as a model for *in vivo* bioavailability of orally administered products was first introduced in the 1960's (64). Yet, both in EU and US specific research projects acknowledge that a lot is still unknown or can be improved. An FDA sponsored project in the US plans to publish a white paper on *in vivo* predictive dissolution testing (IPD) (65, 66). IPD is regarded as a product development tool to be applied as a basis for but not necessarily as an alternative to QC dissolution testing (67, 68).

At the same time, an EU project (see http://www.imi.europa.eu/content/orbito) aims at streamlining and optimizing the development of orally administered drug products with a strong focus to develop novel experimental and theoretical models to increase our knowledge of biopharmaceutical factors and their interplay with the dynamic gastrointestinal physiology (69).

In the context of this OrBiTo project, *in vitro* models for drug absorption testing were reviewed by Kostewicz *et al.*, (70) whereas alternative *in vivo* models were discussed by Sjögren *et al.* (26).

Kostewicz *et al.* describe how the biorelevance of the applied models remains a continuously developing study area. In addition, the *in vitro* models themselves are evolving with the development of new formulation techniques. The authors identified the need to improve the accessibility of the available 'toolbox' to facilitate useful application of the available models. Sjögren *et al.* mention how current understanding of the human GI tract and its effects on the absorption processes of an API from an oral dosage form still has its limitations, which limits the use of *in vitro* models as reliable alternative.

The drug regulatory field could benefit from improvement of the use of such models. Indeed, adequately validated models might be used as a surrogate for an *in vivo* bioavailability or bioequivalence tests in humans.The current regulatory biowaiver guidance limits itself to the first step in the GI absorption of the API from an immediate release solid oral dosage form: drug release tested using an *in vitro* dissolution model (1, 2, 7).

Animal models are commonly applied by pharmaceutical industry to test differences in drug release from pharmaceutical formulations. However, in the context of BE questions, these models do play a limited role: animal models are not approved as models for biowaivers. From a regulatory perspective, a human volunteer is the only acceptable 'model' for comparative bioavailability testing when biowaiver conditions are not met and in absence of an *in vivo in vitro* correlation of the dissolution versus plasma data.

Cell culture models for testing permeability effects are well-known while *in vitro* digestion models and enzyme interaction models also exist. However, none of these are approved to confirm bioequivalence of formulations. The gastrointestinal transit of APIs can be studied using different *in vivo* techniques, but comparative *in vitro* models validated for their biorelevance of this parameter are not known to the authors either. In conclusion, apart from the dissolution test, there is no comparative *in vitro* test model validated and approved for comparative testing of disposition effects.

The regulatory guidance is limited as to allowed (difference in) levels of excipients: as a general rule for BCS-class I and III drug substances well-established excipients in usual amounts should be employed and possible interactions affecting drug bioavailability and/or solubility characteristics should be excluded. Even in the case of Class I drugs it is advisable to use similar amounts of the same excipients in the composition of test like in the reference product. If a biowaiver is applied for a BCS-class III drug substance, excipients have to be qualitatively the same and quantitatively very similar (7). Effects on disintegration or dissolution could be tested by the pharmacopoeial models. However, no specific excipient information or other details are given on the evaluation of any test outcome.

1.3. Practical experience from both scientific and regulatory perspective

Public information on individual bioequivalence studies is scarce. Van der Meersch *et al.* screened publications on BE trials in the time period 2005-2008 and concluded that the quality of the available reports can be improved by inclusion of information on and reference to the applied guidance and acceptance criteria. The authors plead for increased transparency on such studies (71). An exploratory literature study [data not shown] indeed confirmed a publication gap in studies demonstrating bioinequivalence.

Ramirez *et al.* evaluated the outcome of 124 BE trials with a total of 80 APIs at the same study site by BCS class (72). Results not meeting the bioequivalence criteria were available for all BCS classes: 35 failed with respect to C_{max} and for 15 the AUC did not comply. For BCS class 1 about 15% of the trials failed; non-BE was always due to a difference in Cmax. A similar percentage (14%) of BCS class III failed on C_{max} and 9% on AUC. BCS class II compound failed in 50% of the cases on C_{max} and in 25% on AUC. Eight BCS class IV products were tested of which 8% failed on C_{max}. These results were comparable to those presented by Lamouche *et al.* (73).

Cristofoletti and Dressman showed the ratio of failed bioequivalence trials per BCS class as deduced from 500 BE studies in the database of the Brazilian regulatory authorities (74). The relative risk of obtaining a non-BE result was about four times lower for drugs in classes 1 and 3 of BCS or BDDCS when compared with class 2 drugs. All three publications concluded that the outcome of a bioequivalence study seems to be strongly influenced by the solubility of the drug. Cristofolleti and Dressman note that solubility seems to outweigh any effect of the extent of intestinal permeability or extent of metabolism.

On the other hand, Butler deduced from their results that dissolution testing alone can be biopredictive only for BCS class II compounds as these are the only class for which dissolution is the most relevant rate limiting factor(64). The biorelevance of the *in vitro* dissolution test conditions will determine the probability of actually detecting bioinequivalence by an in-vitro test. Indeed, Ramirez et al noted how QC dissolution methods, although not developed for this purpose, were often not sufficiently biopredictive (72). More specifically, BCS class II compound ibuprofen has been under discussion as the usual comparative biowaiver dissolution tests seemed not to be biopredictive (75, 76). With this case in mind, Tsume *et al.* presented a sub-classification of the BCS, based on the pKa of the API (acid, base and neutral) for classes II and IV which would facilitate the selection of biorelevant dissolution conditions (68).

Nair *et al.* reported the BCS class of generic drugs approved by FDA in 2001-2011 (77). They concluded that the majority of the generics for which the BCS class could be confirmed, concerned

BCS class I (42%), followed by BCS class 3 (37%) and BCS class 2 (21%). A list of common deficiencies in applications based on biowaivers was provided. Although the number of biowaivers granted was not stated, the authors hoped to promote application of the biowaiver concept, which suggests a limited number of dossiers including a biowaiver during that decade. In Europe, a limited application of the biowaiver approach was also suggested by the EMA Concept paper on BCS based biowaiver (78).

In 2015, the Global Bioequivalence Harmonisation Initiative was started by the European Federation for Pharmaceutical Sciences (EUFEPS) with the aim of reaching a global harmonized approach in bioequivalence guidance. At the conference in March, it was recognized that joint efforts from industry and regulators are needed to improve the application of the biowaiver concept. In May of this same year, the FDA published an updated draft guidance on the "Waiver of In Vivo Bioavailability and Bioequivalence Studies for Immediate-Release Solid Oral Dosage Forms Based on a Biopharmaceutics Classification System". This version is more aligned with the EMA guidance with regard to the new possibility of biowaivers for BCS class 3 substances and the pH range to be applied for biowaiver purposes. In addition, the criteria for high permeability and high solubility were adjusted.

1.4. Aims and outline of this thesis

This thesis will evaluate the regulatory conditions biowaivers from a risk-based perspective. It has the aim to contribute to a better understanding of the biowaiver concept and to support public health by identifying ways of expanding the application of the biowaiver concept.

As outlined in the introduction above, risk analysis in biowaiver context can be addressed from different perspectives, including scientific and regulatory perspective. The Biothree project originated from a regulatory question: how to improve the application of the biowaiver concept. Scientifically, the project was aligned with the Orbito project. First, the regulatory conditions were studied into more detail from a scientific perspective, in order to identify potential regulatory gaps and possibilities for extension of the biowaiver approach. Subsequently, specific studies focused on potential excipient effects with the aim to expand possibilities for biowaivers in case of differences in excipient content between test and reference product.

2. MATERIALS AND METHODS

2.1. Evaluation of the regulatory context

The regulatory guidance was evaluated in two steps. First, a review of regulatory biowaiver guidance in the US, EU and WHO was performed from a risk-based perspective. Then the effect of deviating regulatory guidance on application of the biowaiver concept was studied based on a recently introduced difference in definition of "Dose".

2.2. Study of potential excipient effects

The potential effect of differences in excipient content between test and reference product was evaluated based on two case studies. A new, top-down approach, using the data of approved products was applied to lactose.

A more traditional bottom-up approach was applied to the potential excipient chitosan hydrochloride. An *in vivo* study was performed to study the actual effect of this substance on the bioavailability of the model API acyclovir. The results of this study were compared to the outcome of *in vitro* and animal model data.

For detailed information on applied methods and materials, reference is made to the individual sections.

3. RESULTS

This chapter describes the results as obtained from the studies of various aspects of the biowaiver approach. The topics are presented as a cumulative overview of the five related publications. The subsections will cover the following topics:

Evaluation of regulatory guidance:

1. Evaluation of regulatory biowaiver guidance from a risk-based perspective:
    - Risk analysis in bioequivalence and biowaiver decisions
2. Effect of deviating regulatory definitions on biowaiver application:
    - The impact of the EMA change in definition of "dose" on the BCS dose/solubility ratio: a review of the biowaiver monographs

Potential excipient effects:

3. A top-down approach - database research:
    - Novel insights into excipient effects on the biopharmaceutics of APIs from different BCS classes: lactose in solid oral dosage forms
4. A bottom-up approach - an *in vivo* study:
    - The influence of chitosan on the oral bioavailability of acyclovir: a comparative bioavailability study in humans
5. A bottom-up approach - *in vitro* and animal studies
    - The effect of chitosan on the bioaccessibility and intestinal permeability of acyclovir

Sections 3.1 to 3.4 are in line with the published manuscripts; section 3.5 represents the manuscript in preparation for publication. Adaptations are limited to those needed to fit the format of this thesis.

3.1. Risk analysis in bioequivalence and biowaiver decisions

Based on:
Kubbinga M, Langguth P, Barends D. Risk analysis in bioequivalence and biowaiver decisions. Biopharm Drug Dispos. 2013;34(5):254-61.

Abstract

This article evaluates the current biowaiver guidance documents published by FDA, EU and WHO from a risk based perspective. The authors introduce the use of a Failure Mode and Effect Analysis (FMEA) risk calculation tool to show that current regulatory documents implicitly limit the risk for bioinequivalence after granting a biowaiver by reduction of the incidence, improving the detection, and limiting the severity of any unforeseen bioinequivalent product. In addition, the authors use the risk calculation to expose yet unexplored options for future extension of comparative *in vitro* tools for biowaivers.

Introduction

The risk of therapeutic inequivalence of two immediate release products can never be reduced to zero, even if a full clinical study would be performed. The conclusion of comparative clinical studies, *in vivo* bioequivalence studies, *in vitro* equivalence tests and biowaivers is based on statistics and scientific data that are assumed to be representative for the products at issue.

The aim of biowaiver guidance is to reduce the risk of bioinequivalence to an acceptable level. Pharmaceutical development work aims at reducing the probability of manufacturing inequivalent formulations taking into account the critical aspects of the product at issue. In this context, the absorption phase is regarded as the critical process determining the equivalence of the pharmacokinetic profiles and thereby the therapeutic equivalence of the test and reference product (13, 79).

The characteristics of the API (Biopharmaceutical Classification System, BCS, class, physicochemical characteristics) affect the probability of incidence of bioinequivalence by affecting solubility, dissolution and permeability. BCS classification combined with available data on bioequivalence studies of a certain drug may be used to consider the risk for bioinequivalence of two products. The International Pharmaceutical Federation (FIP) applies this principle in its biowaiver

monographs (80). Note that studies failing to show bioequivalence may not always or perhaps not usually be published. Therefore, publicly available data should be interpreted with caution, as these may be an underestimation of the actual experience with bioinequivalence. Literature evidence also illustrates how excipients may affect the fraction of dose absorbed by modulating disintegration, solubilization or stabilizing a specific polymorphic form thereby changing the dissolution characteristics of the API (81-83). And, although permeability modulators are not common in marketed oral drug products, these have been described in literature as well (61, 84).Furthermore, the stability and thus the accessibility of an active substance in the gastrointestinal tract could be changed by specific formulations such as nanopharmaceutic dosage forms or by addition of carboxymethyl-starch excipients and enzyme inhibitors to the formulation (44, 45). The transit time of the active pharmaceutical ingredient (API) in the gastrointestinal (GI) tract also influences its availability for absorption; it can be reduced by excipients such as sorbitol and polyethylene glycol (85, 86).

It is evident that modulation of the above-mentioned parameters can affect the fraction of dose absorbed and/or the rate of absorption and thereby increase the risk for bioinequivalence. In this paper four potential causes for bioinequivalence are identified, based on the pharmacokinetic profile of the API: dissolution of the API from the formulation (1), absorption of the API from the GI tract (2), bioaccessibility of the API for absorption (3) and transit time of the API in the gastrointestinal tract (4). In the context of bioequivalence studies, the physiological conditions are considered constant and the pharmacokinetic parameters metabolism and elimination of the API are considered to be independent on the formulation.

This article introduces well-known risk calculation equations in the context of biowaiving. Biowaiver guidance documents published by FDA, EU and WHO will be evaluated from a risk based perspective and yet unexplored options for future extension of comparative *in vitro* tools will be exposed.

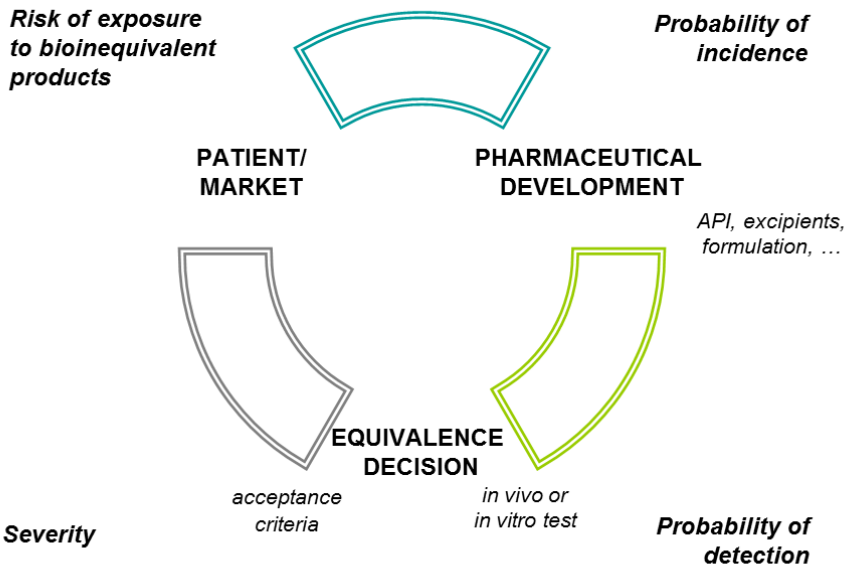


Figure 3.1.1 Evaluation of incidence, detection and severity of bioinequivalence during the life cycle of a pharmaceutical product

Material and methods

Equation (1) describes a general calculation of a risk. It could describe the risk for bioinequivalence of a generic or modified product, as determined by the probability that a bioinequivalent product is released to the market and the potential impact of such bioinequivalence to the patient exposed to it (severity).

risk = probability x severity (1)

The probability that a patient will be exposed to a bioinequivalent product is affected by the actual incidence of a bioinequivalent product and the probability that it is not detected prior to batch release, Equation (2).

probability = probability of incidence x probability of detection (2)

The incidence describes the probability of producing a product that really is bioinequivalent to the comparator. The probability of detection is a measure for the ability to detect (and reject) the bioinequivalent products prior to marketing: the higher the probability of detection, the lower the factor to be included in the risk equation.

Equations (1) and (2) can be combined to Equation (3), which is a well-known equation used to calculate a risk priority number (RPN) in Failure Mode and Effects Analysis (FMEA) (87, 88).

risk = probability of incidence x probability of detection x severity (3)

Severity refers to the potential impact of exposure of a patient to a bioinequivalent product, or the risk to public health. In this paper, Equation (3) will be used in a qualitative way. The probability of incidence and detection and severity will be ranked as 'high' or 'low' without numerical calculation of RPNs.

Results

Figure 3.1.1 shows the relation between incidence, detection and severity and the risk that a patient is exposed to a bioinequivalent product. The guidelines aim at limiting this risk to an acceptable level. Gupta *et al.* reviewed the available guidance in 2006 (89). Table 3.1.1 summarizes the conditions laid down by the current (updated) guidance documents as published by FDA, EMA and WHO (7, 90, 91). The considerations, as laid down in the guidance documents, will be interpreted by the authors in the context of the FMEA calculation: incidence, detection and severity. For the sake of clarity of the exercise, the Japanese guidance will not be reviewed into detail as Japan does not accept biowaivers for generic applications.

Detection

Table 3.1.1 Summary of BCS-based biowaiver conditions in 2014

	EMA		FDA	WHO		
FORMULATION						
API	BCS class I	BCS class III	BCS class I	BCS class I	BCS class II	BCS class III
Excipients	excipients that might affect bioavailability qualitatively the same	excipients that might affect bioavailability qualitatively and quantitatively the same	excipients in FDA-approved IR solid oral dosage forms; not large quantities of excipients that might affect bioavailability	It should be demonstrated that the excipients … are well established for use in products containing that API, and will not lead to differences with respect to processes affecting absorption, or which might lead to interactions that alter the pharmacokinetics of the API.		
Drug type	not for 'narrow therapeutic index (NTI)' drugs		not for NTI drugs and products absorbed in the oral cavity	both indication and therapeutic index are important considerations in determining whether the biowaiver based on BCS can be applied		
Dissolution formulation*	very rapid or rapid dissolution	very rapid dissolution	rapid dissolution	rapid dissolution	D: S ratio ≤ 250mL ánd rapid dissolution at pH 6.8	very rapid dissolution
COMPARATIVE IN VITRO TEST						
In vitro dissolution testing	pH 1 – 6.8 (at least pH 1.2, 4.5, and 6.8). No surfactant. Enzymes for gelatin only.		pH 1.2, 4.5, and 6.8 or simulated gastric or intestinal fluid. No surfactant. Enzymes for gelatin only.	pH 1.2, 4.5, and 6.8		
EQUIVALENCE ACCEPTANCE CRITERIA						
	Similarity (f2 calculation 50-100) or other appropriate statistical method		Similarity (f2 calculation 50-100)	Similarity (f2 calculation 50-100) or other appropriate statistical method; criterion for acceptance (maximum 10% difference between the profiles).		

*very rapid dissolution: > 85 % in 15 min; rapid dissolution: > 85 % in 30 min

Bioequivalence studies were introduced for those circumstances where a full clinical study is not considered ethical: the API is known, and its oral pharmacokinetic properties are considered to be a sufficiently predicting surrogate for clinical effects. Substitution of an *in vivo* test by *in vitro* testing (biowaiving) is meaningful only when *in vitro* testing provides a sufficiently high level of probability to detect bioinequivalence between immediate release drug products. The current guidance documents describe one comparative *in vitro* tool: dissolution testing at three pH levels, to be applied when the conditions listed in Table 3.1.1 are fulfilled. Comparative dissolution testing can be used when differences in the extent or rate of dissolution are expected to determine a potential difference in bioavailability. The API should be highly soluble and show rapid dissolution from the finished product. Rapid dissolution is considered as taking place within the timeframe of

gastric emptying. In addition, the excipients present should not be expected to have an effect on the bioavailability of the API via mechanisms other than dissolution or disintegration.

Incidence

Appropriate pharmaceutical development should limit the actual incidence (or occurrence) of bioinequivalent products; *in vivo* bioequivalence testing will not reduce it. Restrictions posed by the guidelines do however limit the incidence of bioinequivalent products that get access to biowaiving. All guidelines agree that BCS class I APIs are open for biowaivers; BCS class III substances are considered by EMA and WHO guidance as well. Products containing API's with low solubility are considered to have a higher risk of being bioinequivalent and dissolution testing may not be sufficiently discriminating. BCS class II and IV compounds are therefore not open for applying for a biowaiver according the EMA and FDA. Only WHO considers biowaivers for BCS class II compounds with specific characteristics.

Severity

The safety of biowaiving is evaluated based on the therapeutic consequences of a potential difference in bioavailability. Acceptance criteria define the accepted difference between two dissolution profiles and thus indirectly limit the severity of a potential difference. For example, the severity of any occurring and undetected bioinequivalence of two narrow therapeutic index (NTI) drugs is ranked higher than that of other drugs and the dissolution test and limits are not to be applied for such products.

Table 3.1.2. Exemplary test and reference formulations

	Composition (mg)	
	Reference	Test
API	10.0	10.0
Mannitol	50.0	60.0
Croscarmellose sodium	5.0	12.0
Microcrystalline cellulose	15.5	10.0
Magnesium stearate	2.5	3.5

f2> 50; rapid dissolution

Application of the risk calculation

We first consider the situation for highly soluble drugs. Table 3.1.2 contains a theoretical reference product and test product which have the same qualitative composition but a different quantitative composition.

When the API is of BCS class I, say propranolol, a biowaiver could be acceptable based on the guideline of the EMA, FDA and WHO, if appropriately justified. Assuming that mannitol is present in a quantity from which no effect on the bioavailability of propranolol is expected, the risk calculation would then result in low incidence (BCS class I, rapid dissolution, acceptable excipients) × high detection (dissolution testing possible) × low severity (not NTI) = OK. The overview of available FIP biowaiver monographs confirms that, for the APIs categorized as BCS class I, indeed a positive biowaiver recommendation was given (92).

If the API is of BCS class III, say acyclovir, the biowaiver is less straightforward. From the FDA side, the biowaiver will not be accepted due to the BCS classification. From the WHO and EMA perspective, the discussion would probably focus on the presence of mannitol. Is it expected to affect bioavailability? If so, a biowaiver would only be acceptable, in the case that all excipients were qualitatively and quantitatively the same. Alternatively, if it is argued that mannitol is present in a quantity from which no effect on the bioavailability of acyclovir is expected (as assumed above), a biowaiver may be considered acceptable. The risk calculation would then be as above and the discussion would concern the probability of incidence: is it acceptably low? The overview of the available FIP biowaiver monographs includes several APIs classified as BCS class III. For most of these, the authors indeed recommended a biowaiver based on literature reviews, except for those APIs that are narrow therapeutic index drugs.

Lamouche *et al.* (73) conducted a retrospective analysis on 918 bioequivalence studies to determine if the BCS may help predict in vivo bioequivalence (BE) outcome. They showed that BE failure rate was generally low and similar (~11%) for BCS class I and III compounds. In addition, solubility appeared to be the most discriminating factor with regards to BE outcome. These data confirm the approach taken by the EMA and WHO, allowing biowaivers for highly soluble BCS class I and III compounds, while requiring comparative dissolution studies as a surrogate *in vitro* test.

The situation is different for low soluble drugs. If the API is of BCS class II, a biowaiver could be considered based on the WHO guidance only. An example of an API of this class could be ibuprofen. The biowaiver monograph as published by Potthast *et al.*, indeed recommended a biowaiver for this substance (93). However, according Lamouche *et al.*, highly variable Class II compounds showed the highest BE failure rate (54%) (73). For ibuprofen, this is supported by Alvarez *et al.* who showed how ibuprofen formulations were equivalent *in vitro* but did not meet bioequivalence criteria *in vivo* (76). On the other hand, Shohin *et al.* showed that approved ibuprofen tablets may show inequivalent dissolution profiles pointing to a situation that the quality control dissolution method used for ibuprofen tablets may indeed not be biopredictive, as has been pointed out in a recent paper by Tsume *et al.* (75, 94). If a biowaiver for a BCS class II

compound is considered, additional factors such as physical characteristics of the API, excipients and formulation aspects should thus be critically evaluated and the biopredictive power of the applied dissolution media should be reconsidered. In line with these findings, the EMA and FDA consider the potential effects of excipients and formulation on the *in vivo* pharmacokinetics as insufficiently covered by a dissolution test. Translating these aspects to the FMEA calculation, the probability of detection is the risk factor of note for these APIs.

A biowaiver for an API of BCS class IV would not be accepted by any of the guidelines. The potential effects of excipients on the *in vivo* solubility and dissolution of the API from the formulation as well as potential effects on the absorption, accessibility and transit time are considered too complex to allow for a biowaiver approach. The risk of bioinequivalence, when applying the dissolution test only, is considered too high. A low probability of detection thus leads to an unacceptably high outcome of the risk calculation. It is of note that Lamouche *et al.* showed a surprisingly low failure rate (10%) for Class IV compounds when tested in *in vivo* bioequivalence studies. (73) This does not necessarily mean that the assumptions of the guideline are not correct, as their conclusion was not discussed in relation to dissolution data. Furthermore, adequate and perhaps relatively intensive product development studies could reduce the risk for a bioinequivalence of a product containing a BCS class IV API.

Regardless of the BCS classification, all guidelines exclude narrow therapeutic index drugs from biowaiving. The severity of undetected bioequivalence would be higher. In addition, *in vitro* testing is not considered sufficiently sensitive to detect bioinequivalence for narrow therapeutic index drugs and consequentially, narrow therapeutic index drugs are excluded from the option of a biowaiver. Translating this to the risk calculation means that the probability of detection of bioinequivalence of narrow therapeutic index drugs is considered too low with the currently available dissolution test ánd the severity of undetected bioequivalence is considered too high, both factors leading to an unacceptably high risk.

Discussion

A complete waiver for an *in vivo* (clinical or bioequivalence) study can be granted when a suitable *in vitro* alternative or surrogate test is available to allow *detection* of a bioinequivalent formulation. Currently only dissolution testing is described as surrogate test. The *incidence* of a bioinequivalent formulation after granting a biowaiver is limited by defining criteria for API and excipients as well as dissolution of the formulation. The *severity* of a 'passing' bioinequivalent formulation is limited

by exclusion of narrow therapeutic index drugs from the option of a biowaiver and setting appropriate acceptance criteria.

Extending the options for biowaiving may be of interest both from ethical and economic point of view (95). The authors identified several factors that could be improved in order to facilitate application of the biowaiver concept.

The bioequivalence guidelines are based on the assumption that the risk of bioinequivalence is related to the BCS classification of the active substance. To check the validity of this assumption, one would have to consider a sufficiently large but random sample of bioequivalence studies including products of all four BCS classes. Such data may be available at pharmaceutical industry or contract research organizations but reviews in public literature are scarce. Ramirez *et al.* published an overview of 124 bioequivalence studies but were not able to define differential characteristics of each class. The authors took into account the dissolution data obtained using the test dissolution methodology proposed as quality control, but pharmaceutical products with active substances from all four BCS classes showed non-BE studies (72). It is noted that not only BCS classification may be relevant, but also the composition of the formulations compared and the dissolution data of the formulations in the physiological pH range in addition to those obtained at quality control conditions. Lamouche *et al.* presented an overview of data based on 918 studies as discussed above (73). Publication of more such reviews including relevant supporting data would increase the public understanding of the BCS concept in relation to the biowaiver approach.

BCS classification involves testing of solubility and permeability and the outcome may differ depending on the conditions and calculations applied. In addition, the definition of dose used for determination of the BCS classification is ambivalent; maximum dose strength (FDA) and maximum administered single dose (EMA and WHO) are both applied. A generally accepted database on BCS classification of APIs does not exist. This thus introduces an uncertainty factor in this classification and in the risk evaluation. A public database and consensus on BCS classification would also facilitate review of available bioequivalence data and allow improved understanding of the system in the context of biowaivers.

The effect of excipients is strictly limited by the guidance documents: qualitative differences in excipients from which an effect on the bioavailability could be expected, are not accepted whereas scientific reasoning may justify larger and still safe deviations. In the above example, replacing mannitol by lactose in one of the formulations would probably not be of relevance for the bioavailability of the API. There is, however, no generally accepted source to which applicants may refer in this respect: development of a 'safe list' of excipients would be helpful. The inactive

ingredient database as published by the FDA could perhaps serve as a basis, preferably combined with information on the levels of excipients in combination with specific API's (96).

So, the application of biowaivers may be facilitated by the set-up of generally accepted databases based on public scientific data. The eligibility of substances and formulations for biowaivers may thus be clarified and the risk for bioinequivalence will be clarified through better understanding of the probability of incidence.

The probability of detection of bioinequivalence is currently only linked to dissolution testing at three pHs. This method could be improved e.g. for BCS class II APIs. It could also be considered to fine-tune the acceptance criteria. For example, the Japanese guideline adapts the criteria for f2 based on the average dissolution of the reference product: values of 42, 46 and 53 are used as differentiated acceptance criteria in relation to demonstration of similarity of dissolution curves of oral conventional release dosage forms (97). Publication of method details for approved products would facilitate application for specific API's as suggested earlier (95, 98).

The probability of detection of bioinequivalence due to factors other than solubility and dissolution is currently not covered by the guidelines. For example, the risk for bioequivalence due to effect of excipients on permeability is currently limited by restrictions on the difference in excipients between test and reference product. The development of a comparative *in vitro* permeability test could support a biowaiver for those products for which a potential difference in bioavailability may be based on a difference in permeability. Especially BCS class III substances may profit from this, as these have high solubility and low fraction of dose absorbed due to a low permeability.

Other factors for which a surrogate *in vitro* test may be envisaged are the stability and thus the accessibility of an active substance in the gastrointestinal tract and the transit time effects. *In vitro* models could be developed to compare the transit time, *in vivo* degradation and bioaccessibility of test and reference formulations. Existing models may be optimized for this purpose (99-101). The introduction of such comparative models could allow more flexibility with regard to excipient use while limiting the probability of bioinequivalence at an acceptable level and allowing a biowaiver based on *in vitro* testing.

To reduce the severity of an incorrectly applied biowaiver, narrow therapeutic index drugs are excluded. However, the guidelines do not give a clear definition of a narrow therapeutic index drug, leaving room for individual discussion. According to the EMA, it is not possible to define a set of criteria to categorize drugs as narrow therapeutic index drugs and it must be decided case by case. This seems helpful from a scientific point of view and at the same time it may restrict the

number of biowaivers in case a drug is categorized as narrow therapeutic index drug but *in vitro* dissolution testing would be sufficient. It may be possible to develop a biopredictive *in vitro* test with appropriate (tighter) acceptance criteria for narrow therapeutic index drugs as well.

Conclusion

Several authors pointed out that relaxing the biowaiver guidelines, introduction of additional comparative test methods, global harmonization and publication of requirements may facilitate the availability of reliable and affordable (generic) medicines (89, 95, 102). The aim of biowaiver guidance is to reduce the risk of bioinequivalence due to an incorrect biowaiver decision to an acceptable level. Using the FMEA risk calculation approach, the authors showed how clarification of regulatory classifications and definitions could facilitate applications for a biowaiver while still consciously controlling the risk of bioinequivalence based on scientific data. In addition, new options for surrogate *in vitro* testing are open for further investigations, offering possibilities for yet unexplored areas of potential biowaivers. Validation of such models against *in vivo* testing will show which of the options will be of practical value for biowaiving.

3.2. The impact of the EMA change in definition of "dose" on the BCS dose/solubility ratio: a review of the biowaiver monographs

Based on:
Sediq A, Kubbinga M, Langguth P, Dressman J. The impact of the EMA change in definition of "dose" on the BCS dose-solubility ratio: a review of the biowaiver monographs. J Pharm Sci. 2014;103(1):65-70.
The authors A.S. and M.K. both equally contributed to this work.

Abstract

The Biopharmaceutics Classification System (BCS) defines the solubility characteristics of an active pharmaceutical substance based on its Dose-Solubility ratio: for highly soluble drugs, this ratio is less than 250ml over a defined pH-range. Prior to the revision of the EMA guideline in 2010, the "Dose" in this ratio was consistently defined by the FDA, the EMA (formerly EMEA) and the WHO biowaiver guidelines as the highest dosage strength. However, in the revised EMA guideline the Dose is defined as the highest single dose administered according to the Summary of Product Characteristics. The new EMA criterion for highly soluble may be closer to the actual conditions of use, but it is not in line with the Dose that would be used in the *in vivo* bioequivalence study. This paper evaluates the impact on the BCS classification of the APIs of the published biowaiver monographs and discusses the consequences of the possible change in classification on biowaiver recommendations. Using the current definition of Dose by the EMA, the biowaiver recommendations for metoclopramide hydrochloride and verapamil hydrochloride are no longer valid according to EMA criteria. For prednisolone and prednisone, a re-evaluation of the biowaiver recommendation, taking into account usual dosing levels, seems appropriate.

Introduction

Since the introduction of the Biopharmaceutics Classification System (BCS) (13), most regulatory authorities have started to apply this system for bioequivalence (BE) guidance. The BCS is based on two important processes for the absorption of a drug substance, namely its solubility and hence ease of dissolution in the upper gastrointestinal (GI) tract and its permeation through the membrane wall. The BCS classifies the characteristics of these processes by categorizing these parameters as *high* or *low*. The various regulatory authorities have somewhat different criteria for categorizing the solubility and permeability. The solubility of an active pharmaceutical ingredient

(API) is in all cases defined by calculating the Dose-Solubility ratio (D/S) expressed by volume (ml), i.e. the volume sufficient to dissolve the Dose, but the Dose and the range of conditions over which the solubility is determined may differ from jurisdiction to jurisdiction.

An important application of BCS in the regulatory documents is the use of BCS in the guidances for biowaiver procedures. One of the most important criteria for deciding whether a BCS-based biowaiver is appropriate is the BCS class of the API. For instance, products containing BCS class IV APIs are excluded from the BCS-based biowaiver procedure. Additionally, products containing class III APIs cannot, as of this writing, be approved in the USA by the biowaiver procedure. In the EU and countries using the WHO criteria, products containing Class III APIs are only eligible for biowaiving if they are very rapidly dissolving. Class II APIs are only eligible for the biowaiver procedure in countries using the WHO criteria and then only in case of a weak acid which is highly soluble at pH 6.8. By contrast, Class I APIs are eligible for the biowaiver procedure in all jurisdictions which apply it (Japan, notably, is a country which does not yet allow approval of drug products using the BCS-based biowaiver procedure). In general, the regulatory authorities consider an API *highly soluble* if its D/S-ratio is less than 250ml.

The former European Medicines Agency guideline (EMEA, 2001) and the present FDA (2000) Guidance define Dose as the highest dosage strength marketed as an oral immediate release (IR) dosage form, i.e. the tablet or capsule with the highest content of API (8, 103)in. However, the revised EMA (2010) guideline defines Dose as the highest single oral IR dose recommended for administration in the Summary of Product characteristics (also known as the Prescribers' Information) (7). The WHO has a more flexible definition. If the API appears on the WHO Model List of Essential Medicines (EML), the highest dose recommended in that list is to be applied for D/S-ratio calculation. For APIs not on the EML, the highest dosage strength available on the market as an oral solid dosage form is used (104).

Since the BCS classification is an important parameter for biowaiver eligibility, it is important to unambiguously understand how the D/S ratio is calculated. To demonstrate the differences that can arise as a result of the differences in definition of Dose, we evaluated its impact on the BCS classification of the APIs for which biowaiver monographs were published up to 2011. This chapter identifies changes in BCS classification for this set of APIs and discusses the impact of the change on the API's eligibility for the BCS-based biowaiver. The results are also addressed in the context of patient use and in the framework of other regulations relating to bioequivalence.

Methods

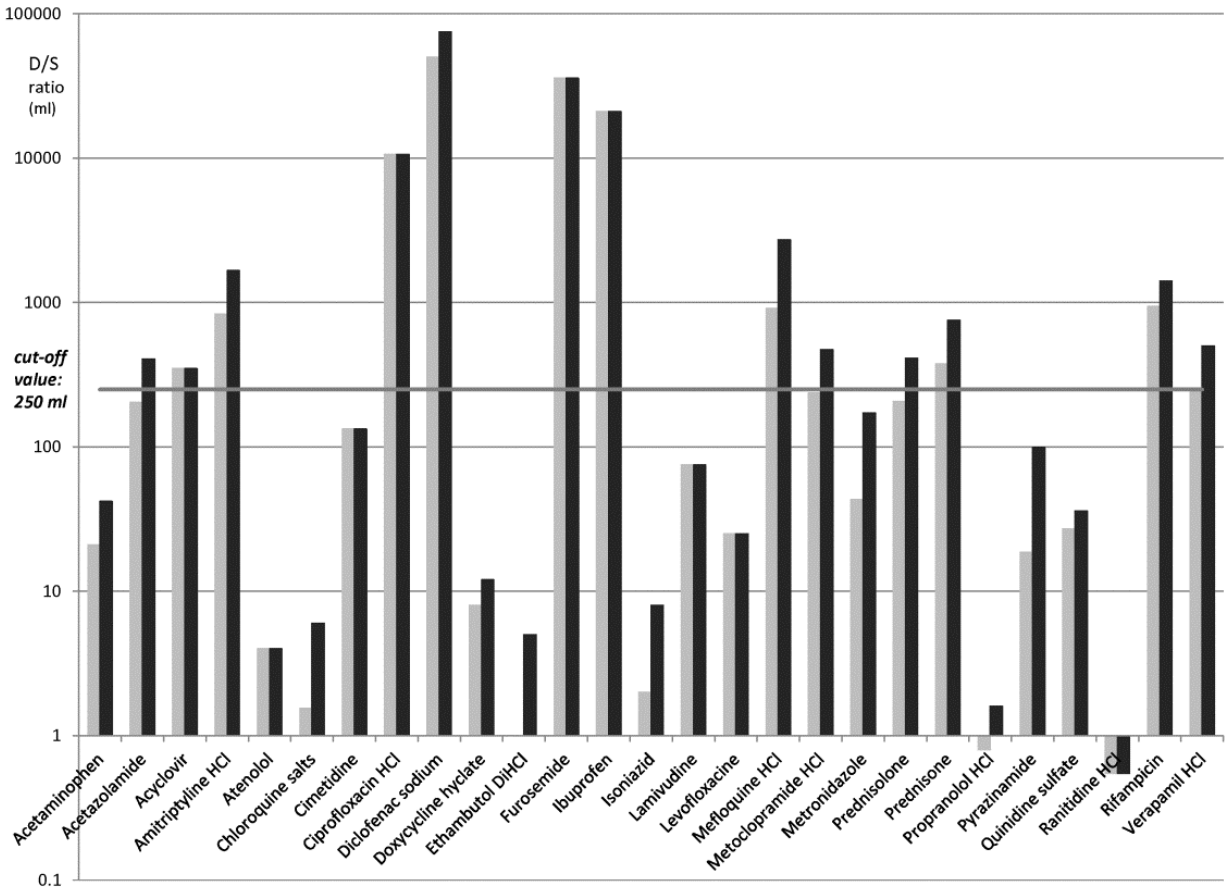
The impact of the change in definition of D on the D/S ratio and hence the BCS classification was evaluated for the 27 APIs for which a biowaiver monograph was published up to June 2011. The highest single dose administered as an immediate release (IR) oral drug product was obtained from Summaries of Product Characteristics (SmPCs) on the website of the Dutch Medicines Evaluation Board and Martindale Extra Pharmacopoeia. Where no SmPC was available from the Dutch Medicines Evaluation Board, the SmPC as published on the company's website was taken and compared with the Martindale information. The solubility value S was taken from the respective biowaiver monographs: to evaluate whether the D/S met the solubility criterion, the worst-case solubility values were used i.e. the lowest value in the pH range 1 to 6.8, the range applied by the EMA. With these values for solubility and dose, the 'new' worst-case D/S ratios were calculated for each active substance.

Results and discussion

Figure 3.2.1 summarizes the results for all APIs evaluated. Table 3.2.1 lists the 24 APIs for which the D/S ratio was recalculated. As the authors of the biowaiver monographs for lamuvidine, levofloxacin and metronidazole had already taken the different dose definition into the calculation of D/S into consideration, results for these three APIs are not tabulated.

For acyclovir, atenolol, cimetidine, ciprofloxacin, furosemide, ibuprofen, lamivudine, levofloxacin, and ranitidine the highest single dose recommended for administration in the SmPC is equal to the highest dosage strength available, so for these drugs, the BCS classification and hence the biowaiver recommendation is not altered (93, 105-112). The solubility values for acetaminophen (paracetamol), chloroquine salts, doxycycline, ethambutol, isoniazid, metronidazole, propranolol, pyrazinamide and quinidine are all very high. Thus, even though the highest single dose recommended in the SmPC is higher than the highest dosage strength available, the D/S-ratio is still beneath 250ml. Therefore, neither the BCS classification nor the biowaiver recommendation is changed for these APIs (106, 113-120).

Figure 3.2.1 D/S ratio of APIs according to the previous definition (grey bars) and the new definition (black bars). The horizontal line shows the cut-off value of 250ml.



The D/S ratio of diclofenac, mefloquine and rifampicin already exceeded the 250ml using the 2001 EMEA definition of Dose, consistent with their classification according to the FDA and WHO criteria. Therefore, the increase in the Dose associated with the new definition, leads to the D/S ratio exceeding the criterion by an even wider margin: the BCS classification of these API is thus not affected (121-124). Although amitriptyline did not meet the criterion for highly soluble at pH 7.5 (FDA criterion) at the highest dose strength, it had been decided to make a positive biowaiver recommendation based on the high solubility at pH values up to 6.8 and the risk analysis. In the EU and Martindale, current recommendations for dosing are up to 150mg/day in divided doses for ambulatory patients and 300mg for patients being treated in hospital. Under the current EMA guideline, even the highest ambulatory *daily* dose falls within the D/S criterion over the pH range 1-6.8. Thus for amitriptyline HCL, no change in the biowaiver recommendation is necessary (125).

Table 3.2.1 Overview of APIs re-evaluated based on updated EMA definition of Dose

a. Highest dose strength versus maximum single dose

Active pharmaceutical ingredient (API)	Highest dose strength (mg)	Maximum single dose (mg)	Corresponding indication
Acetaminophen[a,b]	500	1000	Severe pain
Acetazolamide[a,c]	250	500	E.g. secondary glaucoma
Acyclovir[a,d]	800	800	Herpes zoster infection
Amitriptyline HCl[a,e]	150	300	Severe depressions
Atenolol[a,f]	100	100	E.g. high blood pressure
Chloroquine salts [a,g]	155 (base)	600 (base)	P. falciparum and P. malariae infections
Cimetidine[a,h]	800	800	Gastric and duodenal ulceration
Ciprofloxacin HCl[a,i]	750	750	Bacterial infections
Diclofenac sodium[a,j]	50	75	Rheumatoid arthritis
Doxycycline hyclate[a,k]	200	300	Syphilis infection
Ethambutol DiHCl[a,l]	500	2500	Mycobacterial infection
Furosemide[a,m]	500	500	Acute edemas
Ibuprofen[a,n]	800	800	Severe pain
Isoniazid[a,o]	300	1200	Tuberculosis
Mefloquine HCl[a,p]	250	750	Acute non-immunized patients with malaria
Metoclopramide HCl[a,q]	10	20	Gastroesophageal reflux
Prednisolone[a,r]	50	100	See discussion
Prednisone[a,s]	50	100	See discussion
Propranolol HCl[a,t]	80	160	Hypertension
Pyrazinamide[a,u]	400-500	2000	Tuberculosis
Quinidine sulfate[a,v]	300	400	E.g. cardiac arrhythmias
Ranitidine HCl[a,w]	300	300	E.g. reflux oesophagitis
Rifampicin[a,x]	600	900	Brucellosis
Verapamil HCl[a,y]	120	240	E.g. arrhythmias

a) Sweetman S. 2012. Martindale: The complete drug reference. Pharmaceutical Press., at: http://www.medicinescomplete.com/mc/index.htm,last accessed 17 Jan 2013
b) SmPC Paracetamol 500mg tabletten. http://db.cbg-meb.nl/IB-teksten/h20572.pdf, last accessed 17 Jan 2013
c) SmPC Diamox Tablets 250mg. http://db.cbg-meb.nl/IB-teksten/h00643.pdf, last accessed 17 Jan 2013
d) SmPC Zovirax Tablets: http://db.cbg-meb.nl/IB-teksten/h17156.pdf, last accessed 17 Jan 2013
e) SmPC Tryptizol tablets: http://db.cbg-meb.nl/IB-teksten/h05845.pdf; recommended to take highest dose of 300 mg in divided doses., last accessed 17 Jan 2013
f) SmPC Atenolol Sandoz 100.: http://db.cbg-meb.nl/IB-teksten/h14706.pdf, last accessed 17 Jan 2013
g) SmPC Avloclor Tablets Astra-Zeneca.: http://www.medicines.org.uk/emc/medicine/2272/SPC/Avloclor+Tablets/#POSOLOGY and Nivaquine 100 mg tablets http://db.cbg-meb.nl/IB-teksten/h00303.pdf (chloroquine sulfate) , last accessed 17 Jan 2013
h) SmPC Cimetidine 800mg Teva.: http://db.cbg-meb.nl/IB-teksten/h17233.pdf, last accessed 17 Jan 2013
i) SmPC Ciprofloxacin ratiopharm 750mg.: http://db.cbg-meb.nl/IB-teksten/h25155.pdf, last accessed 17 Jan 2013
j) SmPC Diclofenac Na CF 50.: http://db.cbg-meb.nl/IB-teksten/h17258.pdf, last accessed 17 Jan 2013
k) SmPC Doxycycline 100mg PCH.: http://db.cbg-meb.nl/IB-teksten/h09519.pdf, last accessed 17 Jan 2013
l) SmPC Ethambutol Tablets 400mg (Macleods), TB134.: http://apps.who.int/prequal//WHOPAR/WHOPARPRODUCTS/TB134part4v1.pdf, last accessed 12 April 2013
m) SmPC Furosemide 500mg Teva.: http://db.cbg-meb.nl/IB-teksten/h106750.pdf, last accessed 17 Jan 2013

Table 3.2.1 Overview of APIs re-evaluated based on updated EMA definition of Dose (continued)

b. Comparison of D/S ratios

Active pharmaceutical ingredient (API)	Former highest corresponding D/S ratio (mL)	New highest corresponding D/S-ratio (mL)	Change in BCS classification?	Change in biowaiver decision?
Acetaminophen[a,b]	21	42	No	No
Acetazolamide[a,c]	203	406	Yes	No
Acyclovir[a,d]	348	348	No	No
Amitriptyline HCl[a,e]	832	1664	No	No
Atenolol[a,f]	4	4	No	No
Chloroquine salts [a,g]	1.55	6	No	No
Cimetidine[a,h]	133	133	No	No
Ciprofloxacin HCl[a,i]	10,608	10608	No	No
Diclofenac sodium[a,j]	50,000	75000	No	No
Doxycycline hyclate[a,k]	8	12	No	No
Ethambutol DiHCl[a,l]	<1	5	No	No
Furosemide[a,m]	35714	35714	No	No
Ibuprofen[a,n]	21053	21053	No	No
Isoniazid[a,o]	2	8	No	No
Mefloquine HCl[a,p]	908	2724	No	No
Metoclopramide HCl[a,q]	236	472	Yes	Yes
Prednisolone[a,r]	206	412	Yes	Yes?
Prednisone[a,s]	376	752	No	Yes?
Propranolol HCl[a,t]	0.8	1.6	No	No
Pyrazinamide[a,u]	18.6	99	No	No
Quinidine sulfate[a,v]	27	36	No	No
Ranitidine HCl[a,w]	0.55	0.55	No	No
Rifampicin[a,x]	938	1407	No	No
Verapamil HCl[a,y]	250 (pH7.3)	500 (pH7.3)	Yes	Yes

n) SmPC Ibuprofen Actavis 800mg.: http://db.cbg-meb.nl/IB-teksten/h101818.pdf, last accessed June 2011- this product is no longer marketed in the Netherlands
o) SmPC Isoniazide Apotex 200 mg.: http://db.cbg-meb.nl/IB-teksten/h52497.pdf, last accessed 30 May 2013, dose calculated assuming a body mass of 80 kg and a dose of 15 mg/kg.
p) SmPC: Lariam. http://db.cbg-meb.nl/IB-teksten/h11154.pdf, last accessed 17 Jan 2013
q) SmPC Primperan http://db.cbg-meb.nl/IB-teksten/h05250.pdf. last accessed 17 Jan 2013
r) SmPC Prednisolon http://db.cbg-meb.nl/IB-teksten/h106140.pdf last accessed 17 Jan 2013
s) SmPC Prednison http://db.cbg-meb.nl/IB-teksten/h50970.pdf last accessed 17 Jan 2013
t) SmPC Propranolol HCl 80 PCH, tabletten http://db.cbg-meb.nl/IB-teksten/h10218.pdf last accessed 17 Jan 2013
u) SmPC Pyrazinamide CF 500mg.: http://db.cbg-meb.nl/IB-teksten/h50772.pdf, last accessed 17 Jan 2013
v) SmPC Kinidinesulfaat 200 PCH.: http://db.cbg-meb.nl/IB-teksten/h50909.pdf, last accessed 17 Jan 2013
w) SmPC Ranitidine CF 300mg.: http://db.cbg-meb.nl/IB-teksten/h22509.pdf, last accessed 17 Jan 2013
x) SmPC Rifampicin Sandoz 600.: http://db.cbg-meb.nl/IB-teksten/h07191.pdf , last accessed 17 Jan 2013
y) SmPC Verapamil HCl Sandoz 120mg.: http://db.cbg-meb.nl/IB-teksten/h18015.pdf, last accessed 17 Jan 2013

For the 22 APIs considered above, the new definition of D/S by the EMA has thus no impact on the BCS classification or biowaiver recommendation for the API at all. The five remaining APIs require some additional discussion.

For acetazolamide the BCS classification is affected, as indicated in Table 3.2.1, but the biowaiver decision is not. The highest administered dose of acetazolamide is twice the highest dose strength. This leads to a shift in the classification of acetazolamide to from *highly soluble* to *not highly soluble* i.e. Class I/III to Class II/IV. Because of uncertainty about the permeability and dissolution data, the authors of the biowaiver monograph came to the conclusion that acetazolamide was not a good candidate for the biowaiver procedure (126). Application of the EMA 2010 criterion for D/S would underscore this decision.

At the highest dosage strength for metoclopramide hydrochloride, 10mg, the D/S is 236ml. The maximum single dose recommended in the SmPC, 20 mg, leads to a "new" D/S of 472ml, considerably higher than the cut-off value of 250 ml. According to the EMA guideline, metoclopramide would be reclassified as a class IV drug and therefore would not be eligible for a biowaiver in European jurisdictions (127).

Similarly, while the highest dosage strength for verapamil hydrochloride**,** 120 mg, leads to a D/S≈250ml, the highest recommended single dose is 240 mg, leading to a D/S≈500ml. Thus, verapamil, like metoclopramide, would no longer be eligible for a biowaiver according to the EMA guideline (106).

For prednisolone, the daily dose can vary over a large range: according to Martindale usual oral doses range from 2.5 to 60 mg daily in divided doses, as a single daily dose after breakfast, or as a double dose on alternate days. The maximum dosage strength commercially available in Europe was 50mg according to the biowaiver monograph. At 100 mg, which would be an unusually high dose, the D/S ratio exceeds the cut-off of 250ml, formally rendering it ineligible for a biowaiver, even though it is "highly soluble" over the usual, lower dose range. The situation is similar for prednisone, noting that it is less soluble than prednisolone and thus at the same dose, will have a less favorable D/S ratio. Strictly adhering to the D/S ratio and considering a maximum single dose of about 100 mg, the 250ml threshold is exceeded and prednisone would formally fail to qualify for a BCS-based biowaiver.

In the biowaiver monographs (123, 128), it had been argued that the higher doses may not be the most clinically relevant ones to apply to the D/S ratio calculation, noting that when these APIs are given at the higher doses, these are often split up over the day rather than being given as a single

dose. Basing the calculation on the more commonly applied lower doses would keep the option of biowaiving open for prednisolone and perhaps also for prednisone.

So, the BCS classification of four of the 27 APIs considered was changed when the current EMA definition of Dose was applied. The biowaiver recommendations for two of these are no longer valid according to EMA criteria, one remains negative. For the fourth one, prednisolone, a re-evaluation of the biowaiver recommendation seems appropriate, which may be considered for related API prednisone as well. More recent biowaiver monographs that already took account of the current EMA guideline, identified quinine sulfate as an additional API for which BCS classification may depend on the regional requirements (129). These examples illustrate the relevance of a case-by-case review of biowaivers, especially for substances with borderline solubility characteristics.

It is of note that the new definition of Dose in the EMA regulatory guideline for biowaiving is based on the "worst-case" situation that might occur in clinical practice. The examples show that, quite often, the the maximum single dose recommended for administration is twice as high (or more) than the highest dose strength available on the market. At the highest administered single dose, there will be the greatest level of challenge for the entire dose to be dissolved in the fluids available. The fluid volume used in the BCS calculation is based on fasted state administration. Depending on the recommendation for conditions of administration, this may also be a "worst-case" situation, since when the drug product is administered with or after a meal, volumes available in the stomach will often be considerably higher than the 250 ml used for the calculation. With the EMA definition, access to biowaiving has thus become more conservative, with the result that APIs with borderline solubilities may be transferred from BCS class I to class II or from class III to IV, rendering them ineligible for the biowaiver procedure.

Interestingly, the new definition of Dose is not in line with the dosing requirements for *in vivo* bioequivalence studies, as set out in the very same EMA 2010 guideline. In the section of the guideline addressing *in vivo* studies, it states that these are generally to be carried out with the highest *dose strength* of API commercially available. The overview of comments on the draft guideline clarifies that this was so decided for feasibility (practical and ethical) reasons, although the highest administered dose was originally preferred from a scientific point of view (130). Likewise, in the section dealing with biowaivers for lower doses, it is expected that a bioequivalence study has been carried out at the highest dosage strength, not at the highest single dose recommended in the SmPC. Considering that the biowaiver procedure is clearly to be regarded as a surrogate for an *in vivo* bioequivalence study, it appears that the different

recommendations for the dose to be used in different sections of the guideline are somewhat inconsistent.

The discussion of the deviation of the EMA Dose also raises the question of what is actually the relevant single dose. As illustrated by the case of prednisolone and prednisone, the situation can arise that just a few of the indications or a loading dose could require an exceptionally high dose, whereas for most indications and/or for long term therapy a much lower dose would be appropriate. To select the appropriate dose for calculation of the D/S ratio, one could take into account the prevalence of the various indications to assess how frequently the API would be administered at an exceptionally high single dose level. Another aspect of this risk analysis would be the environment (ambulatory or hospitalized) in which the indication is usually treated. The prevalence of the indication combined with an evaluation of the risk of using a bioinequivalent formulation for that specific indication could be used to define 'unusual' and 'usual' doses, as illustrated above.

Of course, the regulatory consequences of such a risk evaluation on a generic application would need to be taken into account. The bioequivalence guidelines are in principle aimed at obtaining therapeutic equivalence of reference and test product at all claimed indications, including those with for which several doses are administered together. If conclusions are made based on a lower dose, a risk of undetected lack of equivalence for the higher doses will exist. However, the current EMA guideline, as well as the FDA and WHO guidance documents, already implicitly accept this risk as negligible since they all recommend that the in vivo bioequivalence study should in general be conducted at the highest dosage strength, not the highest recommended single dose.

Diverging biowaiver recommendations in the regions do not facilitate the application of biowaivers by pharmaceutical industry in daily regulatory practice. For this and the foregoing reasons, it seems that the dose definition used by FDA and the WHO is more straightforward to implement and is more consistent with the bioequivalence guidelines in general, whose intent after all is to test the therapeutic equivalence of two given drug products.

Conclusion

The change in definition of the Dose, and hence D/S in the BCS classification calculation by the EMA has an impact on the BCS-based biowaiver recommendation for four of the 27 APIs examined. With the change in definition of Dose made by the EMA, the biowaiver recommendations for metoclopramide and verapamil are no longer valid in European jurisdictions. For prednisolone and

perhaps also for prednisone, a re-evaluation of the biowaiver recommendation, taking into account usual dosing levels, would be appropriate.

The new definition of Dose in the EMA regulatory guideline for biowaiving is based on clinical considerations. However, this definition is not yet applied in other bioequivalence guidelines nor is it in line with the dose definition for *in vivo* bioequivalence studies. It would be helpful if the regulatory authorities would clarify these aspects.

3.3. Novel insights into excipient effects on the biopharmaceutics of APIs from different BCS classes: lactose in solid oral dosage forms

Based on:
Kubbinga M, Moghani L, Langguth P. Novel insights into excipient effects on the biopharmaceutics of APIs from different BCS classes: Lactose in solid oral dosage forms. Eur J Pharm Sci. 2014;61:27-31.

Abstract

Excipients encompass a wide range of properties that are of importance for the resulting drug product. Regulatory guidelines on biowaivers for immediate release formulations require an in depth understanding of the biopharmaceutic effects of excipients in order to establish bioequivalence between two different products carrying the same API based on dissolution tests alone. This paper describes a new approach in evaluating biopharmaceutic excipient effects. Actually used quantities of a model excipient, lactose, formulated in combination with APIs from different BCS classes were evaluated. The results suggest that companies use different (relative) amounts depending on the characteristics of the API. The probability of bioinequivalence due to a difference in lactose content between test and reference products was classified as low for BCS class I APIs and medium for BCS class II and III APIs, whereas a high probability was assigned to the combination of lactose and BCS class IV APIs. If repeated for other excipients, this retrospective, top-down approach may lead to a new database and more widespread applications of the biowaiver approach.

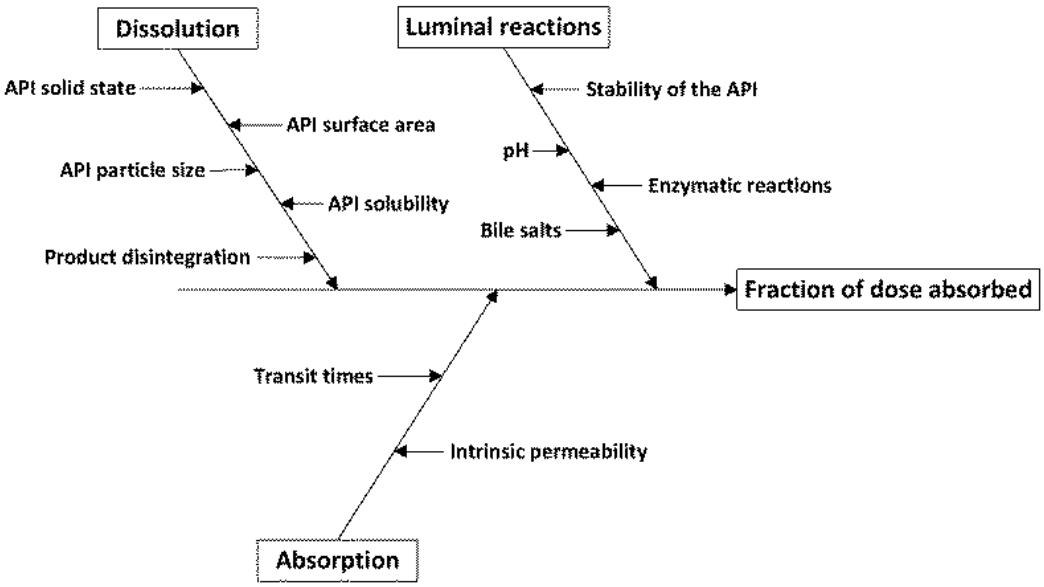
Introduction

Excipients encompass a wide range of properties that are of importance for the resulting drug product. The properties of the final dosage form, such as bioavailability and stability of the active pharmaceutical ingredient (API) are highly dependent on the excipients chosen and their concentrations (24, 25). No longer can excipients be regarded simply as inert or inactive ingredients and a detailed knowledge of these materials is essential for formulators throughout the world.

European and US regulatory guidelines on biowaivers for immediate release formulations require an in-depth understanding of the biopharmaceutic effects of excipients in order to establish bioequivalence between two different products carrying the same API based on dissolution tests

alone (7, 8, 103). Figure 3.3.1 summarizes biopharmaceutic processes that may be affected by excipients. The guidance documents mention some critical excipients like sweeteners or surfactants. However, many excipients are not mentioned and new mechanisms for potential interactions are still being discovered (47, 131-138).

Figure 3.3.1: Biopharmaceutic processes that may be modulated by excipients



There is a frequent lack of well-controlled studies regarding modulation of biopharmaceutic drug properties by excipients**.** Scientific literature describing the effects observed in laboratories, in vitro studies, animal studies and mechanistic studies is available (**bottom-up approach**). However, a systematic evaluation of these data versus the clinically relevant effects in (approved) medicinal products is less common and literature data confirming unwanted excipient effects in in vivo human studies is scarce.

The **top-down approach** presented in this paper involves retrospective analysis of approved generic drug products containing different amounts of the same excipient. Lactose was used as a

model excipient since it is commonly applied and information on its quantitative content is publicly available from the Summary of Product Characteristics (SmPC). This paper illustrates how information on the actually applied ranges of an excipient like lactose may be used for improved biopharmaceutic understanding in pharmaceutical product development.

Methods

Literature

Literature evidence on the potential effects that lactose may have on the bioavailability of active substances from solid oral dosage forms was collected using Pubmed and Scopus.

Product selection

Immediate release solid oral dosage forms (tablets, film-coated tablets and capsules) containing lactose and approved after the enforcement of the relevant bioequivalence requirements (January 2002) were selected from the database of the Dutch Medicines Evaluation Board (MEB). From the resulting lists, a random selection of APIs was made, representing all four BCS classes. The BCS class was determined using public literature and the database published by TSRL(www.tsrlinc.com).

Lactose content: data collection

The quantitative amount of lactose per product was obtained from paragraph 2 of the SmPC, as available on the website of the MEB (www.cbg-meb.nl) in September 2013. The lactose ranges were then grouped per API, using all available product strengths for all available manufacturing authorization holders. These data were grouped per BCS class, taking the minimum and maximum values found.

The differences in lactose content between the generic products and the corresponding innovator products were determined based on the highest comparable dose strengths. The absolute difference in lactose content in milligrams and the relative lactose content compared to the selected dose strength of the API (set as 100%) were calculated. Where differences between the available dose ranges for the innovator and the generics or between the different ranges of generic products exist, the highest matching dose strength was used for comparative evaluation.

Results

Lactose

Lactose is a well-known excipient, widely used as diluent, filler or bulking agent in solid oral dosage forms. Various grades are available, with different physical properties that allow selection of the most appropriate grade for a specific formulation (139-142). In view of chemical interactions, lactose cannot be used in combination with all APIs or excipients (24, 142). Literature data show potential biopharmaceutic effects of lactose in the field of solubility and dissolution of APIs (143-147). Effects of lactose on the permeability of an API were not reported when tested, nor were effects on luminal reactions or transit time of specific APIs reported in literature (148). Lactose intolerance may cause gastrointestinal and systemic effects and affect an individual patient's preference for a specific product (without lactose) (149, 150).

Lactose ranges per BCS class

The database search resulted in 1103 tablets, 1940 film-coated tablets, and 208 capsules authorized since 2002. A random selection of 13 APIs was made, representing all four BCS classes and covering 302 generic products. The SmPC of 268 of these products contained quantitative information on lactose content. Table 3.3.1 summarizes the ranges of lactose per BCS class. The ranges for the BCS class I and class III APIs overlap almost completely (1-254 and 4-232mg respectively). The observed values for the BCS class IV APIs are lower: 34-171mg, whereas the absolute ranges for BCS class II APIs are higher than for the other BCS classes, up to 576mg.

Table 3.3.1: Quantity of lactose in generic products

BCS class	API	Nr of groups[a] (nr of products)	Range per API (mg)	Range per BCS class (mg)
I	Amlodipine	4 (8)	140 -151.60	1 - 254
	Bisoprolol	6 (26)	1.26 -136	
	Enalapril	3 (8)	78 -253.60	
II	Clozapine	3 (7)[b]	32.44 -281.62	32 - 576
	Loratadine	3 (3) [b]	62.5 - 75	
	Nevirapine	3 (3)	168 - 464	
	Simvastatin	23 (78)	35 -576.24	
III	Capecitabine	16 (36)	7 - 68.95	4 - 232
	Fluconazole	7 (27) [b]	16.6 -210	
	Levofloxacin	3 (6)	3.6 -26.54	
	Losartan	18 (57)	4.5 - 231.6	
IV	Azathioprine	3 (6)#	34.36 -116	34 - 171
	Allopurinol	1 (3)	57 - 171	

[a] Definition of group: Range of strengths with the same product name e.g. the amlodipine Mylan 5 mg and 10 mg tablets are considered as one group. This number generally corresponds to the number of different manufacturing authorization holders.

[b] Excluded are 12 clozapine products, 8 loratadine products, 10 fluconazol products and 4 azathioprin products which were selected but did not mention lactose content in the SmPC.

Comparison of generics and innovator

Table 3.3.2 provides comparative data for the highest comparable dose strength. The maximum absolute difference of lactose content between the generics and the respective innovators for BCS class I, II and III ranges from 145 to180mg. The maximum difference for the BCS class IV compounds is lower: 5-21 mg. Expressed in percentage, the maximum difference is infinite for two of the three BCS class I substances (the innovators do not contain lactose). The maximum difference varies from 6-47% for BCS class II APIs and from 52% to infinity for the four BCS class III APIs considered. The percentage difference for BCS class IV APIs azathioprine and allopurinol is within the range of 7-14%.

The two last columns of Table 3.3.2 show the relative lactose in percent to that of the dose of the API: varying from 0 to 1452% in BCS class I products, 84-750% for BCS class II, 0-232% for BCS class III and 50-148% for BCS class IV.

Table 3.3.2: Quantity of lactose in generics compared to the innovator

BCS class	API	Highest dose strength (mg)	Nr of generic products	Lactose in generic (mg)	Lactose in innovator (mg)	Max. difference (mg)	Max. difference (w/w % of innovator lactose content)	Rel. lactose content in generic (w/w % of API dose)	Rel. lactose content in innovator (w/w % of API dose)
I	Amlodipine	10	4	140 - 145.2	-	145	∞	1452	0
	Bisoprolol	7.5[a]	3	1.77-30	-	30	∞	24 - 400	0
	Enalapril	20[b]	1	159	154	5	3.2	795	770
II	Clozapine	100[c]	2	129.76 - 140.8	192	62	32	130 - 141	192
	Loratadine	10	3	62.5 - 75	71.3	9	13	625 - 750	713
	Nevirapine	200	3	168 - 464	318	150	47	84 - 232	159
	Simvastatin	80	13	525.84 - 576.24	565.8	40	7	657 - 720	707
III	Capecitabine	500	16	25 - 68.95	52	27	52	5 - 14	10
	Fluconazole	200	7	66.4 - 210	198.82	132	67	33 - 105	99
	Levofloxacin	500	3	7.2 - 26.54	-	27	∞	1-5	0
	Losartan	100	17	18 – 231.6	51	180	353	18 - 232	51
IV	Azathioprine	50	1	68.72 - 70.5	74	5	7	137 - 141	148
	Allopurinol	300	1	171	150	21	14	57	50

[a] Bisoprolol generics are available up to 10 mg, while the innovator Emcor film-coated tablets are currently available up to 7.5 mg. The comparison is made based on the common highest strength. Note that some generic 5 and 10 mg bisoprolol products contain lactose up to 136 or 131 mg respectively. [b] Enalapril generics are available up to 40 mg, containing up to 253,60 mg lactose, while the comparable Renitec innovator tablets are currently available up to 20 mg. [c] Clozapine generics are available in the range of 25 mg to 200 mg. The innovator product Leponex is currently only available in 25 and 100 mg strengths.

Discussion

Lactose as potential bioavailability modulator

Literature data identify lactose as a potential solubility or dissolution modulator. Individual considerations or preferences for a product without lactose, e.g. due to lactose intolerance, are not taken into account in the context of (population) bioequivalence questions.

As described earlier, the probability that a patient will be exposed to a bioinequivalent product is determined by the actual incidence of a bioinequivalent product and the probability that it is not detected prior to batch release (151). The comparative dissolution test described in regulatory guidance is suitable to detect the potential effects of lactose identified. Therefore, the probability of detection of bioinequivalence due to a difference of lactose content is considered high (low probability number). For lactose, development of additional comparative in vitro test methods does not appear necessary.

Lactose ranges per BCS class

The observed range of lactose (1 – 576mg) is considered valid as it is well within the maximum potency values listed for immediate release solid oral dosage forms in FDA's Inactive Ingredient Database (35.19 - 1020mg) and the average of the maximum contents of the individual APIs listed is 212mg compared to 256mg of the relevant products in that database (96).

Lactose is present in products from all BCS classes. The relatively high upper value used with BCS class II APIs may be due to formulation strategies that aim at aiding the dissolution of the low solubility of this class, as expected based on literature. Overlapping ranges do not suggest a difference in formulation strategies for BCS class I and III APIs. For BCS class IV APIs, no difference compared to the other three classes is identified either.

Comparison of lactose content of generics and innovator

The highest dose strength was used as this strength is generally used in a bioequivalence study. The relative content of lactose vs. the API is usually the same or very similar for a dose range from the same manufacturer. Moreover, in case of a dose-dependent effect, the highest strength represents the worst-case situation.

The variability and overlap of the absolute and relative differences in lactose content between generics and innovator suggest limited dependence of the BCS class. The large differences observed for BCS class I and III APIs suggest a low criticality of any difference in lactose content between formulations containing APIs from these classes, as expected for highly soluble drugs. The data for BCS class II and IV APIs show smaller relative differences, which may be due to the potential effects on the solubility and/or dissolution of the API.

Relative lactose content related to the dose of the API

The relative lactose content in percent compared to that of the dose of the API suggests a decrease with increasing BCS class. However, the dose strengths of the BCS class I APIs are relatively low, whereas higher values are e.g. observed for BCS class III APIs. The values thus seem to largely depend on the absolute value of the dose of the API and confirm absence of a dose-dependent effect of the excipient.

Probability of bioinequivalence due to lactose per BCS class

Table 3.3.3 summarizes the biopharmaceutic classification of lactose. The probability of bioinequivalence of an immediate release solid oral dosage form containing a BCS class I API and lactose is classified as low, due to low occurrence. A change in lactose content of 145 mg or 1452% did not affect the bioequivalence. Lactose levels up to 254 mg did not show a relevant effect on bioavailability, irrespective of the lactose content of the innovator and the dose of the API. In case of larger differences, comparative dissolution studies seem sufficient to support any change.

The probability of bioinequivalence of a formulation containing a BCS class II API and lactose is classified as medium. The absolute and relative content of lactose with respect to the API were both found to be quite high. However, the relative difference between generic and innovator remained at 47% suggesting limited freedom of changes, translated to medium probability of occurrence of bioinequivalence. Lactose seems to be usable within a range of about X ± 150 mg, where X denotes the absolute amount of lactose used in the innovator product. Comparative dissolution studies seem advisable to support any change.

Table 3.3.3 Probability of bioinequivalence due to lactose

Probability of bioinequivalence	BCS class I	BCS class II	BCS class III	BCS class IV
P (Occurrence)	Low	Medium	Medium	High
P (1-Detectability)	Low	Low	Low	Low
P (Bioinequivalence)	**Low**	**Medium**	**Medium**	**High**
Conditions				
Max Q	254mg	576mg	232mg	171mg
Max Δref-test	145mg or ∞%	150mg or 47%	180mg or ∞%	21mg or 14%
Max rel. content	1452%	713%	232%	148%
***In vitro* dissolution test**	No	Yes	Yes	Yes
BE study	No	No	No	No

Explanation of terms:

- P (Occurrence) : probability that a bioinequivalent product is manufactured
- P (1-Detectability) : probability that bioinequivalence is *not* detected by the selected detection method (e.g. in vitro dissolution or in vivo bioequivalence study)
- P (Bioinequivalence) = P (Occurrence) * P (1-Detectability): probability of releasing a bioinequivalent product
- Max Q : maximum quantity used in bioequivalent product
- Max Δref-test : maximum difference observed between quantity used in generic (test) product and corresponding innovator (reference) product
- Max rel. content : maximum percentage of lactose relative to the dose of the API
- *In vitro* dissolution test: need to perform a comparative dissolution test if quantity of lactose is changed within aforementioned ranges
- BE study: need to perform a bioequivalence study to support a change of lactose within the aforementioned ranges

The probability of bioinequivalence of a formulation containing a BCS class III API and lactose is considered of medium level as well. Lactose is not known to affect the low permeability of this class of APIs and differences up to 180 mg did not result in bioinequivalence. The maximum quantity used (232 mg) and the maximum relative content compared to the dose of the API (232%) do not directly point to an increased probability of bioinequivalence either. However, two of the four products showed an absolute difference of only 27 mg compared to the innovator and the relative contents do generally not differ to a large extent. Considering these limitations, the probability of occurrence of bioinequivalence is rated as medium and comparative dissolution studies seem advisable to support any change.

The probability of bioinequivalence of a formulation containing a BCS class IV API and lactose is classified as high, always requiring additional justification in the form of relevant in vitro dissolution tests. The lactose contents varied within a small range and the absolute values were lower than for the other three classes. In addition, the differences with the innovator were below 20% both compared to the innovator content as well as to the API dose. The limited data

considered thus provide insufficient supporting evidence of safety of differences in lactose between innovator and generic.

Evaluation of the presented methodology

The applied methodology combines knowledge contained in the databases of approved medicinal products with literature evidence for evaluation of critical biopharmaceutic effect levels of specific excipients. This data mining is more specific than FDA's database on inactive ingredients, which lists levels of excipients for specific routes of administration and dosage forms, but does not mention the API. It is also of additional value to the FDA Guidance for industry on Scale-Up and Postapproval Changes (SUPAC) of immediate release solid oral dosage forms, which prescribes comparative studies depending on the type of excipient and the total drug product weight. E.g. the difference of 145.2 mg observed for BCS class I APIs exceeds the criterion of 10% tolerable variation of lactose (as a filler), while there seems to be no added value of a confirmatory bioequivalence study (152). The approach is of complementary value to FIP's biowaiver monographs, which list excipients in multisource drug products with the API at issue, but do not address specific excipient levels in relation to bioequivalence.

The methodology focuses on the probability of bioinequivalence due to differences in lactose content between two supposedly bioequivalent products. The risk of bioequivalence was previously defined as *probability × severity* (151).The severity of any occurring bioinequivalence and the assessment of the risk related to a difference in AUC and/or C_{max} depends on the therapeutic characteristics of the API and is to be evaluated case-by-case.

Conclusion

Progress in biowaiver opportunities requires the definition of critical biopharmaceutic excipient parameters affecting drug bioavailability (including regulatory acceptable preclinical test systems) and safe levels of excipients in drug products need to be defined. The methodology applied in this paper could be extrapolated to more products and other excipients, building a database of biopharmaceutic understanding of excipients. This may lead to more widespread applications of the biowaiver approach as information on biopharmaceutic role of excipients will increase formulation opportunities and limit unsuccessful trial-and-error approaches.

3.4. The influence of chitosan on the oral bioavailability of acyclovir – a comparative bioavailability study in humans

Based on:
Kubbinga M, Nguyen MA, Staubach P, Teerenstra S, Langguth P. The influence of chitosan on the oral bioavailability of acyclovir-a comparative bioavailability study in humans. Pharm Res. 2015. Jul;32(7):2241-9.

Abstract

Purpose: The effects of chitosan hydrochloride on the oral absorption of acyclovir in humans were studied to confirm the absorption enhancing effects reported for in vitro and rat studies, respectively.

Methods: A controlled, open-label, randomized, 3-phase study was conducted in twelve healthy human volunteers. Zovirax 200 mg dispersible tablets co-administered with doses of 400 mg and 1000 mg chitosan HCl were compared with Zovirax only.

Results: The expected increased absorption of acyclovir was not observed. On the contrary, mean area under the plasma concentration-time curve ($AUC_{0\text{-}12h}$) and maximal plasma concentration (C_{max}) decreased following concomitant chitosan intake (1402 versus 1017 and 982.0 ng·h/ml and 373 versus 208 and 235 ng/ml, respectively). In addition, T_{max} increased significantly in presence of 1000 mg of chitosan from 1 to 2 hours.

Conclusions: The results of this study in human volunteers did not confirm an absorption enhancing effect of chitosan. Reference values were comparable to literature data, whereas addition of chitosan resulted in significant opposite effects on C_{max}, T_{max} and AUC. Additional studies are needed to investigate the cause of the discrepancy. The observed variability and complex potential interactions may complicate the use of chitosan HCl in oral pharmaceutical formulations.

Introduction

Permeation enhancement has been a research topic in pharmaceutics for decades. Increasing the absorption of drugs with low permeability may for instance aid the reduction of the variability in bioavailability as well as reduce the administered dose of an active substance. Examples of

absorption modulators are acetylcystein, supposedly acting via reduction of the mucous layer by disrupting disulfide bridges; surfactants, polymers and chelating agents potentially interfering with the tight junctions, and substances interfering with absorption or efflux transporters (56, 61, 153).

Chitosan and its derivatives have been described as potential permeability enhancers acting via a disruptive effect on the tight junctions between the epithelial cells (59, 154-158). Chitosan is a heteropolysaccharide derived from natural source; characterized by variable molecular weights, degrees of deacetylation (DD) and salt forms (159).

Acyclovir is a BCS class III substance that is predominantly absorbed via the paracellullar route and has a low and variable bioavailability of 10-30% (160, 161). Some studies suggest the existence of a saturable carrier system or a limited absorption window. Most of the drug is renally excreted in untransformed state (162, 163). Chitosan was shown to increase the *in vitro* permeability of acyclovir across Caco-2 monolayers and the absorption of acyclovir in the rat (164-166). In the absence of clinical studies in humans, the translation of these effects in cell culture and animal models to humans currently remains unclear. The aim of the study presented here was to test the effects of chitosan hydrochloride on the oral absorption of acyclovir in human volunteers.

Materials and methods

Materials

Zovirax 200 mg dispersible tablets (GlaxoSmithKline, Austrian license number 1-18043) were used as reference product. Chitosan hydrochloride (chitosan HCl) of pharmacopoeial quality was obtained from Heppe Medical, Halle; degree of deacetylation (DD) 93.05%, viscosity 1% in water at 20°C 5.9 mPas, molecular weight (MW) 30-400kDa. It was dispensed into quantities of 400 mg and 1000 mg and labeled by Löwen Apotheke in Hochspeyer, Germany. The powders released by the Department of Pharmaceutical Technology and Biopharmaceutics of the Johannes Gutenberg University in Mainz, Germany. Acyclovir reference material was purchased from Fagron. All other materials were purchased from Sigma Aldrich.

Study design

A controlled, open-label, randomized, 3-phase study was conducted at the Clinical Research Center of the University Hospital in Mainz, Germany. The clinical trial protocol (EudraCT-Nr. 2010-023882-22) was approved by both the relevant Ethics Committee (Landesärztekammer Rheinland-Pfalz)

and the German competent authorities (Bundesinstitut für Arzneimittel und Medizinprodukte, BfArM) and the study was performed in accordance with the Declaration of Helsinki. Adult, male and female healthy volunteers were included after a health check based on interview, blood pressure, blood parameters and ECG. Taking into account the variation coefficients as presented previously by Vergin *et al.* in a bioequivalence study with 200 mg acyclovir products and considering an estimated minimum effect size of 30% as potentially clinically relevant, a number of twelve volunteers was selected for this exploratory study (167). Participants did not take part in another clinical study in parallel and did not take part in one within the preceding 90 days. Other exclusion criteria were: alcohol abuse or medication dependency in anamnesis, known hypersensitivity to study medication, active liver disease or unexplained increased levels of serum transaminases, intake of prescription drugs in the last 1-2 months, a recent (90 days) history of cytomegalovirus or systemic herpes infection(s) or recurrent systemic infections of herpes viruses, pregnancy and lactation, renal dysfunction. The subjects received Zovirax on the first trial day and were then randomly assigned to either the sequence 1000mg followed by 400 mg chitosan HCl or vice versa. This resulted in a 2-treatment (400 vs 1000 mg chitosan HCl), 2-sequence crossover design with pretreatment by Zovirax. This study design allowed evaluation of the pharmacokinetics of acyclovir, without potential interference of chitosan. A wash-out phase of 7 days was applied for each treatment.

Zovirax tablets were used as reference and the same tablets, co-administered with a known quantity of chitosan HCl were used as test 'formulations'. All products were dispersed in 100 mL water and administered with another 150 mL, resulting in a total volume of 250 mL water. Start concentrations of 1.6 g/L (0.16%) and 4.0 g/L (0.4%) chitosan HCl were thus obtained, in line with those described in literature (0.1%-0.5%) (164, 165). The maximum chitosan dose was limited to 1 g in view of the practical feasibility of oral intake of the quantity as a single dose and the related limited relevance of higher quantities as a potential excipient in an actual solid oral dosage form.

Subjects entered the studies fasting for at least 9.5 hours. The first meal was offered 4 hours post-dose. The medication was administered with a total of 250 mLwater; subjects had access to more water from 1 hour post-dose on and consumed at least 1.4 L mineral water during the 12 hour trial. Blood samples were collected prior to administration of the product(s) and at 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 6, 8 and 12 hours after dosing. Blood samples were centrifuged for separation of plasma and stored at -20°C or lower prior to analysis.

HPLC assay

Plasma samples of 500µl were prepared for analysis by solid phase extraction, using Oasis HLB 1cc cartridges (30mg) supplied by Waters. The column was washed with 500ml water and then eluted with 500µl acetonitrile. The extract was centrifuged at 14000 rpm for 10 minutes at -5°C and the concentration of acyclovir was determined by LC-MS/MS using a sample injection volume of 10 µl. The bioanalytical method was modified from previously reported methods (165, 168, 169). Validation data are included as supplementary material. The HPLC consisted of a Prontosil C18; 100*2,00mm; 5µm column, using an Agilent 1100 LC binary pump. A gradient elution was used with mobile phase A and B where A consisted of 15mM ammonium acetate + 0.1375% formic acid at pH 3.5 and B was acetonitrile + 0.1375% formic acid. Details of the gradient:

Step	Total Time(min)	Flow Rate(µl/min)	A (%)	B (%)
0	0.00	300	97.0	3.0
1	0.50	300	97.0	3.0
2	0.60	300	5.0	95.0
3	1.20	300	5.0	95.0
4	2.00	300	97.0	3.0
5	5.50	300	97.0	3.0

Detection took place by a triple quadrupole LC-MS/MS mass spectrometer, API 3000 manufactured by AB Sciex Instruments, using multiple reactions monitoring with transitions Q1/Q3: 255,992→ 151,873. Source temperature was 500°C, overall run time was 5.5 minutes. The method was linear in a range of 10 to 800 ng/ml, with a detection limit of 1 ng/ml. QC samples were analyzed with the plasma samples to monitor method accuracy and precision.

Pharmacokinetic and statistical analysis

The data were analyzed using Microsoft Excel 2010 and IBM SPSS Statistics version 19. Maximum plasma concentration, $C_{max,}$ and the time point at which this was measured, $T_{max,}$ were manually selected from the data. The areas under the curve, $AUC_{(0-t)}$ and $AUC_{(0-\infty)}$, were estimated using the linear trapezoidal method as available in PKSolver2.0 with automatic calculation of the terminal elimination slope using the regression with the largest adjusted R^2 based on at least three of the last time points (170).

The complete dataset was analyzed for sequence or period effects on C_{max} and AUC using a general linear model with period, treatment, sequence and subject within sequence as fixed effects. The difference between related parameters was considered statistically significant if $p \leq 0.05$.

Parametric 90% confidence intervals were calculated based on the univariate ANOVA of the mean ratio of test versus reference using log-transformed C_{max} and AUC data. An increase of the bioavailability of more than 30% was predefined as clinically significant. In addition, the products were evaluated based on the European Guideline on bioequivalence. Test and reference products were considered bioequivalent if the ln-transformed ratios of C_{max} and AUC and their confidence intervals were within the equivalence range of 80-125% (7).

T_{max} was analysed using the nonparametric Wilcoxon signed rank test, where $p \leq 0.05$ was considered statistically significant.

Tolerability

Tolerability was assessed by monitoring electrocardiogram and blood parameters before and after the study and by subject interviews during the study.

Results

Tolerability

The formulations were well tolerated. No adverse events were reported.

Table 3.4.1 P- values for effects on $AUC_{(0-12)}$, $AUC_{(0-\infty)}$ and C_{max}

	p-values (α= 0.1)		
Source	$AUC_{(0-12)}$	$AUC_{(0-\infty)}$	C_{max}
Treatment	0.883	0.713	0.913
Period	0.900	0.975	0.544
Sequence	0.285	0.208	0.218
Subject within sequence	0.686	0.487	0.594

Study design and data evaluation

Table 3.4.1 shows the p-values of the potential treatment, period, sequence and subject within sequence effects obtained for the parameters $AUC_{(0-12)}$, $AUC_{(0-\infty)}$ and C_{max}, which were all above 0.05. These data confirmed absence of period or sequence effects and justified pooling of the data of each of the two doses of chitosan obtained at the two different time points. The pooled data were compared to the data obtained with reference product Zovirax.

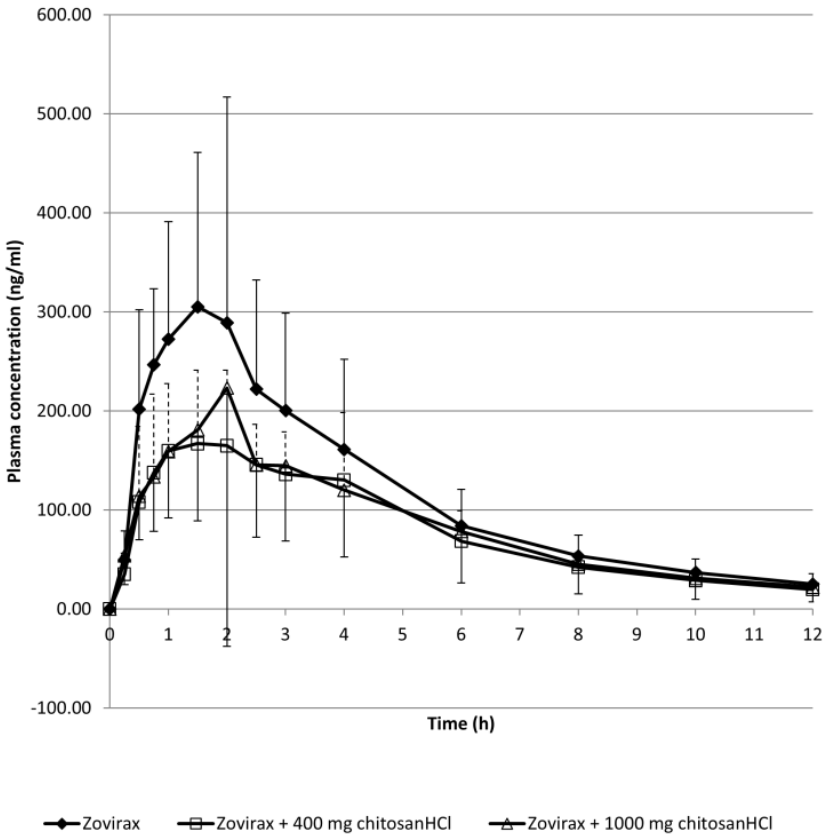


Figure 3.4.1 Mean acyclovir plasma profiles.

For clarity of the figure, only one-way error bars representing the standard deviation are shown. Positive bars are shown for the Zovirax reference and for Zovirax with the 400 mg chitosan dose (dashed line). Negative bars are shown for Zovirax with the 1000 mg chitosan dose. The actual standard deviations were equal in both positive and negative direction.

Figures 3.4.1 to 3.4.3 show the mean plasma curves obtained and an overview of individual data. The individual plasma profiles are accessible as supplementary material. The individual AUC data were considered sufficiently reliable as $AUC_{(0-12)}$ was > 80% $AUC_{(0-\infty)}$ in all cases, except for one in a

test situation with 400mg chitosan HCl where $AUC_{(0-12)}$ was 67% of $AUC_{(0-\infty)}$. All data were used in the analysis.

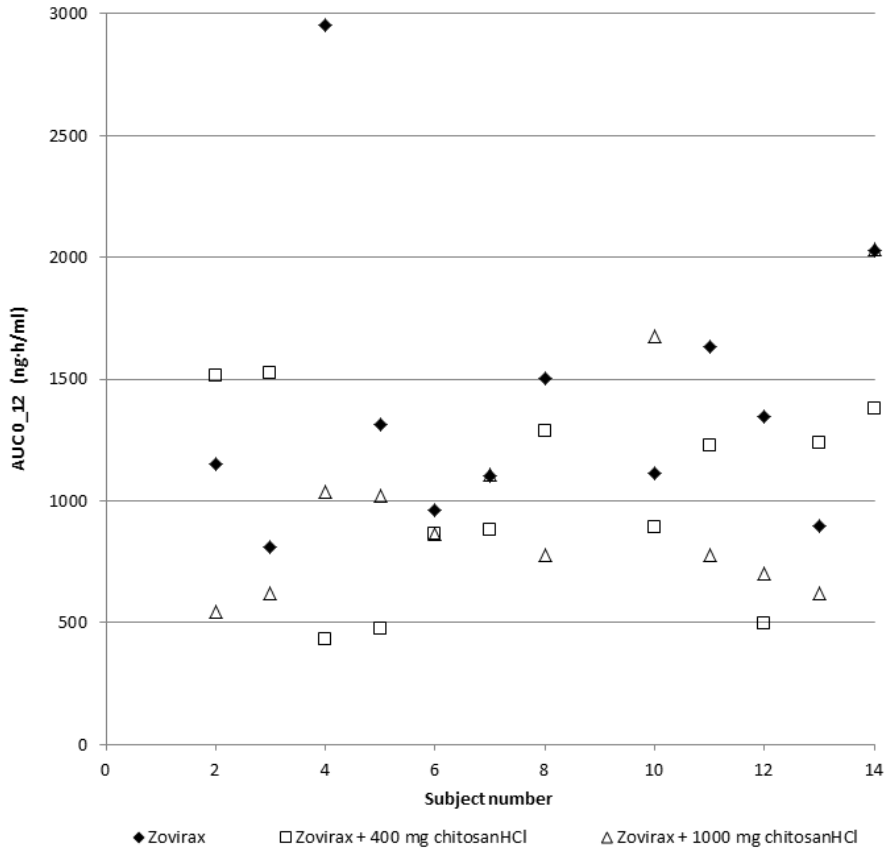


Figure 3.4.2 Individual data for $AUC_{(0-12)}$ per subject.

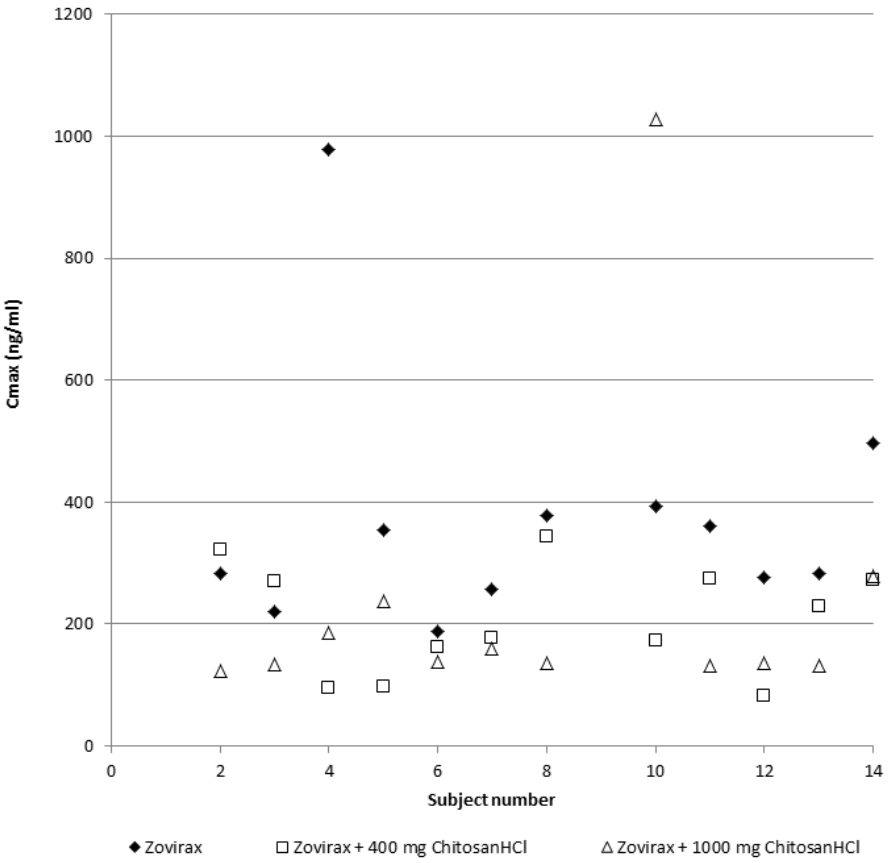


Figure 3.4.3 Individual data for C_{max} per subject.

Comparative evaluation of pharmacokinetic data

Table 3.4.2 Pharmacokinetic data (mean values ± sd) of a 200 mg dose of acyclovir, including comparison with literature values

Parameter	Zovirax	Zovirax + 400 mg Chitosan HCl	Zovirax + 1000 mg Chitosan HCl	Lit ref [a]	Lit ref [b]	Lit ref [c]
C_{max} (mg/ml)	0.373 ± 0.209	0.208 ± 0.090	0.235 ± 0.255	0.3 ± 0.1 (c, s)	0.5 ± 0.2 (t) 0.5 ± 0.2 (r)	0. 7 ± 0.2 (t) 0.7 ± 0.2 (r)
T_{max} (h)	1.2 ± 0.4	1.5 ± 0.9	1.8 ± 0.9	1.5 ± 0.6 (c) 1.2 ± 0.3 (s)	1.6 ± 0.7 (t) 1.6 ± 0.7 (r)	1.5 ± 0.5 (t) 1.5 ± 0.4 (r)
$AUC_{0-\infty}$ (mg·h/ml)	1.528 ±0.627	1.132 ± 0.418	1.074 ± 0.509	1.5 ± 0.5 (c) 1.6 ± 0.5 (s) (AUC_{0-24})	2.4 ± 0.7 (t) 2.6 ± 0.9 (r)	3.4 ± 1.3 (t) 3.5 ± 1.2 (r)
$t_{1/2}$ (h)	3.4 ± 1.1	3.5 ± 1.0	3.2 ± 0.6	2.9 ± 0.8 (c) 2.9 ± 0.7 (s)	3.2 ± 0.6 (t) 3.0 ± 0.6 (r)	4.5 ± 2.4 (t) 3.9 ± 2.7 (r)

(Lit ref = literature reference; t= test, r= reference tablet; c= capsule, s = solution)

[a] de Miranda P, Blum MR. Pharmacokinetics of acyclovir after intravenous and oral administration. J Antimicrob Chemother. 1983;12 Suppl B:29-37.
[b] Vergin H, Kikuta C, Mascher H, Metz R. Pharmacokinetics and bioavailability of different formulations of aciclovir. Arzneimittelforschung. 1995;45(4):508-15.
[c] Rojanasthien N, Teekachunhatean S, Kumsorn B, Chaichana N, Hay YK. Bioequivalence study of generic acyclovir compared with the brand name acyclovir. J Med Assoc Thai. 2002;85(10):1121-9.

Table 3.4.2 shows the pharmacokinetic data for each of the treatments; including comparative data of previously reported pharmacokinetic data of 200 mg doses of acyclovir. C_{max}, T_{max} and AUC values of the reference product were of the same magnitude as the data reported previously, both with regard to the mean values as well as to standard deviations (162, 167, 171). The mean values obtained for the C_{max} and AUC in presence of chitosan are lower than reference values reported in literature, whereas the standard deviations for C_{max} and T_{max} in presence of chitosan are higher. The elimination half-lives are also similar to those reported by others.

Table 3.4.3 and 3.4.4 list the point estimate and the 90% confidence interval for AUC and C_{max} for each of the two doses of test product versus the reference product. Comparison of reference acyclovir with the two situations of concomitant chitosan intake show that both the arithmetic mean of the original data and the geometric mean of the ratios (GMR) of the AUC and C_{max} decreased in presence of chitosan. The individual data show how in nine out of twelve cases the C_{max} and AUC values of reference Zovirax are indeed higher than those of the combinations with chitosan.

Table 3.4.3 Comparative evaluation of 200 mg Zovirax p.o. without (reference) and with (test) concomitant 400 mg chitosan HCl

Pharmacokinetic parameter	Geometric mean ratio test/reference	90% Confidence intervals	CV (%)
$AUC_{(0-12)}$	0.72	0.51-1.13	46
$AUC_{(0-\infty)}$	0.73	0.54-1.00	42
C_{max}	0.59	0.39-0.90	57

Table 3.4.4 Comparative evaluation of 200 mg Zovirax p.o. without (reference) and with (test) concomitant 1000 mg chitosan HCl

Pharmacokinetic parameter	Geometric mean ratio test/reference	90% Confidence intervals	CV (%)
$AUC_{(0-12)}$	0.70	0.50-0.99	46
$AUC_{(0-\infty)}$	0.69	0.51-0.94	42
C_{max}	0.58	0.38-0.88	57

The confidence intervals of the geometric mean ratios for $AUC_{(0-12)}$ and $AUC_{(0-\infty)}$ concerning 400 mg chitosan HCl, included 1.00 and could thus not confirm a significant effect. However, those for the 1000 mg dose of chitosan did not include 1.00, pointing to a significant negative effect. The lower boundary of the 90% confidence intervals for $AUC_{(0-12)}$ and $AUC_{(0-\infty)}$, 0.50 resp. 0.51, show how this decrease could be up to about 2-fold compared to the reference value.

For C_{max}, neither 90% confidence interval includes 1.00, thereby demonstrating a significant decrease in C_{max} of acyclovir in presence of both doses of chitosan. This effect is at least 10% for the 400 mg dose and 12% for the 1000 mg dose. The confidence intervals for the 400 mg and 1000 mg doses almost completely overlap: the median effect is a decrease by 41-42% to values of 58-59% of the reference value (see GMR values). The decrease could be at maximum 61-62%, i.e. to values as low as 38-39% of the reference C_{max}, considering the lower boundaries of the confidence intervals.

The T_{max} obtained with 400 mg chitosan HCl is not significantly different from the T_{max} of the reference Zovirax. However, T_{max} increased significantly in presence of 1000 mg of chitosan from 1 to 2 hours (p=0.029).

Discussion

The results obtained with the acyclovir reference product were in line with those published by others, both regarding the magnitude of the mean values as well as the standard deviations (see Table 3.4.2).

The effects of chitosan on the bioavailability of acyclovir from Zovirax were evaluated in the context of a pretreatment with Zovirax, as administered to all volunteers on the first trial day. In view of the half-life of acyclovir of about 3 hours, a carry-over effect of pretreatment with Zovirax is not expected to play a role and the order of administration of the test and reference products is not expected to affect the results (172).

Locally administered chitosan increased acyclovir's permeability across Caco-2 membranes and facilitated the absorption of acyclovir in rats (164-166). The clinical trial protocol was based on an expected increase of C_{max} and AUC and defined that an increase by 30% would be considered as relevant. However, chitosan HCl did not act as absorption enhancer of acyclovir in the human study presented here. None of the confidence intervals includes a value of 1.3 or more, so an increase by 30% is highly unlikely.

Masuda et al. postulated a cut-off value of 10 kDa as minimal chain length of the chitosan polysaccharide to facilitate acyclovir's absorption. Opanasopit et al. showed an increasing negative effect on the transepithelial electric resistance of Caco-2 monolayers with increasing molecular weight in the range of 20-460 kDa (173). The applied chitosan had a molecular weight of 30-400 kDa which is well within these ranges. Masuda *et al.* also found a dose dependent effect and the authors explained its transience by dilution of the concentration at the site of action and by neutralization of the acidified perfusate by the intestinal fluids. An enhancing effect due to changes in viscosity or cationic charges only was not found likely. Here, chitosan HCl and acyclovir were administered orally and their concentrations were diluted by the luminal fluids while progressing through the gastrointestinal tract, probably resulting in insufficiently high concentrations at acyclovir's absorption site. Moreover, the actually dissolved and protonated fraction of chitosan may have been too low at gastrointestinal pH, thereby limiting a charge-based interaction with the epithelial membrane (174).

Chitosan is metabolized in the colon, a property that has been used to produce colon targeted formulations (175). Although evidence suggests that chitosan metabolism is likely to mainly take place in the colon, the concentration at acyclovir's absorption site may also have been decreased by enzymatic degradation in stomach (pepsin) and/or duodenum/ileum (pancreatic enzymes) (176-181). In addition, the presence of mucus may represent an unfavorable microenvironment and have

hampered the access of chitosan to the mucosa to exert its permeability enhancing effect (182, 183).

If the data were to be evaluated as a bioequivalence study, a confidence interval contained within 0.8 to 1.25 would be considered as bioequivalent and confirm absence of clinically relevant difference. Considering the lower values of each of the 90% confidence intervals, which are all well below 0.8, the data do not confirm bioequivalence of the combination of Zovirax with chitosan to Zovirax only. In fact, the data unexpectedly show a reduced bioavailability of acyclovir in presence of chitosan HCl. C_{max} was significantly reduced by both chitosan doses, and AUC and T_{max} only by the 1000 mg chitosan dose. Dose dependency could not be demonstrated as point estimates for AUC and C_{max} were close and the confidence intervals for both doses of chitosan almost completely overlap. Confirmatory studies may clarify the actual significance and magnitude of the observed negative effects on the means.

Acyclovir is known for its variable absorption. De Miranda and Blum described how a 200 mg acyclovir outperformed a 200 mg acyclovir solution in as many subjects as vice versa thereby demonstrating how the variability in absorption is a substance related characteristic and not product related (23). Figure 3.4.2 and 3.4.3 also show variable individual effects in absence and presence of chitosan: contrary to the results of the means, three volunteers actually showed an increase of the bioavailability for one of the chitosan doses. The standard deviations calculated for AUC and C_{max} remain comparable to literature data in presence of chitosan (see Table 3.4.2). However, the standard deviation for T_{max} is higher than literature values for the reference product. Several mechanisms may have influenced the bioavailability of acyclovir. Considering the effects on the variability of T_{max}, the significant effects on C_{max} and AUC, and other hand, the similarity of the terminal half-lives with reference values, chitosan's effect seems to mainly take place in the absorption phase.

Literature evidence does not point to interaction between chitosan HCl and acyclovir leading to degradation of acyclovir, either in a formulation or in the gut lumen. An effect of chitosan HCl on the dissolution (rate) of acyclovir at the time of administration is conceivable. The tablets were dispersed in 100 mL water or 100 mL chitosan solution and the glass was rinsed with another 150 mL of water. In view of acyclovir's solubility of $\geq$ 2.3 mg/mL in pH range 1.2–7.4 at 37°C and the expected final concentration 2 mg/mL in the first 100 mL water at room temperature, not all acyclovir may have been dissolved (105, 184). However, after addition of 150 ml water and assuming the presence of additional volume of aqueous fluids and a temperature of 37°C in the stomach, acyclovir was expected to dissolve sufficiently well. Experiments [data not shown] indeed confirmed the visual compatibility of 1g chitosan HCl with 200 mg pure acyclovir after 1 minute of

stirring in 250 mL water at room temperature, resulting in a clear solution. In addition, any residues of undissolved acyclovir were expected to be similar for reference and test situations.

Acyclovir's solubility is at the borderline of BCS class III and IV. Increasing its solubility in the lumen may improve its bioavailability, as demonstrated by the effects of a self-microemulsifying drug delivery system (185). Vice versa, its solubility may become limiting if it is chemically or physiologically reduced. Allam *et al.* showed how chitosan-acyclovir co-crystals prolonged the release of acyclovir, however, crystal formation was not observed when acyclovir and chitosan were dissolved in 250 mL water [data not shown] (186). Nadai et al. showed how chitosan (25 mg/kg) prolonged T_{max} of orally administered suspensions of indomethacin and griseofulvin in rats, and reduced C_{max} and AUC. Association of the anionic indomethacin to the tertiary amino groups of chitosan did not play a role according to those authors (187). The molecular structures of chitosan (pKa ~6-6.5) and acyclovir (pKa's 2.3 and 9.3) do not seem to favor charge based complex formation or adsorption either (188).

Effects on T_{max} and C_{max} may be related to the gastric emptying time; whereas an effect on intestinal transit time may be relevant for the absorption of the BCS class III compound acyclovir. It was previously found unlikely that chitosan caused alterations in the gastric emptying rate, as the delayed T_{max} was not observed for other compounds (187). However, in the current case, the difference in stomach content after ingestion of the chitosan dispersion (increased osmotic value, lower pH, perceived nutrient density) compared the reference dispersion may have influenced the gastric emptying rate in this study which may explain the variable effect on C_{max} and T_{max}.

Nadai *et al.* postulated an indirect effect as a cause for the prolonged T_{max}: binding of bile acids to chitosan resulting in inhibition of the solubilisation of the drug substances (187). Heinen et al. indeed described how presence and reduction of bile salts affected the absorption of BCS class III drug trospium chloride (138). In fact, the absorption of acyclovir increased in presence of conjugated trihydroxy bile salts and bile salt-acylcarnitine mixed micelles (189, 190). The 1000 mg chitosan HCl used in the clinical study presented here corresponds to about 14 mg/kg for a human being of 70 kg and is thus of the same order of magnitude as the dose used by Nadai *et al.* Although amphoteric acyclovir is in neutral state at physiological pH and is not known for having a significant food effect, chitosan may have interacted with luminal bile components indirectly resulting in a reduced bioavailability of acyclovir (163, 184).

Finally, chitosan may act at the site of absorption. Chitosan's mucoadhesive properties have been studied in potentially absorption enhancing formulations of acyclovir (191-193). Although no literature evidence is known to the authors, theoretically, chitosan may have physically or biochemically delayed or prevented access of acyclovir to its paracellular permeation route by

binding to mucus at the absorption site and/or changing the microclimate e.g. by formation of a protective viscous layer. Derivatives of chitosan, quercetin and DM72 have been shown to inhibit the efflux of acyclovir via P-gp, with the aim of improving its bioavailability (194-196). An opposite effect, i.e. induction of (expression of) P-gp by chitosan has not been described and seems an unlikely effect of a single dose of chitosan.

An effect of chitosan on the metabolism of drugs has not been described. As acyclovir is eliminated mainly in unchanged form, such interaction is not considered likely either (163).

As chitosan is currently not in use as an excipient in approved drug products, the clinical impact of the results seems limited. However, the observed variability and complex potential interactions may complicate the use of chitosan HCl in oral pharmaceutical formulations. In addition, chitosan is advertised as food supplement in high doses, up to 6 grams a day; interactions with concomitant pharmacotherapy seem possible (197).

Conclusion

The results of this study in human volunteers did not confirm the data obtained in in vitro experiments and in rat studies that suggested that chitosan would improve the bioavailability of acyclovir. Chitosan increased the variability of the absorption of acyclovir and significantly reduced its absorption. Mechanistic studies are needed to investigate the cause for the discrepancy in these results. A luminal interaction of chitosan with acyclovir, bile salts or the epithelial membrane may be responsible for the negative effects on acyclovir's absorption.

3.5. The effect of chitosan on the bioaccessibility and intestinal permeability of acyclovir – *in vitro* studies

Based on:
Kubbinga M, Augustijns P, Heinen C, Wortelboer HM, Verwei M, Langguth P. The effect of chitosan on the bioaccessibility and intestinal permeability of acyclovir, *<publication in progress>*

Abstract

Chitosan is object of pharmaceutical research as a candidate permeability enhancer. However, chitosan was recently shown to reduce the oral bioavailability of acyclovir in humans. The effect of chitosan on two processes determining the oral bioavailability of acyclovir, bioaccessibility and intestinal absorption, was now investigated. Acyclovir's bioaccessibility was studied using the dynamic TNO gastro-Intestinal Model (TIM-1). Several epithelial models were used for permeability experiments: a Caco-2 cell model in absence and presence of mucus and both rat and porcine excised intestinal segments. Study concentrations of acyclovir (0.8 g/L) and chitosan (1.6 g/L and 4 g/L) were in line with those used in the aforementioned human study. No effect of chitosan was measured on the bioaccessibility of acyclovir in the TIM-1 system, which confirmed absence relevant of intraluminal interactions. The Caco-2 model was not biopredictive, neither in absence nor in presence of a mucus layer. The rat and porcine intestinal tissue models showed a negative trend of acyclovir's permeation in presence of chitosan, including a statistically significant effect for the rat tissue at 1.6 g/L chitosan. These tissue segment models thus suggested being the most biopredictive, although – in absence of an established correlation - *in vivo* pharmacokinetic studies remain necessary to determine the actual clinical effect of chitosan on the absorption of acyclovir.

Introduction

Chitosan is an unbranched binary heteropolysaccharide consisting of the two units N-acetyl-d-glucosamine and d-glucosamine, obtained by partial deacetylation of the natural product chitin (198-202). The absorption of a variety of pharmaceutical substances has been studied in combination with chitosan. Besides its employment as a conventional excipient (e.g. filler, disintegrant, release modifier), chitosan has been tested as a candidate drug bioavailability modulator. Various modulating effects of chitosan were shown in preclinical research including improved dissolution of poorly water-soluble drug ECU-01 and naproxen (203, 204), reduced

intraluminal degradation or metabolism of calcitonin and mesalazine (205-207), increased gastric residence time of melatonin and acyclovir due to mucoadhesion and floating of the formulation (191, 192, 208, 209) or enhanced permeability of acyclovir, cyclosporine-A and hydrophilic marker molecules like mannitol (157, 166, 191, 210). However, the data of these *in vitro* and animal experiments were not confirmed by human data tested at identical dose levels and have not yet resulted in a marketed pharmaceutical product.

The *in vivo* relevance of chitosan's effects is still subject to further research (211-213). Chitosan salts have been shown to increase the *in vitro* permeability of acyclovir across Caco-2 monolayers and the oral absorption of acyclovir in the rat at concentrations varying from 0.1% to 3% (164, 165). The mechanism for permeation enhancement is postulated to occur through an interaction of the positively charged chitosan molecules and negative charges in the cavity of the epithelial tight junctions resulting in opening of these tight junctions (16, 30-33). However, a recent study in healthy human volunteers performed by the authors showed how chitosan hydrochloride actually reduced the bioavailability of 200 mg acyclovir when orally administered at a concentration of 0.16% or 0.4% (1.6 g/L or 4 g/L; given as 400 mg and 1000 mg chitosan hydrochloride respectively in 250 mL water) (214). The data obtained from other available studies are often difficult to compare as the characteristics of the applied chitosan (molecular weight, degree of deacetylation (DD), salt form) as well as the test compounds and dose levels differ between the studies. The current paper describes the effect of chitosan on acyclovir absorption using different studies all performed with chitosan of the same or very similar quality at identical dose levels as applied in the human study. The studies improve the understanding of chitosan's effects on two kinetic processes underlying the oral bioavailability of the high solubility and low permeability model drug acyclovir (BSCIII). In addition, this research allows evaluation of the different intestinal permeability models for pharmaceutical development or biowaiver purposes.

Materials and methods

Materials

Chitosan hydrochloride (degree of deacetylation (DD) 92.7%; viscosity 1% in water at 20°C 4-5 mPas for TIM-1 studies and Ussing type rat model and DD 93.05%, viscosity 1% in water at 20°C 5.9 mPas for Caco-2 and the inTESTine study) was obtained from Heppe Medical GmbH, Halle, Germany. Zovirax 200 mg dispersible tablets (GlaxoSmithKline) were purchased in the Netherlands. Acyclovir was obtained from Fagron (The Netherlands). For the Caco-2 studies, Hanks' balanced salt solution (HBSS), Dulbecco's modified Eagle's medium (DMEM), 10,000 IU/mL penicillin and

10,000 µg/mL streptomycin, nonessential amino acid medium (100 x) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were obtained from Lonza (Verviers, Belgium). Fetal bovine serum (FBS) was purchased from Biological Industries (Beit Haemek, Israel). 2-(N-morpholino)ethanesulfonic acid (MES) was obtained from Sigma–Aldrich (St. Louis, MO, United States). For rat ligated loop studies, ketamin (Ketavet, Pfizer, Germany), and xylazin (Rompun, Bayer, Germany) were obtained via the Pharmacy of the Medical Center of the Johannes Gutenberg University, Mainz, Germany. For the InTESTine study, 14C-Antipyrine (55 mCi/mmol) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, Missouri, United States). 3H-Atenolol (1.79 Ci/mmol), and 14C-acyclovir (440 mCi/mmol) were purchased from Moravek biochemicals Inc. (Brea, California, United States). All other chemicals were purchased at Sigma-Aldrich, Schnelldorf, Germany.

TIM-1 model

The *in vitro* dynamic TIM-1 system consists of one gastric compartment and three intestinal compartments (duodenum, jejunum and ileum) connected with valves simulating the gastric and small intestinal passage of food and pharmaceutical products. The TIM-1 systems (215-217) have a simulated pyloric sphincter for controlled gastric emptying of liquids and solids (particles less than 3-5 mm) with specific settings for fasted and fed conditions. The conditions in the compartments are computer-controlled via pH electrodes, temperature and pressure sensors. The secretions into the gastric compartment consist of artificial saliva with electrolytes and α-amylase and gastric juice with hydrochloric acid, pepsin and lipase. In the small-intestinal compartments, the secretion fluids consist of bicarbonate, electrolytes, pancreatic juice with digestive enzymes, and bile.

The TIM-1 studies were performed with acyclovir (0.8 g/L) in absence or presence of chitosan (1.6 and 4.0 g/L) to provide information on the effect of chitosan on the bioaccessibility (i.e. availability for absorption) of acyclovir during gastrointestinal passage. Preparation of the dispersions containing Zovirax tablets in presence and absence of the two levels of chitosan took place in line with the instructions applied during the human study (214): a total volume of 250 mL water was introduced in the gastric compartment. The model conditions simulated the fasted state including gastric pH profile, enzyme levels, gastric emptying etc. A gastric emptying half-time of 20 minutes (default fasted state) was used for the three conditions (acyclovir in absence of chitosan, in presence of 1.6 g/L or in presence of 4.0 g/L chitosan). The jejunum and ileum compartments were connected with dialysis membranes (cut-off 5kDa) to remove the released and water-dissolved compounds. Jejunum and ileum dialysate samples were collected every 30 minutes during the first three hours and every 60 minutes during the next 2 hours till a total of five hours. The amount of

acyclovir in these dialysate samples was considered as the fraction available for absorption from the upper gastrointestinal tract, i.e. the bioaccessible amount, within a given time period. In addition, ileum effluent was sampled every hour. These ileum effluent samples provide information on the non-bioaccessible fraction during transit through the upper GI tract, and which will enter the colon. After five hours, the experiments were ended and the residues were collected to be able to calculate the mass balance of acyclovir in each individual TIM-1 experiment. All samples were stored at or below -18°C until analysis.

Caco-2 cell permeation studies (n=3 wells)

Caco-2 cells were obtained from American Type Culture Collection (Manassas, VA) and were grown in $DMEM^+$ at 37 °C in an atmosphere of 5% CO_2 and 90% relative humidity. Cells were passaged every 3–4 days (at 80–90% confluence) at a split ratio of 1:6. For transport experiments, cells were seeded at a density of 90,000 cells/cm^2 on Costar Transwell membrane inserts (3 µm pore diameter, 12 mm diameter; Corning Inc., Corning, NY, United States) and were used for experiments 17–18 days after seeding. Only monolayers with transepithelial electrical resistance (TEER) values higher than 400Ω * cm^2 were used for transport studies.

Caco-2 cell culture medium consisted of DMEM supplemented with 10% FBS, 1% nonessential amino acids, 100 IU/mL penicillin and 100 µg/mL streptomycin ($DMEM^+$).

Transport medium consisted of HBSS containing 25 mM glucose and was buffered with 10 mM HEPES to pH 7.4 or with 10 mM MES to pH 6.0 ($HBSS^+$ pH 7.4 or pH 6.0).

Three conditions were tested in absence or presence of mucus: 0.8 g/L acyclovir, 0.8 g/L acyclovir + 1.6g/L chitosan and 0.8 g/L acyclovir + 4 g/L chitosan based on the concentrations for chitosan which effects have been described previously (164, 165). The mucus used as a protective barrier in the Caco-2 assay consisted of type III mucin derived from porcine stomach dissolved in $HBSS^+$ pH 6.0. Mucus was used in a concentration of 50 mg/mL (218).

Prior to the transport study, Caco-2 cells were washed twice with pre-warmed $HBSS^+$ pH 7.4 and placed in a shake incubator (Thermostar, BMG Labtech, Offenburg, Germany) at 37 °C and 300 rotations per minute (rpm) for 30 min. After the pre-incubation, 100 µL of mucus was applied to the apical compartment of the transwell plates for the corresponding conditions; in the basolateral compartment fresh $HBSS^+$ pH 7.4 was added. The transport experiment was initiated by adding the corresponding incubation medium (0.5 mL) to the donor compartment. Plates were incubated in the shake incubator at 300 rpm for 2 h at 37 °C. 200µL samples were taken from the basolateral

compartment at t =15, 30, 45, 60, 90 and 120 min and were replaced by fresh buffer. 10 µL apical samples were taken at t = 0 and 120 min and diluted 100x in $HBSS^+$ pH 7.4. Samples were analysed immediately. Monolayer integrity after the transport experiment was confirmed by comparing the measured TEER at t = 0 min with the TEER at t = 120 min.

Ussing-type chamber permeation studies using rat jejunal segments (5-6 rats)

Rats were purchased from Charles River (Sulzfeld, Germany). Rat excised jejunal segments were obtained and permeation studies in an Ussing-type chamber were performed as described by Heinen *et al.* 2013 (137). On the apical side, a 5 mL Krebs-Ringer-Bicarbonate-Buffer (KRB) containing 4-morpholine ethane sulfonic acid (MES) was used. The reference solution was 200mg acyclovir dissolved in 250mL buffer. For the respective other solutions, increasing quantities of chitosan hydrochloride were added, corresponding to 1.6 g/L, 4 g/L, 10 g/L, 30 g/L and 50 g/L, respectively, to test whether a dose related effect would be observed. Samples of 600 µL were taken at 30, 60, 90 and 120 min from the acceptor chamber, each replaced with fresh KRB buffer and at 0 and 120 min from the donor chamber.

Porcine excised segment InTESTine permeation studies (2 individual studies each with 4 replicates)

Porcine excised jejunal segments were obtained from healthy pigs, mounted in a newly developed InTESTine™ system, and permeability studies were performed as described by Westerhout *et al.* (219). On the apical side, a 1 mL pre-warmed (37°C) Krebs-Ringer Bicarbonate buffer (containing 10 mM glucose, 25 mM HEPES, 15 mM sodium bicarbonate, 2.5 mM calcium chloride, pH 7.4, and saturated with oxygen using a 95%/5% O_2/CO_2 mixture by gassing for 120 minutes, further indicated as KRB-HEPES) dose solutions containing 10 µM acyclovir (containing [14C]-acyclovir, 2 kBq/mL) in the absence and presence of chitosan (0, 1.6, 4, 10, 30, and 50 g/L) and 50 µM fluorescein isothiocyanate-dextran (FD4) as a membrane integrity marker was used. The basolateral compartment contained 7.5 mL pre-warmed KRB buffer (37°C). In parallel, the permeability of 3H-atenolol and 14C-antipyrine (both 10 µM) was determined in the absence and presence of chitosan as a control for the permeability of a low and high permeability marker, respectively. The InTESTine samples were taken from both the apical and basolateral compartment after a pre-incubation time of 45 minutes in order to measure the linear phase of the transport over 60 min incubation. Recovery of active substance compared to added quantity at the beginning of the study was determined at the end of the studies by determination of the mass balance. It was previously

shown that intestinal permeability of a wide range of compounds is comparable between (adult) human and porcine intestinal tissue (219).

Analytical methods

Liquid chromatography

- Acyclovir by HPLC-FID (TIM-1)

Acyclovir in ileum and jejunum dialysate, ileum effluent and residue samples from the TIM-1 experiment was analysed by HPLC with fluorescence detection (excitation wavelength 260 nm, emission wavelength 375 nm). A Waters Sunfire 150 x 3,0 mm 3,5 µm column was used at ambient conditions, with an injection volume of 10 µl. A gradient elution method was applied involving solvent A (0.1% formic acid in acetonitrile) and solvent B (0.1% formic acid in purified water) at a flow rate of 0.6 mL/min. Solvent A and B were used in a ratio of 99:1 during the first 5 min, followed by 2 min of 100% solvent B. The run time of 14 min was completed using the 99:1 ratio again. Low, middle and high QC samples in two TIM matrices were analyzed in duplo in parallel with the samples. Linearity was demonstrated in a range of 25 mg/mL to 250 mg/mL ($R^2 \geq 0.998$). Regarding precision, intraday variation coefficients per concentration level varied from 0.03 to 5.1%, which is acceptable considering the complex matrix. The results for accuracy showed for QC low (25 µg/mL) an average deviation of +15% in both matrices. Considering that the deviation varied from +5% to +23% and that the results for overall recovery are limited to 110%, a correction factor was not applied. The mean QC middle (100 µg/mL) and high (250 µg/mL) showed an average deviation of the nominal values of -1% and 4%, respectively.

- Acyclovir by HPLC-UV (Caco-2)

Acyclovir concentration in media samples from the Caco-2 cell experiments were analysed by HPLC consisting of a Waters 600 pump and a Waters 717 auto injector (Waters, Milford, MA). For chromatographic separation, a Waters Novapak C18 column under radial compression was used. UV absorbance was monitored using a Waters 2487 detector at 254 nm. The observed peaks were integrated using Empower Pro (Empower 2) software. The mobile phase consisted of a 25 mM acetate buffer (pH 3.5) (95%) / methanol (5%) and a flow rate of 1 mL/min was applied. Retention time of acyclovir was 6.0 min. The calibration curve of acyclovir was linear over the concentration range of 0.12 – 1000µM. The assessment of repeatability at concentrations of 250, 25, 2.5 and 0.25 µM resulted all in RSD's below 2.5%. Samples from the acceptor compartment were not diluted; the

10 µL samples from the apical department were diluted with 990 µL HBSS+ pH 7.4. The injection volume amounted to 50 µL.

- Acyclovir by HPLC-UV (Ussing-type chamber)

Acyclovir concentration in media samples from Ussing-type chamber experiments were determined using isocratic HPLC with UV detection at 254 nm. A Lichrospher 10 RP 18 (5 µm), 250-4 column was used for chromatographic separation, at 40°C and ~133 bar. The mobile phase consisted of 10 mM acetic acid and acetonitrile (95:5; V/V) and a flow rate of 1 mL/min was applied (retention time acyclovir ~ 4.6 min). Linearity was demonstrated in a range of 0.050 µg/mL to 50.0 µg/mL acyclovir. Intraday precision resulted in RSD values <5.5% for concentrations ≥ 0.50 µg/mL and <11% for the lower concentrations. Interday precision resulted in RSD values <5.5% for concentrations ≥ 0.10 µg/mL and 13% for the lowest concentration of 0.050 µg/mL. Samples from the acceptor compartment were not diluted; 10 µL of the samples from the apical department were diluted with 390 µl buffer. Injection volumes were 50 µL.

Liquid scintillation counting (InTESTine)

Concentrations of radioactive labeled compounds in The InTESTine samples were measured on a Tri-Carb 3100TR Liquid Scintillation counter (LSC, Perkin Elmer, Boston, Massachusetts, United States) after adding scintillation liquid (Ultima Gold, Perkin Elmer Inc., Boston, Massachusetts, United States) to samples of the InTESTine experiments.

Fluorescence spectrophotometry (InTESTine)

FD4 levels in media samples from both the apical and basolateral compartments of the InTESTine system were determined using a FLUOstar OPTIMA fluorescence spectrometer (BMG Labtech, Ortenberg, Germany) at excitation wavelength 490 nm and emission wavelength 520 nm.

Data analysis

Bioaccessibility

The amount of acyclovir in each sample collected during the TIM-1 experiment was calculated by multiplying the measured concentration by the total volume of the individual samples collected in the time periods. The bioaccessibility of acyclovir is given as percentage of the intake dose of

acyclovir and expressed as the mean and range of duplicate TIM-1 experiments. The mass balance (recovery) was calculated as the sum of the bioaccessible fraction (ileum and jejunum), the ileum effluent and the residues in the system after ending the experiments.

Apparent permeability

The apparent permeability coefficient (P_{app}) was calculated based on the linear part of the curves according to the following equation:

$P_{app} = dQ/dt * 1/A * C_0$

where Q is the amount of drug appearing in the acceptor compartment as a function of time (t), A is the surface area of the Transwell membrane (1.13 cm^2) or the exposed surface of the intestinal segment in the Ussing-type chamber (0.67 cm^2) , or InTESTine system (0.79 cm^2), and C_0 is the initial drug concentration in the donor (apical) compartment.

Statistical analysis

The TIM-1-results for cumulative bioaccessibility were compared using unpaired t-tests at each time point. Differences were considered statistically significant when $p < 0.05$.

Statistical analysis of the permeation studies was performed using GraphPad Prism 6.03. The mean P_{app} values obtained from the Caco-2 study, Ussing-type chamber, InTESTine experiments as well as the AUCs of the ligated loop experiments were compared with the references using one-way ANOVA and Dunnett's multiple comparison test. P-values below 0.05 were considered significant at a confidence level of 95%.

Results

Bioaccessibility in TIM-1

Figure 1 shows the jejunal, ileal and total bioaccessible fractions of acyclovir tested in absence (reference) and presence of chitosan (1.6 g/L and 4.0 g/L). The total bioaccessibility of the high solubility compound acyclovir tested under fasted state conditions in TIM-1 was found to be high, as values above 90% were measured. No effect of chitosan on the bioaccessibility of acyclovir was observed. Equal bioaccessible fractions were measured in the TIM-1 runs in absence of chitosan

(93.6% ±0.9%, mean±range expressed as % of intake, n=2); in presence of 1.6 g/L chitosan (90.6%±6%, mean±range, n=2) and in presence of 4.0 g/L chitosan (93.2%%±3.2%, mean±range, n=2), respectively. Overall recovery of acyclovir in the six TIM-1 runs was 108.6%± 5.1% (mean±rsd; n=6).

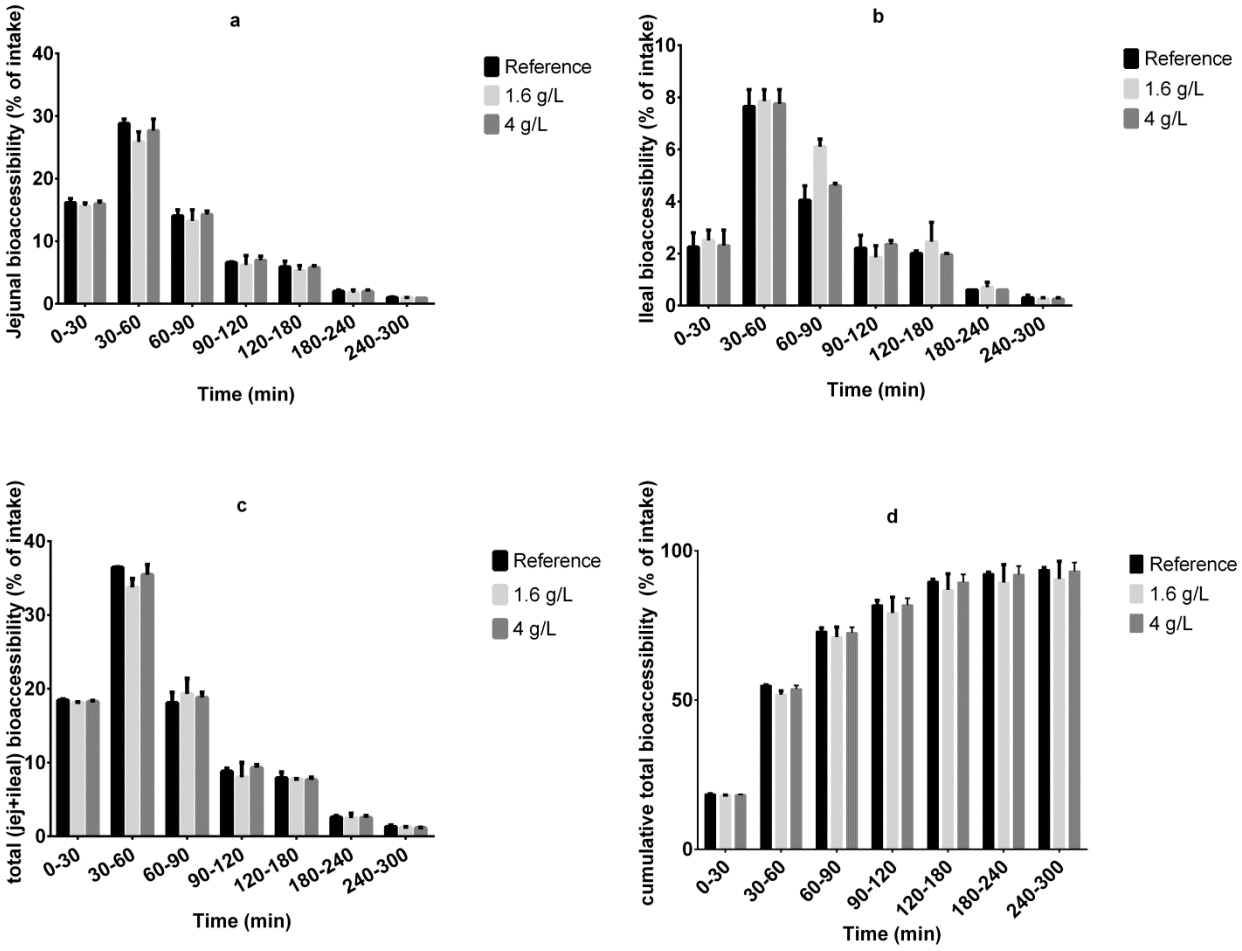


Figure 3.5.1 Bioaccessibility of acyclovir in absence (reference) or presence of chitosan (1.6 and 4.0 g/L) as measured in TIM-1 in hourly time intervals a. jejunal bioaccessibility (% of intake, mean ± range, n=2); b. ileal bioaccessibility (% of intake, mean ± range, n=2); c. total bioaccessibility (= sum of jejunum and ileum; % of intake, mean ± range, n=2); d. cumulative total bioaccessibility (% of intake, mean ± range, n=2)

Values above 100% could be related to possible overestimation of the acyclovir content at low concentrations as observed in the analysis of the low QC samples (in contrast to the mid and high levels).

Intestinal permeability

Table 3.5.1 shows an overview of the results of the intestinal permeability of acyclovir measured in results of the different models. The mean relative effect on the absorption is reflected by the ratio of test versus reference condition. The p-values indicate the statistical significance of the observed effect compared to the reference.

Table 3.5.1 Apparent permeability values (P_{app}) of acyclovir in the presence of chitosan as measured in different permeability models

Model and concentration chitosan hydrochloride[#]		**Permeation of acyclovir**		
Caco-2	Nr of inserts	P_{app} in 10^{-6} cm/s (sd)	Ratio test vs reference	p-value
0 g/L	3	0.17 (0.01)		
1.6 g/L	3	21 (1.08)	124	p<0.0001*
4 g/L	3	24 (1.31)	143	p<0.0001*
Caco-2+mucus	Nr of inserts			
0 g/L	3	0.12 (0.01)		
1.6 g/L	3	0.13 (0.003)	1.08	0.96
4 g/L	3	0.28 (0.15)	2.33	0.11
Ussing type (rat)	Nr of segments[&]			
0 g/L	5	7.4 (1.5)		
1.6 g/L	5	5.4 (9.3)	0.73	0.034*
4 g/L	5	6.2 (9.8)	0.84	0.23
InTESTine (pig)	Nr of segments[$]			
0 g/L	2x4	0.54 (0.30)		
1.6 g/L	2x4	0.49 (0.12)	0.91	0.96
4 g/L	2x4	0.38 (0.05)	0.70	0.67

[#] All conditions included 0.8 g/L acyclovir
[&] from 5 rats
[$] from two pigs, each 4 slices
* p<0.05, statistically significant

Caco-2 cell permeation studies

Monolayer integrity was measured through the TEER values, see Table 3.5.2. At reference conditions, the monolayer integrity was well preserved both in absence and presence of a mucus layer. In the unmodified model, the addition of 1.6 g/L and 4 g/L chitosan resulted in a complete loss of monolayer integrity after 120 min of incubation. Due to this loss of barrier function, the transport of acyclovir from apical to the basolateral compartment increased at these conditions. This effect was confirmed in a second study at the University of Mainz with the same test substances at the same concentrations following the same study protocol (data not shown): a reduction of TEER values to 2-3% of the original value was found in presence of chitosan, accompanied by a 30 to 40-fold increase in acyclovir's permeability.

In absence of chitosan, P_{app} values of acyclovir in the Caco-2 system in absence and presence of mucus were comparable. This indicates that the mucus layer had no influence on the permeation of acyclovir. When 1.6 g/L chitosan was added in the apical compartment of the Caco-2 model in presence of a mucus layer, the monolayer integrity was maintained during the experiment. Addition of 4 g/L chitosan showed that the monolayer integrity was partly compromised after 120 min, which was accompanied by increased variation in TEER results. In presence of mucus, 1.6 g/L chitosan resulted in a P_{app} value (i.e. 0.13 $*10^{-6}$ cm/sec) similar to the reference value (i.e. 0.12 $*10^{-6}$ cm/sec). Increasing the concentration of chitosan to 4 g/L led to an elevated P_{app} value (i.e. 0.28 $*10^{-6}$ cm/sec) compared to the reference conditions, although this difference was not statistically significant.

Table 3.5.2 Effect of acyclovir and the addition of chitosan on monolayer integrity of Caco-2 cells as measured in the absence and presence of mucus

Concentration chitosan hydrochloride (g/L)	TEER (0 min) (%)	TEER (120 min) (%)	SD	RSD
Caco-2				
0	100	115.0	6.0	5.3
1.6	100	4.1	0.2	5.9
4	100	2.8	0.5	18.4
Caco-2 +mucus				
0	100	124.5	2.4	1.9
1.6	100	132.4	1.6	1.2
4	100	55.7	27.8	49.9

Rat jejunal tissue mounted in the Ussing-type chamber

Two concentrations of chitosan hydrochloride (1.6 g/L, 4 g/L) were applied in the Ussing type chamber with rat intestinal segments to test their effect on the permeability of acyclovir (0.8 g/L). The results were compared with acyclovir alone (reference). In all incubations, the transsegmental electrical resistance (Rf) values decreased during the experiment as presented in Table 3.5.3. At reference conditions, the Rf decreased by 20%; in presence of chitosan, the membrane integrity diminished less than the reference. A relatively high P_{app} value for the permeability of acyclovir in absence of chitosan was found (i.e. 7.4 *10^{-6} cm/sec) in the Ussing type chamber with rat intestinal segments. This mean P_{app} was reduced by both concentrations of chitosan, which was statistically significant only for 1.6 g/L chitosan in the donor solution.

Table 3.5.3 Effect of acyclovir and the addition of chitosan on monolayer integrity of rat intestinal tissue as mounted in Ussing-type chamber

Concentration chitosan hydrochloride (g/L)	Rf (0 min) (%)	Rf (120 min) (%)	SD	RSD
0	100	79.7	6.6	8.3
1.6	100	97.3	5.6	5.8
4	100	88.2	6.6	7.5

Porcine intestinal tissue

The permeability of acyclovir, atenolol and antipyrine was measured across jejunal porcine tissue mounted in the InTESTine system. The tissue of two rats (each 4 slices) was subjected to the same test concentrations with respect to chitosan as used in the rat tissue Ussing-type chamber experiments. Recovery of all compounds based upon media alone were > 95%. FD4 leakage remained below 1% indicating no effect of the test solutions on intestinal integrity. The P_{app} values of acyclovir, atenolol, and antipyrine in the absence of chitosan were 0.54*10^{-6} cm/sec, 0.46*10^{-6} cm/sec and 6.58*10^{-6} cm/sec, respectively. The addition of concentrations of 1.6 and 4 g/L chitosan resulted in a negative trend on the permeation of acyclovir, but the overall effect of both concentrations was not statistically significant. The permeability of the paracellular transport marker atenolol enhanced with higher concentrations of chitosan from ~0.5 * 10^{-6} cm/s up to ~ 1 * 10^{-6} cm/s at 4.0 g/L (data not shown). No effect of chitosan was observed on the permeability of the transcellular transport marker antipyrine, which varied in the range of 6 to 9 * 10^{-6} cm/s (data not shown).

Discussion

This work aimed to understand chitosan's effects on kinetic processes underlying the oral bioavailability of BCS class III drug acyclovir. A recent study in humans, summarized in Table 4, revealed that both acyclovir's mean area under the plasma concentration-time curve (AUC_{0-12} and $AUC_{0-\infty}$) and its maximal plasma concentration (C_{max}) decreased following concomitant intake of 400 and 1000 mg chitosan, whereas its T_{max} increased. The effect of chitosan on C_{max} was statistically significant for both doses of chitosan; the effects on AUC and T_{max} were statistically significant for the 1000 mg of chitosan co-administration only. The chitosan doses of 400 and 1000 mg were administered as solutions of 1.6 and 4.0 g/L, respectively, in water (214).

Table 3.5.4 Oral bioavailability data of acyclovir as measured in humans (214)

	C_{max} (mg/ml)	Ratio#
Reference Zovirax 200	0.37 ± 0.21	
Ref +400 mg chitosan	0.21 ± 0.09*	0.56
Ref +1000 mg chitosan	0.24 ± 0.26*	0.63
	$AUC_{0-\infty}$ (mg·h/ml)	
Reference Zovirax 200	1.53 ± 0.63	
Ref +400 mg chitosan	1.13 ± 0.42	0.74
Ref +1000 mg chitosan	1.07 ± 0.05*	0.70
	T_{max} (h)	
Reference Zovirax 200	1.2 ± 0.4	
Ref + 400 mg chitosan	1.5 ± 0.9	1.25
Ref + 1000 mg chitosan	1.8 ± 0.9*	1.50

ratio of absolute values of test vs reference

* $p<0.05$ comparing 90% confidence intervals of the ratio of test and reference product

Chitosan may exert its effect on oral bioavailability through modulation of different elements of the absorption process including the dissolution of the active substance from the dosage form, the gastrointestinal transit process and interactions in the intraluminal compartment or at the permeation site. In the human study, the test products were dispersed prior to use and as such, an effect of chitosan on release and dissolution of acyclovir from the dosage form can be ruled out. In the present study, two of the other individual processes were investigated using different *in vitro* techniques that could provide a mechanistic understanding of previously obtained *in vivo* observations: bioaccessibility and intestinal permeability. The same dose levels as applied in the human study were used.

The selected *in vitro* intestinal models have previously been described as tools to study the individual processes underlying oral bioavailability and identify the critical process(es) hampering the oral bioavailability of a specific compound. Based on the physico-chemical properties of acyclovir and available data, acyclovir was previously classified as a BCS III compound indicating that intestinal permeability, not solubility, is the rate limiting process in oral bioavailability.

The TIM-1 system simulating the human physiological conditions in the stomach and the three parts of the small intestine was applied to investigate the effect of chitosan on the bioaccessibility of acyclovir. A high bioaccessibility of acyclovir was measured (>90%) in absence and presence of chitosan which indicates a large fraction of the acyclovir dose added to the gastric compartment appears to be available for absorption irrespective of the presence of chitosan. These observations are in line with the high solubility characteristics of acyclovir. Luminal interactions affecting both acyclovir and chitosan might possibly explain the *in vivo* results. For example, an interaction of chitosan with bile acids in the gut lumen, reducing the solubility of acyclovir was hypothesized (187, 214). In the study presented here, no effect of chitosan on the bioaccessibility of acyclovir was observed, indicating that the possible luminal interactions did not affect the bioaccessibility of acyclovir.

The current set-up of the TIM-1 experiments simulated the average human adult fasted state conditions concerning enzyme levels, pH values, transit times, etc. Gastric emptying time was fixed and identical in the TIM-1 runs testing the three applied experimental conditions. An effect of chitosan on the gastric emptying time was thus not tested in current study. As the TIM-1 system is a computer-controlled *in vitro* system, individual parameters as gastric emptying rate could be changed to investigate the effect of this specific parameter. Including known variation in the human population concerning specific simulated parameters in TIM-1 is also possible and could be relevant in explaining *in vivo* results related to processes in the human GI tract. However, a physiological basis to assume that the gastric emptying rate would change in the presence of chitosan has not been confirmed so far. Rat experiments using similar doses of chitosan (5 mg/kg and 25 mg/kg) in combination with several drugs with different absorption characteristics showed that delayed gastric emptying is an unlikely effect of chitosan (38). However, the recently performed human study showed an increased T_{max} value at 4g/L chitosan which could be related to delayed gastric emptying of acyclovir.

Mucosal interactions of chitosan causing a change in permeation of acyclovir were tested in four different permeability models. Chitosan is thought to act as a potential permeability modulator through interaction with the tight junctions between the epithelial cells, resulting in redistribution of cytoskeletal F-actin and translocation of tight junction proteins ZO-1 and occludin from the membrane to the cytoskeleton (157, 220, 221). Smith *et al.* showed activation of PKC-dependent

signal transduction pathways using a Caco-2 model (222). The mucus layer was also reported to play a role in the access of chitosan to the epithelial membrane and the subsequent effect on the paracellular permeation route (183).

In the unmodified human intestinal Caco-2 cell model, chitosan enhanced the permeability of acyclovir to a statistically significant extent. This was caused by the disruption of the monolayer integrity as shown by the reduction of the TEER values. This loss of intestinal integrity due to exposure to chitosan is in line with literature data (164, 165, 173, 223). The almost complete reduction of the TEER values suggested a cytotoxic effect. The consequential relatively large increase in P_{app}-values (124 and 143-fold respectively) compared to literature may partly be due to the low reference value of $P_{app,}$ as compared to other studies (84, 164, 165). The low pH of the current study, applied to overcome precipitation of chitosan, and the general interlaboratory variability of the Caco-2 system (224, 225) may explain absolute differences compared to literature values.

The modified Caco-2 model with mucus demonstrated that the disruptive effect of chitosan on the integrity of the epithelial cells is much lower in presence of mucus (183, 226). Both TEER values and acyclovir's permeation were less affected than in the unmodified model when 1.6 g/L chitosan was added, suggesting that the mucus layer prevented damage to cell monolayer integrity and consequential increased permeation of acyclovir. The higher concentration of chitosan (4 g/L) caused reduced monolayer integrity accompanied by an elevated permeation of acyclovir, although this was not statistically significant. The observed results thus suggest a protective effect of the mucus layer, that should be taken into account as relevant characteristic of the intestinal wall when using biorelevant permeation models. In addition, the observed precipitate suggests a direct interaction of chitosan with the mucus reducing the available concentration of the modulator in solution. Therefore, in the presence of mucus, no effect of chitosan (1.6 and 4.0 g/L) on the absorption of acyclovir was detected in this modified Caco-2 cell model.

In the rat jejunal segments mounted in the Ussing-type chamber, the permeability of acyclovir was statistically significantly reduced in presence of a concentration of 1.6 g/L chitosan, while the reduction remained a non-significant trend at 4.0 g/L. The Rf values decreased during the experiment and the limited reduction in presence of chitosan suggested a stabilizing effect of mucus on the epithelial integrity. Interactions of chitosan with mucus may again explain these observations. The mucus production of intestinal tissue segments in the Ussing-type chamber is enhanced by the experimental conditions (183, 227), as was also observed in the present study. Charge interactions of chitosan in the mucus-producing model may neutralize the reactivity of chitosan and prevent an absorption enhancing effect. In addition, the positive charges of chitosan

may have undergone an interaction with the negative glycoproteins in the mucus layer, thereby increasing the rigidity of the protective layer and enhancing its protective effect (183).

Both concentrations of chitosan (1.6 and 4 g/L) suggested a negative effect on the absorption of acyclovir across porcine jejunal segments of both pigs mounted in the InTESTine system. The effect was quite pronounced in one of the two pigs (ratio test versus reference of 0.44), however, the overall effect was not statistically significantly different from the reference. The effect of chitosan on paracellular transport marker compound atenolol was in line with that for acyclovir, which confirmed an effect of chitosan on the paracellular absorption route. The absence of an effect of chitosan on transport of antipyrine confirmed the absence of an effect on the transcellular route and of a non-specific effect on the mucus barrier.

The reference P_{app} values of the Caco-2 experiments and porcine intestinal segments were comparably low and in line with earlier observations that permeability is the limiting process in the oral bioavailability of the BCS class 3 compound acyclovir (37, 46). The reference values for the two Caco-2 models were similar. The absolute P_{app} values of acyclovir measured with the rat intestinal segments were higher than those obtained with the Caco-2 and porcine models. Both interspecies differences between the characteristics of the human Caco-2 model and the two animal tissue models, as well as differences resulting from the applied test conditions may play a role.

The unmodified Caco-2 model does not resemble the true human anatomic intestinal lining due to the lack of a mucus layer. The results obtained with the other permeation models employed indeed suggest that the presence of mucus in the *in vivo* GI tract prevented chitosan to exert an overall enhancing effect on the bioavailability of acyclovir, as discussed above. The application of the Caco-2 model as biorelevant tool to predict *in vivo* permeation is further limited by differences in expression of tight junctions and transporters as compared to healthy human colon (67, 225). Especially the differences in protective mucus layer and tight junctions are of relevance, as the modulation of membrane transport by cationic chitosan is postulated to occur through an interaction with negative charges in the cavity of the tight junctions.

Porcine intestinal segments have proven to be physiologically similar to human intestinal segments with mucus production, high CYP3A metabolic activity, and active transporter processes. Detailed research on metabolic differences and expression of transporters compared to humans is ongoing (26, 219). Rat intestine mounted in an Ussing chamber has shown to be a useful model in predicting human intestinal absorption too, although differences in transporter expression and metabolic characteristics apply as well (26, 228, 229). For the current test substance acyclovir, a difference in transporter expression or metabolism between the cell-based Caco-2 models and the rat and porcine tissue models was not expected to play a major role as acyclovir is mainly passively

absorbed and intestinal metabolism has not been reported. The higher reference P_{app} values measured in the studies with rat segments as compared to the porcine intestinal segments and the Caco-2 model were thus most likely due to differences in passive absorption.

The absorption of acyclovir in rats was shown to decrease along the GI tract from stomach to colon (230). Consequently, the human colon-based Caco-2 model would be expected to show a relatively low permeation compared to the two small intestinal tissue models, which was indeed observed in the current study. Both animal tissue models made use of jejunal segments. The rat model contained proximal jejunal tissue , whereas the porcine model used four mid jejunal segments per rat. The use of more distal tissue segments for the inTESTine model, compared to the proximal rat tissue segments mounted in the Ussing type chamber, may partly explain the higher reference P_{app} values in the rat tissue model.

However, differences in the (preparation of) the intestinal tissue as mounted in the experimental setting of the two models constitute the most prominent cause of the relatively low basal P_{app} of the porcine intestinal segments compared to that observed for the rat tissue. The relatively long villi and thicker tissue, compared to that of the stretched rat tissue, presented a larger permeation barrier. In addition, the relatively thick mucus layer located between the villi limited the ease of access of the model substances to the basolateral cell layer of the porcine tissue.

Our human study showed a relatively high intra-individual and interindividual variability of the absorption of acyclovir itself, which augmented in presence of chitosan (214). Both increased and decreased AUC and C_{max} values were observed when acyclovir was co-administered with chitosan. The animal tissue models also showed interindividual differences. Further evaluation of potential intra- and interindividual differences in physiological processes in the absorption of acyclovir and the potential effect of chitosan on these processes, may thus be relevant. Integration of different *in vitro* datasets in a PBPK model can also be helpful in this perspective.

An evaluation of the biorelevance of the applied models is also of relevance in the context of biowaiving. Based on human studies with acyclovir and cimetidine, Vaithianathan *et al.* recently proposed widened biowaiver possibilities for changes in the content of 12 common excipients combined with BCS class III substances (231). In the current case of potential excipient chitosan, the contrary can be concluded. The unmodified Caco-2 cell model showed a loss of membrane integrity, which has no correlation to the *in vivo* human situation. The mucus-containing Caco-2 model showed a non-significant positive trend, which is not line with the *in vivo* data. These models thus seem unsuitable to replace *in vivo* testing. The overall trends observed for the tissue segment models (rat and porcine intestine) were negative and correctly predicted a statistically significantly reduced absorption of acyclovir at two conditions. These models thus seems most

promising for further development as a tool for biowaiver purposes. However, none of the models has so far been sufficiently validated as suitable to fully exempt from *in vivo* testing. An *in vivo* pharmacokinetic study remains necessary to determine the magnitude of chitosan's clinical effect on C_{max}, AUC and T_{max} and its consequential relevance for drug absorption.

Conclusion

This study presents for the first time a comparison of various preclinical models run under comparative conditions with the aim of testing the predictive power of each model. The overall *in vivo* effects of chitosan pointed to a reduced rate and extent of the absorption of acyclovir. Acyclovir's bioaccessibility in TIM-1 was not affected by chitosan; this model thus confirmed absence of intraluminal interactions hampering the solubility and availability for absorption of acyclovir. Chitosan's influence on intestinal permeability of acyclovir differed per model. The Caco-2 model was not biopredictive, neither in absence nor in presence of a mucus layer. The rat and porcine intestinal segments showed an overall negative trend of acyclovir's permeation in presence of chitosan, including statistically significant results for the rat. These tissue segment models thus showed to be the most biopredictive models and can be considered as the most promising candidates for further development as biowaiver tool. As a follow-up, PBPK modelling may be applied to more specifically correlate the outcome of the models to the *in vivo* data. In absence of an established correlation, *in vivo* pharmacokinetic studies remain necessary to determine the actual clinical effect of chitosan on the absorption of acyclovir.

4. GENERAL DISCUSSION

4.1. Biowaiver extensions from a risk-based perspective.

Regulatory authorities in EU and US as well as the WHO offer the alternative option for biowaiving based on the Biopharmaceutic Classification System. The overall aim of biowaiver guidance is to reduce the need for human studies while keeping the risk of bioinequivalence at an acceptable level, as described in **Chapter 1** of this thesis. The common principle lies in the use of a comparative *in vitro* dissolution test as a surrogate for the *in vivo* bioequivalence test that would otherwise be required.

Section 3.1 introduced the following risk calculation:

risk = probability of incidence x probability of detection x severity.

The incidence describes the probability of producing a product that really is bioinequivalent to the comparator. The probability of detection is a measure for the ability to detect (and reject) the bioinequivalent products prior to marketing. Severity refers to the potential impact of exposure of a patient to a bioinequivalent product, or the risk to public health. The relevance of the different factors in this equation in improving the accessibility of the biowaiver route is illustrated by examples in the **Chapter 3, sections 3.2 to 3.5**

Incidence

The incidence or occurrence of bioinequivalence i.e. the probability that a bioinequivalent product is manufactured depends on the prior knowledge and understanding of the manufacturer. Public databases of scientific experience may facilitate sharing and availability of information to improve this understanding. Such databases may be set-up for BCS classification of APIs, outcomes of bioequivalence trials and classification of excipient effects.

From a regulatory perspective, the probability of incidence of a bioinequivalent formulation is limited by the access criteria based on the BCS. As **Section 3.2** of this thesis shows, the regional differences in biowaiver guidance can be of pivotal importance for the regulatory hurdles to take in

the approval of a generic medicinal product *via* this route. The assignment of the BCS class has recently been changed by the EMA by a change in definition of the Dose based on which Solubility is determined. The subsequent eligibility for a biowaiver, depending on the BCS class, also differs per guidance. Such regional differences are likely to hinder application of the biowaiver concept by globally operating pharmaceutical industries. Harmonization seems preferable.

The application of the risk analysis to biowaiver guidance as described in **Section 3.1** shows an obvious gap in applied understanding of excipient effects on bioavailability of the API. This may be due to the absence of knowledge on these effects, but also to the lack of initiatives to make the available experience accessible for evaluation. In the (pre)formulation stage, the focus seems to be on the API. After approval, the quantitative composition of individual products remains mostly confidential. A systematic approach to characterize and analyze excipient effects seems not to be available at either stage.

The **top-down** database research described in **Section 3.3** exemplifies how much information ('big data') is hidden in the databases of regulatory agencies. Data mining may disclose many interesting formulation development decisions. The case study with lactose showed how its quantity is varied to specified extents without affecting the bioequivalence of the generic product. This database research, perhaps supplemented by comparison of pharmacokinetic data, may be helpful in increasing overall knowledge of excipient effects and be used to estimate safety levels excipients. A limitation of this one-dimensional approach is the implicit assumption that the excipients itself do not interact and that manufacturing steps have limited influence. Furthermore, the database at regulatory agencies offers a view on approved, bioequivalent products. It would be helpful if these data could be complemented by results on bioinequivalent products as available at research centers.

Chitosan's discovery has been attributed to Rouget in 1859 (177). A search in Pubmed including 'chitosan' as key word retrieves more than 14 000 publications. Still the results presented in **Sections 3.4 and 3.5** demonstrate how little we actually know about its effects after oral intake in general and more specifically about its potential modulation of the bioavailability of acyclovir. The outcome of the comparative bioavailability trial in **Section 3.4** was not as foreseen, even though the conditions were based on publications on chitosan's *in vitro* effects on permeation of acyclovir in a Caco-2 model. Publication of these studies might help reduce trial and error approaches with the combination of acyclovir and chitosan by other researchers.

The results thus underline the continued relevance of the classical bottom-up approach while developing a new formulation and estimating excipient effects. Application of biorelevant models facilitates optimal substance and product understanding and consequential decision making.

However, the application of models at the stage of drug discovery and development should not be confused with the use of models for biowaiver purposes or for QC testing. Drug development aims at promoting promising drug candidates or formulations to the next level of development and at excluding only truly bad candidates. False negative outcomes are not favourable in this case as this would exclude the wrong candidates, while a certain probability of false positives may be acceptable if these can be identified at the next development stage. For biowaiver purposes or for QC testing, however, it is critical that bioinequivalent formulations will not pass the test. Discriminatory capacity is, thereby, more important than biopredictability.

Current regulatory guidance offers very limited possibilities to exempt from *in vivo* studies (biowaiver) for BCS class 3 compounds like acyclovir in case of changes in excipient content. Identification of a biorelevant model might offer new biowaiver options, as discussed below.

So far, a public, systematic approach to classify excipient effects and model outcomes is lacking. Such an excipient-centered approach could include an evaluation of the characteristics of the excipient, its effects on the physiological system of the GI tract and the relevance of this effect in combination with specific APIs. Ideally, such a system would include standardized biorelevant test models and criteria for evaluation of the obtained results covering all or most relevant mechanisms by which an excipient could influence the bioavailability of an API. As Kostewicz *et al.* note it may be valuable to invest in complex models at the beginning of drug development and apply more simple modes for later stages (70).

Table 4.1 provides a simplified outline of such a toolbox. A systematic approach highlighting the strengths and limitations of a justified set of models is recommended. The approach of a decision tree in combination with a toolbox, resulting in a case by case selection of the most appropriate test method, as suggested by Kostewicz et al for product development in general could be applied in this context too.

Further evaluation and validation of the models in **Section 3.5** is needed to determine their value in drug development. The biorelevance of the models depends on the model characteristics and on the applied conditions. The search for biorelevant media, for example, is ongoing. Guidance on relevant dilution factors is also lacking. Concentration profiling to determine biorelevant luminal concentrations is an interesting topic for further research in that context (232). Work on shearing forces has further be reported as interesting topic for further research (70).

*Table 4.1 Excipient evaluation toolbox for development of oral IR dosage forms with systemic action**

Parameter in oral drug absorption	Mechanism of interaction with formulation or GI physiology	In vitro model
Drug release	Formulation: disintegration and dissolution	dissolution test at 3 pH levels
Luminal interactions		
	Degradation due to enzymes	Model simulating enzymatic conditions e.g. enzyme test
	Degradation due to physical conditions (e.g. pH, temperature)	Model simulating physical conditions e.g. TIM-1, in vitro tests
	Transit time	?
Absorption		
	Opening of tight junctions	Caco-2 model
	Interaction with transporter	Adapted tissue model with relevant transporter

*here limited to *in vitro* models

Detection

A biowaiver can be granted on condition that there is a model that is capable of excluding bioinequivalence. The probability of correctly detecting a bioinequivalent formulation depends on the model conditions and on the applied acceptance criteria. As noted above, the discriminatory capacity of the model is essential: it should correctly identify potential bioinequivalent formulations.

Currently, there is only one approved model for biowaiver testing: the dissolution test. The concept of dissolution testing at 3 pH levels is generally accepted for biowaiving immediate release oral formulations containing BCS class I APIs and, depending on the region, BCS class III and specific BCS class II APIs. The biorelevance of the dissolution test is thus considered sufficient for biowaiver purposes.

Addition of new models could offer new options for biowaiving. **Section 3.5** describes application of four models measuring the permeation of acyclovir in absence and presence of chitosan hydrochloride. The unmodified Caco-2 model showed the highest sensitivity to chitosan. The increased permeation of acyclovir was not seen *in vivo*, and the model is thus not biopredictive. Although the Caco-2 model has its limitations in correctly predicting human intestinal permeability

of paracellularly absorbed compounds (225), this sensitivity to excipient effects may have its utility in biowaiver context. Because of the observed cytotoxicity, the Caco-2 model functioned as an (over)discriminatory comparative *in vitro* test. Although the (extent of the) actual effects differed per model, the results obtained with the other permeability models discussed in section 3.5 confirm potential intestinal permeability modulating characteristics of chitosan . All these models thus suggest that a BCS-based biowaiver for a change in the content of potential excipient chitosan hydrochloride is not a viable option.

If more tools for comparative testing with the aim of biowaiving become available, their regulatory application will need to be discussed. Some specific variations can currently be supported by dissolution data only. The availability of a tool(box) should not automatically lead to its mandatory application for any variation or bioequivalence issue. Otherwise, this could inadvertently complicate the registration route for products that are currently categorized as low risk. Unjustified introduction of more mandatory tests will not only discourage development of new tools and models, but also be incorrect from a scientific perspective.

To avoid unnecessary strict requirements, a risk-based approach in implementation of new and/or tightened guidance is advisable and use of a risk-based decision tree can be considered to select the appropriate test method on a case-by-case basis.

Transparency in available knowledge on excipient effects may change the risk analysis. For example, **Section 3.3** concludes how lactose can be varied within usual ranges without the need for a bioequivalence study, if supported by an adequate comparative dissolution study. Considering the available knowledge on the potential effects of lactose, additional *in vitro* tests – even if available – may not be relevant for this specific excipient. This is illustrated by Table 4.2 which summarizes the risk analysis on changes in lactose content while retaining a bioequivalent product.

Table 4.2 Risk analysis – lactose in oral IR tablet

Failure mode	Target	Probability	Severity	Detectability	Overall Risk	Test?[$]
Dissolution	equivalent	Medium*	High	High	Medium*	Yes
Permeability	equivalent	Low	High	Zero	Low	No
Intraluminal fate	equivalent	Low	High	Zero	Low	No
Transit time	equivalent	Low	High	Zero	Low	No

*Depending on API characteristics; see also Chapter 4.
[$]Yes= use validated comparative test method or avoid/refuse difference in excipient

The chitosan case could have started by an initial risk evaluation as summarized in Table 4.3.a. With the knowledge as obtained from the *in vivo* study and the results from the models, this risk analysis can change to Table 4.3b.

Table 4.3a. Initial risk analysis – chitosan

Failure mode	Target	Probability	Severity	Detectability	Overall Risk	Test? [$]
Dissolution	equivalent	Zero[#]	High	N/A	N/A	No
Permeability	equivalent	High	High	Medium	High	Yes
Intraluminal fate	equivalent	Medium	High	Medium	High	Yes
Transit time	equivalent	Medium	High	Low	High	Yes

[$]Yes= use validated comparative test method or avoid/refuse difference in excipient

Table 4.3b. Final risk analysis – chitosan

Failure mode	Target	Probability	Severity	Detectability	Overall Risk	Test? [$]
Dissolution	equivalent	Zero#	High	N/A	N/A	No
Permeability	equivalent	Medium	High	Medium	Medium	Yes
Intraluminal fate	equivalent	Low	High	Medium	Medium	Yes
Transit time	equivalent	Medium	High	Low	High	Yes

[#] API was in solution

[$]Yes= use validated comparative test method or avoid/refuse difference in excipient

Severity

In the above examples in Table 4.2 and 4.3.a/b, the severity has been ranked as high, independent of the API. However, regulatory requirements not only need to consider reducing the probability of incidence or non-detection as much as possible but also take account of the severity of the potential bioinequivalence.

The severity of the possible bioinequivalence depends on the API. Improved access to safety evaluations and therapeutic windows of APIs may help definition of specific acceptance criteria. **Section 3.2** provides an example of an API for which, depending on the criteria, a biowaiver may

or may not be applied. Prednisolone's usual doses would classify it as a BCS class I drug, while strict adherence to the maximum administered dose – which is usually not intended for chronic use - would rule out this option.

4.2. Regulatory perspective

The available guidance on biowaivers shows that EU and US regulators are in principle open to submissions including adequate justification of full BCS based biowaivers or waivers in the context of a change in composition of the product. For BCS class I biowaivers 'qualitative differences' are acceptable, if appropriately justified and provided that those excipients that might affect the bioavailability are qualitatively and quantitatively the same. In case of a change in composition, 'minor changes' in excipient content may be accepted based on dissolution data only i.e. without addressing other steps in the absorption process. In such a case, the classification of the change as 'minor' is to be assessed on a case-by-case basis. To avoid the need for comparative testing of each difference in composition and clarify the classification of changes as 'minor' or 'major', publication of information of the effects of specific excipients in combination with specific APIs seems useful.

In Quality by design terminology, the quality target product profile includes 'pharmacokinetics' as quality attribute with target 'bioequivalent' (i.e. .compliant with bioequivalence criteria'). Dissolution may be identified as a critical quality attribute that determines the bioequivalence. Factors like 'permeability', 'intraluminal fate' or 'transit time' are normally not mentioned as (critical) quality attributes and no specific tests are included in the control strategy. Still, besides the dissolution test, many other models and many relevant experience with excipient effects must exist. Little of this knowledge obtained in drug development studies is currently translated into regulatory guidance on excipients. Authorities may, therefore, seem unnecessary restrictive in the acceptance of differences in excipients. However, it should also be noted that authorities do not dispose of these, mostly confidential, company data and development of regulatory acceptable models depends on the availability of public data and shared knowledge.

Regulatory agencies do have an archive of application files. However, just as pharmaceutical industry focuses on bringing a product to the market, regulatory agencies are generally organized and financed in a way to process individual application procedures. Although regulatory agencies acknowledge the relevance of scientific research as illustrated by the implementation of the quality by design concept and references such as the MEB's website www.regulatoryscience.nl or the EMA's

logo ("science, medicine, health"), financial and organizational restrictions may not easily allow time-consuming product-independent evaluation.

4.3. Overall evaluation and future perspectives

This thesis combines scientific evidence with regulatory reality on a topic that is part of public discussion: generics. The idea was to support public health by reducing the regulatory burden of new generic applications by identifying new biowaiver options. Application of the biowaiver concept requires definitions and evaluation of acceptance criteria for the risk of bioinequivalence. The perception of the risk associated with a biowaiver depends on the expertise and experiences of the risk evaluator, e.g. as a scientist, regulator or patient. Considering the subjectivity of risk assessments, a generally acceptable balance between increased uncertainty of bioequivalence and the risk mitigation measures applied, can be difficult to define. However, all these potential evaluators would benefit from increased understanding of excipient effects to improve or confirm their assessment.

In general, increased transparency of available scientific data as well as regulatory decision-making is encouraged. In addition, harmonization of evaluation criteria of BCS classification, biowaiver access criteria, biowaiver test conditions and acceptance criteria as well as clarification of the definition of narrow therapeutic index drugs would enhance insight in the regulatory possibilities.

This thesis also showed how the top-down and bottom-up approach may be of complementary value in helping improve excipient understanding. New options for surrogate *in vitro* testing are open for further research, while data mining may disclose available knowledge. Future research may be used to consciously apply these approaches for individual excipients in relation to biowaiver discussions.

Current international efforts joining industry and regulatory forces and aiming at improving the understanding of oral drug absorption, development of biorelevant tools and optimization of the use of the biopharmaceutic classification system (e.g. regulatory harmonization, consideration of subclasses) may all contribute to optimization of the biowaiver concept. Although scientific evidence is not always sufficient to establish or change regulatory guidance, this thesis provides concepts that can help systematic extension of our knowledge and facilitate translation of this evidence to regulatory science.

5. SUMMARY

5.1. Summary

For orally administered products with systemic action, the plasma profile of the active pharmaceutical ingredient is generally considered as a measure of both efficacy and safety of the medicinal product. In case of changes to an existing product or development of a generic version, confirmation of equivalent plasma profiles is sought through a so-called bioequivalence study. Such an *in vivo* bioequivalence study in human volunteers may be waived if there is sufficient *in vitro* evidence of the equivalence of the test and reference products at issue. Biowaiver options depend on the characteristics of the API and the formulation.

This thesis evaluates international (US, EU and WHO) regulatory conditions for biowaiving from a risk-based perspective with the aim to identify new biowaiver options. Regional differences in biowaiver conditions are obvious. Deviating definitions of the substance characteristic 'solubility' may have important consequences for the required evidence to show bioequivalence of a generic product. Harmonisation of the criteria as a common effort of authorities, academia and industry may facilitate application of the biowaiver approach and thus reduce the number of human studies.

Understanding of the biopharmaceutic effects of excipients may extend biowaiver options. Lactose, for example, is used in highly variable quantities in similar tablets and capsules in combination with APIs from different BCS classes. In addition, an effect of lactose on other parameters than dissolution is not known from literature. Therefore, a comparative *in vitro* dissolution study is considered sufficient to support a change in lactose content if varied within the approved range.

The biopharmaceutic effects of potential excipient chitosan on the absorption of acyclovir were explored both in humans and in several models. In a human study, chitosan showed a significant negative effect on C_{max}, T_{max} and AUC of acyclovir. The effect of the same chitosan on acyclovir was then tested in a bioaccessibility model as well as in four permeation models. The results showed potential intestinal permeability modulating characteristics of chitosan. A BCS-based biowaiver for a change in this potential excipient does thus not seem a viable option. Further studies are necessary to determine the suitability of the models for application as a tool for biowaiver purposes.

5.2. Zusammenfassung

Bei peroral eingenommenen Arzneimitteln wird das Plasmakonzentrations-Zeit-Profil des aktiven Stoffes im Allgemeinen als Maß für die Wirksamkeit und Sicherheit des Arzneimittels angesehen. Bei Veränderungen an bestehenden Arzneimitteln oder der Entwicklung eines Generikums kann durch Bioäquivalenzstudien an gesunden Probanden untersucht werden, ob das Plasmakonzentrations-Zeit-Profil gleich bleibt. Auf eine solche Studie kann verzichtet werden (sog. Biowaiver), wenn ausreichend bewiesen ist, dass das Test- und das Referenzprodukt in vitro äquivalent sind. Die Möglichkeit für einen Biowaiver hängt von den Eigenschaften des aktives Stoffes und der Formulierung ab.

Die vorliegende Doktorarbeit untersucht die internationalen (US, EU und WHO) regulatorischen Bedingungen für einen Biowaiver aus einer risikoorientierten Perspektive mit dem Ziel, neue Möglichkeiten für einen Biowaiver zu identifizieren. Offensichtlich sind die Bedingungen für einen Biowaiver weltweit verschieden. Unterschiedliche Definitionen der Löslichkeit eines Arzneistoffes können entscheidende Konsequenzen haben für die Daten die für den Nachweis der Bioäquivalenz nötig sind. Eine Harmonisierung der Kriterien durch die Zulassungsbehörden, akademische Welt und Industrie könnte die Anwendung des Biowaiveransatzes vereinfachen und die Zahl der klinischen Studien verringern.

Das Verständnis der biopharmazeutischen Effekten von Hilfsstoffen könnte die Möglichkeiten für einen Biowaiver erweitern. Laktose wird zum Beispiel in sehr unterschiedlichen Mengen in vergleichbaren Tabletten und Kapseln mit Arzneistoffen aus unterschiedlichen Klassen des Biopharmaceutics Classification Systems (BCS) kombiniert. Daneben ist aus der Literatur bekannt, dass Laktose höchstens einen Effekt auf die Freisetzung des aktiven Stoffes hat und nicht auf andere Parameter. Daher könnte eine vergleichende In-vitro-Wirkstoff-Freisetzungsstudie ausreichend sein um eine Veränderung des Laktosegehaltes im zugelassenen Bereich zu unterstützen.

Die Möglichkeiten der Anwendung von neuen Modellen für Biowaiver wurden anhand von Chitosan untersucht. Chitosan ist bekannt als ein potentieller Modulator der intestinalen Absorption. In einer klinischen Studie hatte Chitosan einen signifikant negativen Effekt auf auf die Maximalkonzentration (C_{max}), den Zeitpunkt ihres Auftretens (t_{max}) und die Fläche unter der Kurve (AUC) von Acyclovir. Der Effekt der gleichen Chitosankonzentration wurde danach in einem Biozugänglichkeitmodell und in vier Permeationsmodellen untersucht. Auch wenn die Ergebnisse variabel waren, wurde in allen Permeationsmodellen bestätigt, dass Chitosan potentiell die intestinale Permeabilität beeinflussen kann. Ein Biowaiver auf der Basis des biopharmazeutischen Klassifikationssystems ist daher nicht gerechtfertigt bei einer Veränderung dieses Hilfsstoffes.

5.3. Samenvatting

Voor het op de markt brengen van een nieuw geneesmiddel moet de farmaceutische industrie onderzoek doen naar werkzaamheid en veiligheid van het product. Dit onderzoek gebeurt eerst in laboratoria en in dierproeven en daarna in mensen (klinische studies). Voor generieke (merkloze) geneesmiddelen is het niet nodig dat alle klinische studies herhaald worden. En ook bij aanpassingen van een bestaand geneesmiddel, bijvoorbeeld het wijzigen van een hulpstof, zijn niet volledig nieuwe studies nodig. Voor geneesmiddelen die via de mond worden ingenomen en hun werking uitoefenen via de bloedbaan, volstaat in zulke gevallen een onderzoek waarbij aangetoond wordt dat de bloedspiegels gelijkwaardig zijn. Dit wordt een bioequivalentiestudie genoemd. Dergelijk onderzoek wordt uitgevoerd in gezonde vrijwilligers die zowel het nieuwe product innemen als het bestaande product.

In sommige gevallen is een bioequivalentiestudie niet nodig (biowaiver), maar volstaat een vergelijkende laboratoriumtest. Dit proefschrift gaat over regelgeving rond biowaivers. Deze regelgeving houdt het risico dat producten onterecht slagen voor de laboratoriumtest zo klein mogelijk door strenge beperking van de mogelijke verschillen. De eisen die aan een biowaiver gesteld worden, wijkenechter in Europa en de Verenigde Staten op een aantal punten van elkaar af. Om het nodeloos uitvoeren van studies in mensen te verminderen, is het gewenst dat overheid en industrie hun krachten bundelen om eenduidige, geharmoniseerde afspraken te maken.

De regelgeving biedt ruimte om nieuwe criteria en testmodellen te ontwikkelen die studies in mensen kunnen vervangen, bijvoorbeeld als de producten verschillen in hulpstoffen. De criteria voor hulpstof lactose zouden op grond van dit proefschrift verruimd kunnen worden. Uit de database van in Nederland geregistreerde geneesmiddelen blijkt namelijk dat lactose in zeer diverse hoeveelheden voorkomt in vergelijkbare tabletten en capsules. Als de toegepaste hoeveelheid binnen gebruikelijke marges blijft, lijkt het daarom niet nodig een bioequivalentiestudie uit te voeren bij een verandering in hoeveelheid lactose, maar kan worden volstaan met vergelijkende oplosbaarheidsstudies.

We hebben daarnaast de toepasbaarheid van een vijftal nieuwe modellen onderzocht aan de hand van modelstof chitosan. Chitosan is een stof van natuurlijk oorsprong die de opname van geneesmiddelen zou kunnen bevorderen. Uit onze studies in mensen blijkt dat de stof chitosan de bloedspiegels van geneesmiddel acyclovir juist kan verlagen. Geen van de geteste modellen voorspelde de uitkomst van de humane studie precies. Als er sprake is van een verschil in chitosan, is het daarom niet mogelijk om de toegepaste laboratoriummodellen in te zetten ter vervanging van de studie in mensen. De resultaten wijzen er juist op dat in dit geval een studie in mensen aan te bevelen is om het daadwerkelijke effect te achterhalen.

6. REFERENCES

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7. APPENDICES

7.1. Supplementary information to section 3.4: Bioanalytical method validation

Description

The bioanalytical method was modified from previously reported methods. Plasma samples of 500µl were prepared for analysis by solid phase extraction, using Oasis HLB 1cc cartridges (30mg) supplied by Waters. The column was washed with 500ml water and then eluted with 500µl acetonitrile. The extract was centrifuged at 14000 rpm for 10 minutes at -5°C and the concentration of acyclovir was determined by LC-MS/MS using a sample injection volume of 10 µl. The HPLC consisted of a Prontosil C18; 100*2,00mm; 5µm column, using an Agilent 1100 LC binary pump. A gradient elution was used with mobile phase A and B where A consisted of 15mM ammonium acetate + 0.1375% formic acid at pH 3.5 and B was acetonitrile + 0.1375% formic acid. Details of the gradient:

Step	Total Time(min)	Flow Rate(µl/min)	A (%)	B (%)
0	0.00	300	97.0	3.0
1	0.50	300	97.0	3.0
2	0.60	300	5.0	95.0
3	1.20	300	5.0	95.0
4	2.00	300	97.0	3.0
5	5.50	300	97.0	3.0

Detection took place by a triple quadrupole LC-MS/MS mass spectrometer, API 3000 manufactured by AB Sciex Instruments, using multiple reactions monitoring with transitions Q1/Q3: 255,992→ 151,873. Source temperature was 500°C, overall run time was 5.5 minutes. The method was linear in a range of 10 to 800 ng/ml, with a detection limit of 1 ng/ml. QC samples were analyzed with the plasma samples to monitor method accuracy and precision.

Table 7.1 .1 Summary of bioanalytical method validation data

Parameter	Control
Short description of the method	HPLC/MS/MS – see above
Biological matrix	Plasma
Analyte	Acyclovir
Internal standard (IS)	See selectivity
Calibration concentrations (ng/ml) – 10; 12.5; 25; 50; 200; 400; 800	
Lower limit of quantification (ng/ml)	10 ng/ml, accuracy 106%, CV 26.0%
QC concentrations (ng/ml)	
Between-run accuracy	By QC
Between-run precision	By QC
Within-run accuracy	By QC
Within-run precision	By QC
Matrix Factor (MF) (all QC)	Not applicable
Short term stability of the stock solution and working solutions (Observed change %),	By QC
Short term stability in biological matrix at room temperature or at sample processing temperature. (Observed change %)	By QC
Long term stability in biological matrix (Observed change %)	Not available, see short term stability
Autosampler storage stability (Observed change %)	By QC
Post-preparative stability (Observed change %)	By QC
Freeze and thaw stability (Observed change %)	Not available, confirmed by literature

Selectivity

The analytical method is able to differentiate acyclovir from endogenous components in the matrix or other components in the sample. Ganciclovir was added to the samples as optional internal standard. However, the CV and correlation coefficient of the method were not improved by addition of ganciclovir. Calculation of acyclovir concentrations was therefore performed not taking into account the ganciclovir. No other interfering peaks were observed.

Carry-over

Calibration curves were obtained using increasing and decreasing concentration ranges. Accuracy and precision remained acceptable independent of the order of injection. No carry-over was observed.

Calibration curve

The back calculated concentrations of the calibration standards/QC samples were generally within 15% of the nominal value. The intraday precision data in Table 2 showed a CV<15% for 6 out of 7 concentrations, with a CV 26% for the LLOQ, while the interday precision (11 days) data in Table 3 showed a CV <15% for 6 out of 7 concentrations and CV<20% for one concentration. This was considered acceptable.

Table 7.1.2 Intraday precision and accuracy

Nominal Concentration [ng/ml]	Number of Values	Mean Calculated Concentration	Accuracy [%]	Std. Deviation	%CV
10	3	10.64	106.4	2.77	26.0
12.5	3	10.48	83.8	1.54	14.7
25	3	25.24	101.0	3.34	13.2
50	3	47.53	95.1	3.58	7.5
200	3	191.33	95.7	30.79	16.1
400	3	413.49	103.4	25.96	6.3
800	3	795.59	99.4	59.82	7.5

1. overall regression equation: y = 0.0123 x (r = 0.9978)
2. ascending 1: y = 0.0114 x (r = 0.9980)
3. descending: y = 0.0131 x (r = 0.9998)
4. ascending 2: y = 0.0124 x (r = 0.9997)

Table 7.1.3 Interday precision and accuracy

Nominal Concentration [ng/ml]	Mean calculated concentration at day 1-11	Accuracy [%]	Std. Deviation	%CV
10.0	10.12	101.15	0.39	3.85
12.5	9.99	79.90	0.90	8.98
25.0	19.93	79.73	3.68	18.46
50.0	49.38	98.76	1.80	3.65
200.0	204.53	102.27	26.27	12.85
400.0	406.23	101.56	1.93	0.47
800.0	797.40	99.68	5.83	0.73

Accuracy

Within-run/intraday accuracy

See Table 2. Within-run accuracy was determined by analyzing in a single run 3 samples per level at 7 concentration levels covering the calibration curve range. The mean concentration was well within 15% of the nominal values for the QC samples, except for the LLOQ which was within 26% of the nominal value. Considering the other lower values this was considered sufficient for this study.

Between-run/interday accuracy

See Table 3. For the validation of the between-run accuracy, LLOQ, low, medium and high QC samples from at least seven runs analysed on a total of 11 different days were evaluated. The mean concentration was generally well within 15% of the nominal values for the QC samples, except for 12.5 and 25.0 ng/ml which remained within 20% of the nominal value.

Precision

Within-run precision

For the validation of the within-run precision, there should be a minimum of five samples per concentration level at LLOQ, low, medium and high QC samples in a single run. The within-run CV value should not exceed 15% for the QC samples, except for the LLOQ which should not exceed 20%.

Between –run precision

For the validation of the between-run precision, LLOQ, low, medium and high QC samples from at least three runs analysed on at least two different days should be evaluated. The between-run CV value should not exceed 15% for the QC samples, except for the LLOQ which should not exceed 20%.

Matrix effect

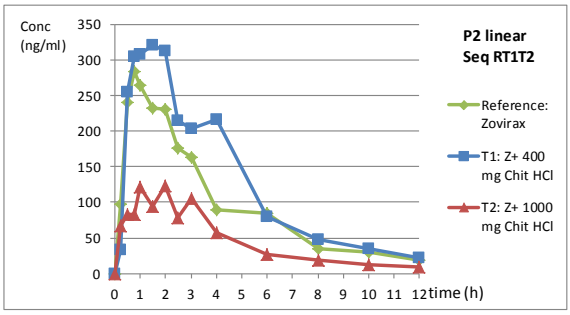
Matrix effects of plasma were excluded by comparison of samples of two different donors which both resulted in calibration curves with individual and mean r > 0.999 and acceptable CV and accuracy values; see also Table 4.

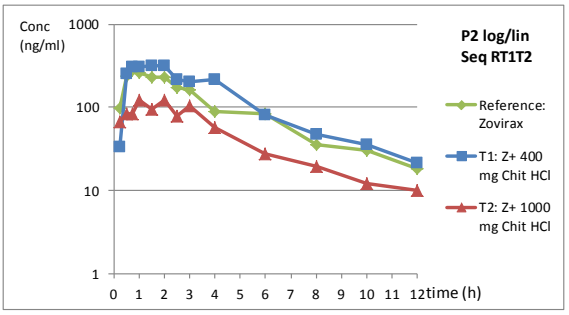
Table 7.1.4 Matrix effects in two different donor plasma samples

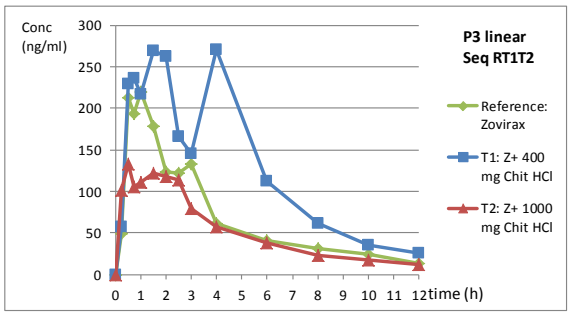
Expected Concentration [ng/ml]	Number of Values	Mean Calculated Concentration	% Accuracy	Std. Deviation	%CV
6.25	2	7.21	115.4	0.04	0.6
12.5	2	13.02	104.2	1.65	12.7
25	2	25.68	102.7	0.04	0.2
50	2	50.39	100.8	8.54	17.0
100	2	102.97	103.0	1.50	1.5
200	2	198.27	99.1	3.74	1.9

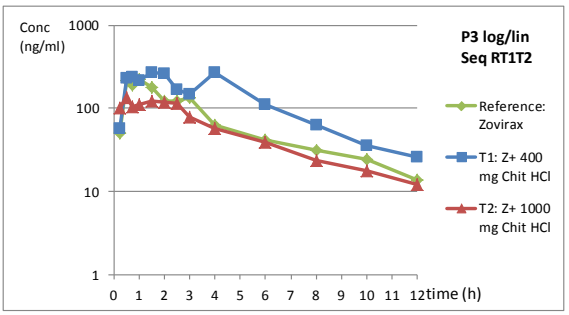
7.2. Supplementary information to section 3.4: Individual plasma profiles

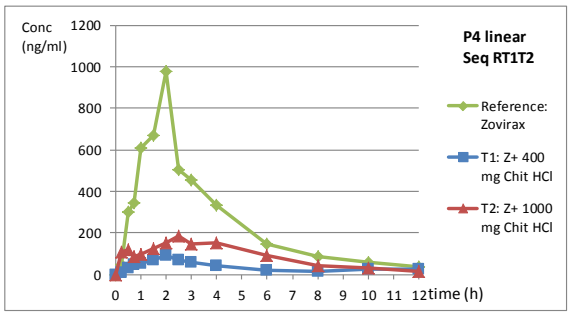
In the charts below, P indicates the volunteer number; per volunteer both the linear and log/linear graph are presented.

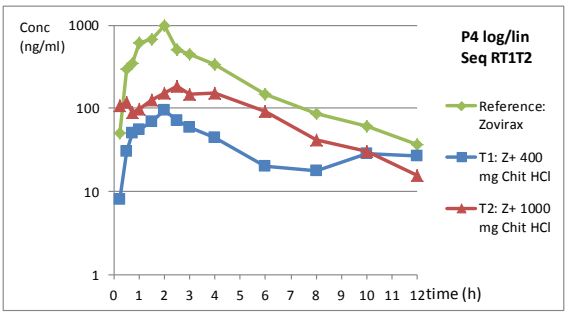


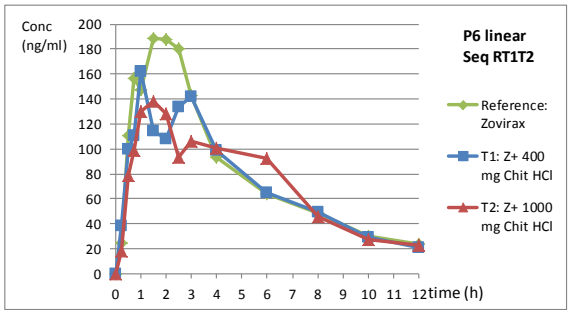


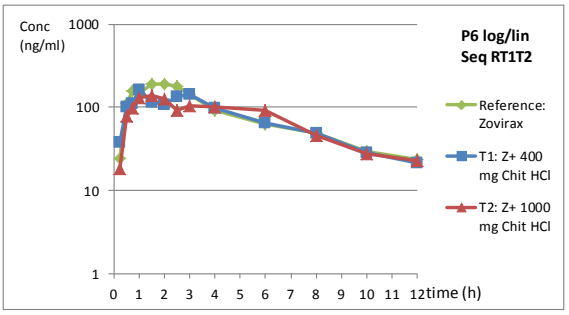


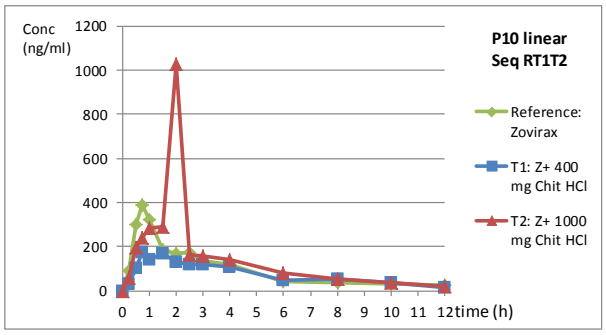


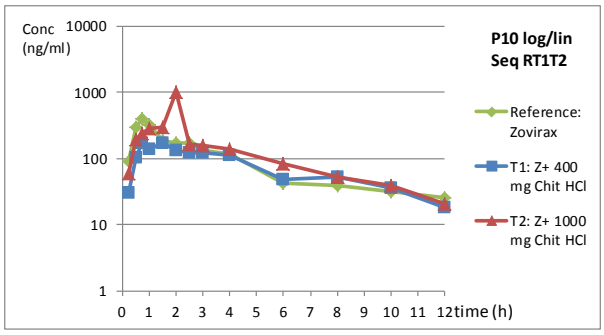


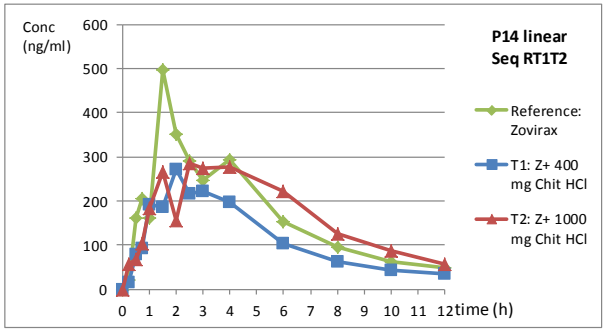







Conc (ng/ml)
P10 linear
Seq RT1T2
Reference: Zovirax
T1: Z+ 400 mg Chit HCl
T2: Z+ 1000 mg Chit HCl
time (h)


Conc (ng/ml)
P10 log/lin
Seq RT1T2
Reference: Zovirax
T1: Z+ 400 mg Chit HCl
T2: Z+ 1000 mg Chit HCl
time (h)


Conc (ng/ml)
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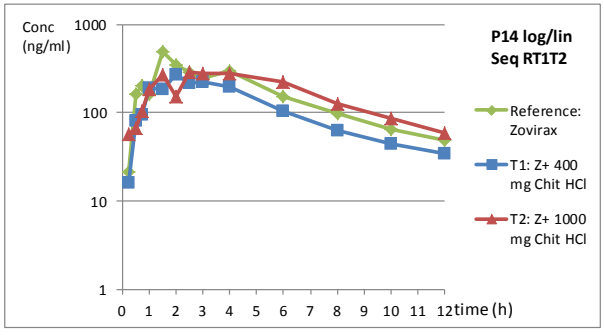
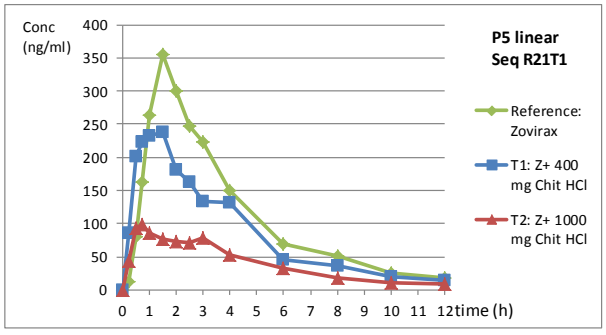
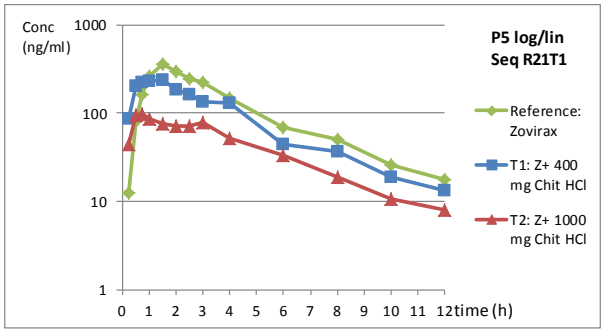
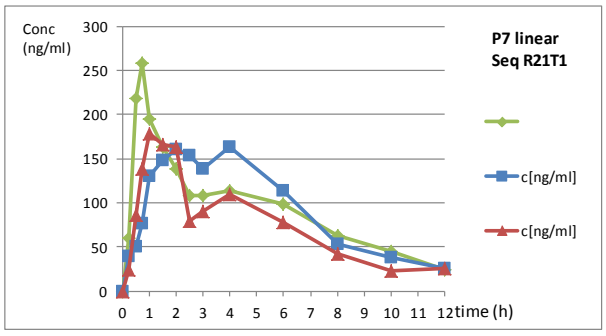
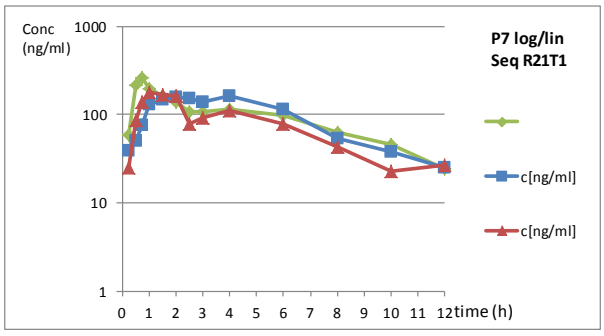
Seq RT1T2
Reference: Zovirax
T1: Z+ 400 mg Chit HCl
T2: Z+ 1000 mg Chit HCl
time (h)

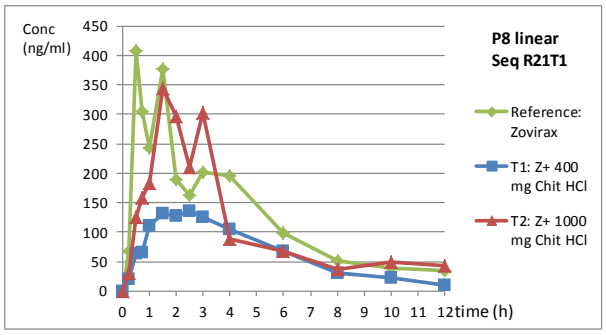

Conc (ng/ml)
P14 log/lin
Seq RT1T2
Reference: Zovirax
T1: Z+ 400 mg Chit HCl
T2: Z+ 1000 mg Chit HCl
time (h)


Conc (ng/ml)
P5 linear
Seq R21T1
Reference: Zovirax
T1: Z+ 400 mg Chit HCl
T2: Z+ 1000 mg Chit HCl
time (h)


Conc (ng/ml)
P5 log/lin
Seq R21T1
Reference: Zovirax
T1: Z+ 400 mg Chit HCl
T2: Z+ 1000 mg Chit HCl
time (h)


Conc (ng/ml)
P7 linear
Seq R21T1
c[ng/ml]
c[ng/ml]
time (h)


Conc (ng/ml)
P7 log/lin
Seq R21T1
c[ng/ml]
c[ng/ml]
time (h)


Conc (ng/ml)
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Reference: Zovirax
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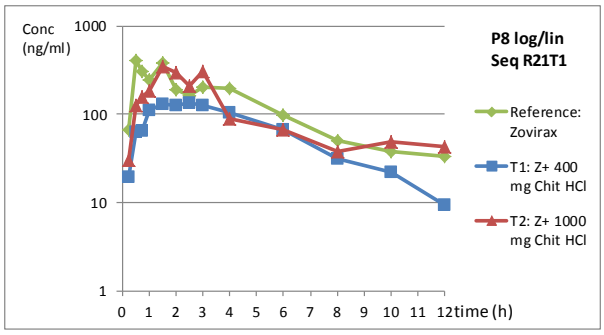
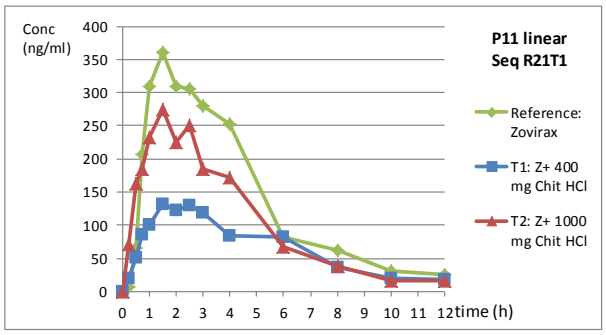
T2: Z+ 1000 mg Chit HCl
time (h)


Conc (ng/ml)
P8 log/lin Seq R21T1
Reference: Zovirax
T1: Z+ 400 mg Chit HCl
T2: Z+ 1000 mg Chit HCl
time (h)


Conc (ng/ml)
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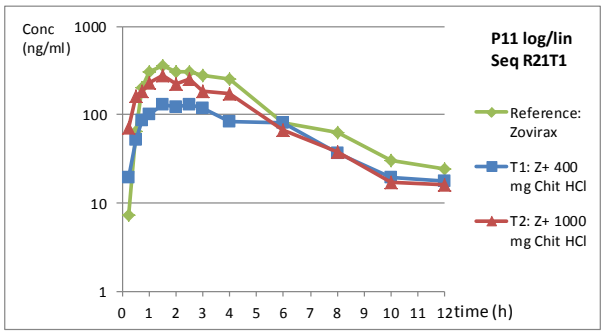
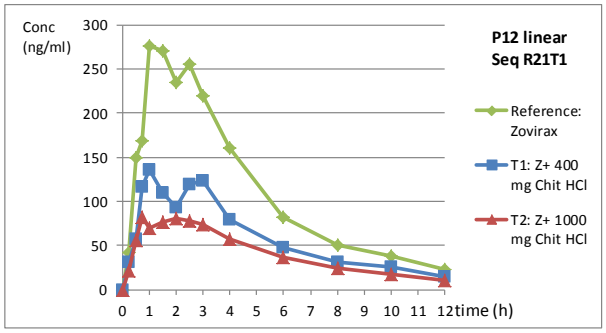
Reference: Zovirax
T1: Z+ 400 mg Chit HCl
T2: Z+ 1000 mg Chit HCl
time (h)


Conc (ng/ml)
P11 log/lin Seq R21T1
Reference: Zovirax
T1: Z+ 400 mg Chit HCl
T2: Z+ 1000 mg Chit HCl
time (h)


Conc (ng/ml)
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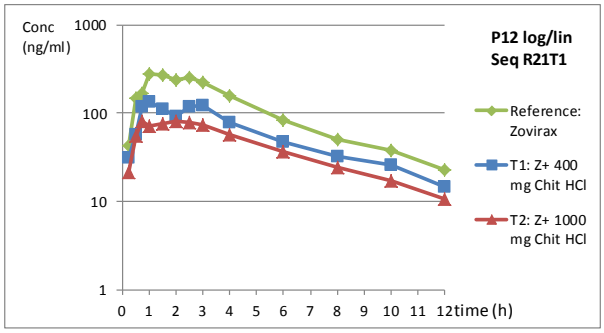
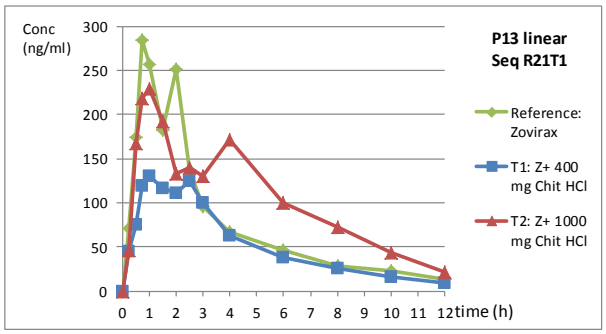
Reference: Zovirax
T1: Z+ 400 mg Chit HCl
T2: Z+ 1000 mg Chit HCl
time (h)


Conc (ng/ml)
P12 log/lin Seq R21T1
Reference: Zovirax
T1: Z+ 400 mg Chit HCl
T2: Z+ 1000 mg Chit HCl
time (h)


Conc (ng/ml)
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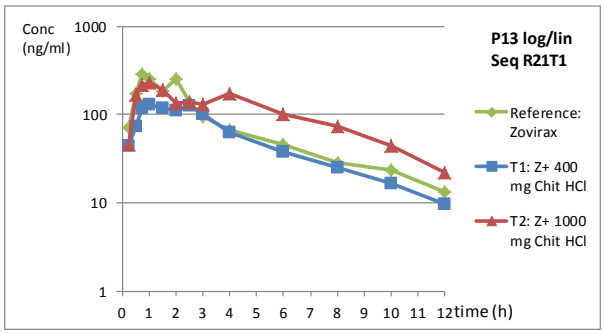
Reference: Zovirax
T1: Z+ 400 mg Chit HCl
T2: Z+ 1000 mg Chit HCl
time (h)


Conc (ng/ml)
P13 log/lin Seq R21T1
Reference: Zovirax
T1: Z+ 400 mg Chit HCl
T2: Z+ 1000 mg Chit HCl
time (h)

8. LIST OF PUBLICATIONS

Publications in relation to this thesis:

- Kubbinga M, Nguyen MA, Staubach P, Teerenstra S, Langguth P. The influence of chitosan on the oral bioavailability of acyclovir-a comparative bioavailability study in humans. Pharm Res. 2015; 32: 2241-2249.
- Kubbinga M, Moghani L, Langguth P. Novel insights into excipient effects on the biopharmaceutics of APIs from different BCS classes: Lactose in solid oral dosage forms. Eur J Pharm Sci. 2014;61:27-31.
- Sjogren, E., Abrahamsson, B., Augustijns, P., Becker, D., Bolger, M.B., Brewster, M., Brouwers, J., Flanagan, T., Harwood, M., Heinen, C., Holm, R., Juretschke, H.P., Kubbinga, M., Lindahl, A., Lukacova, V., Munster, U., Neuhoff, S., Nguyen, M.A., Peer, A.V., Reppas, C., Hodjegan, A.R., Tannergren, C., Weitschies, W., Wilson, C., Zane, P., Lennernas, H., Langguth, P., In vivo methods for drug absorption - comparative physiologies, model selection, correlations with in vitro methods (IVIVC), and applications for formulation/API/excipient characterization including food effects. Eur J Pharm Sci. 2014;57:99-151
- Sediq A, Kubbinga M, Langguth P, Dressman J. The impact of the EMA change in definition of "dose" on the BCS dose-solubility ratio: a review of the biowaiver monographs. J Pharm Sci. 2014;103(1):65-70.
- Kubbinga M, Langguth P, Barends D. Risk analysis in bioequivalence and biowaiver decisions. Biopharm Drug Dispos. 2013;34(5):254-61.

9. CONFLICTS OF INTEREST AND FINANCIAL DISCLOSURES

The research described in this thesis was initiated by RIVM in the framework of RIVM Strategic Research Programme (SOR, currently SPR), in which expertise and innovative projects prepare RIVM to respond to future issues in health and sustainability. The project received financial support from the Fédération Internationale Pharmaceutique (FIP). The work on excipients described in Chapter 3 received support from and contributes to the Innovative Medicines Initiative Joint Undertaking (http://www.imi.europa.eu) under grant agreement no. 115369. The clinical study as described in Chapter 3 was sponsored by the Product Quality Research Institute (PQRI).

Marlies Kubbinga is employed as a quality assessor at the Medicines Evaluation Board of the Netherlands, but the views presented in this thesis do not necessarily reflect the opinion of the Board.

10. ACKNOWLEDGEMENTS/DANKWOORD/DANKSAGUNG

This thesis is the result of efforts and patience from many sides. I hereby wish to thank everyone who allowed the project and me to move forward. I am grateful for each of your supporting words or actions. Some personal words:

11. CURRICULUM VITAE