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UNIVERSITY OF ZAGREB
FACULTY OF PHARMACY AND BIOCHEMISTRY

MARIJANA ERCEG

EFFECTS OF SALT FORMS ON THE ORAL ABSORPTION OF HIGHLY PERMEABLE WEAK BASE DOXAZOSIN

DOCTORAL THESIS

Zagreb, 2011

SVEUČILIŠTE U ZAGREBU
FARMACEUTSKO-BIOKEMIJSKI FAKULTET

MARIJANA ERCEG

UTJECAJ RAZLIČITIH SOLI VISOKO PERMEABILNE SLABE BAZE DOKSAZOSINA NA ORALNU APSORPCIJU

DOKTORSKI RAD

Zagreb, 2011.

UNIVERSITY OF ZAGREB
FACULTY OF PHARMACY AND BIOCHEMISTRY

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EFFECTS OF SALT FORMS ON THE ORAL ABSORPTION OF HIGHLY PERMEABLE WEAK BASE DOXAZOSIN

DOCTORAL THESIS

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Zagreb, 2011

Rad je predan na ocjenu Fakultetskom vijeću Sveučilišta u Zagrebu Farmaceutsko-biokemijskog fakulteta radi stjecanja akademskog stupnja doktora znanosti u znanstvenom području Biomedicina i zdravstvo, polje farmacija, grana farmacija.

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SUMMARY

In this study, the usefulness of biorelevant *in vitro* data and of canine data in forecasting early exposure after the administration of two phases of a BCS Class II compound, i.e., doxazosin base (DB) and its mesylate salt (DM) was evaluated. DB, DM, and doxazosin hydrochloride (DH) were prepared and extensively characterized.

The solubility of prepared substances was tested *in vitro* in various media, including human aspirates, using the shake flask method. Dissolution experiments were performed in simple buffer media and biorelevant media simulating gastric and intestinal fluid in the fasted and fed state. Pharmacokinetic (PK) studies were performed in dogs with DB and DM tablets in the fasted and fed state, while the results form human PK study on DM tablets in the fasted state were available from previous Pliva´s study. Analytical method for determination of doxazosin in canine plasma was developed and validated using canine samples collected in the fed state in order to assure the suitability of the method for measurement of low concentrations of doxazosin in plasma and high specificity of the method despite the number of interfering compounds in fed state plasma samples.

Solubilities of DB and DM in human gastric fluid were forecasted by data in the fasted state simulating gastric fluid containing physiological components (FaSSGF-V2) but not by data in $HCl_{pH1.8}$. Unlike data in FaSSGF-V2, dissolution of DB and DM tablets in $HCl_{pH1.6}$ is rapid. Dissolution of DB tablet in FaSSGF-V2 is incomplete and conversion to DH seems to occur. Differences between DB and DM in dissolution in the small intestine are overestimated in the absence of physiological solubilizers. Using the *in vitro* data and previously described modelling procedures, the cumulative doxazosin profile in plasma was simulated and 0–2 h profile was used for evaluating early exposure. Individual cumulative doxazosin profiles in plasma, after single DM tablet administrations to 24 adults in the fasting state were constructed from corresponding actual plasma profiles. Compared with *in vitro* DM data in aqueous buffers, DM data in biorelevant media led to better prediction of early exposure. Based on intersubject variability in early exposure after DM administration and simulated profiles, the administered phase, DB or DM, does not have a significant impact on early exposure in fasting state. Early exposure in dogs (evaluated based on partial *AUC*s) was significantly higher after administration of DM to dogs. Therefore, dog is not a good model for predicting differences between DB or DM in the fasted state, but it may be a good model for predicting food effects and differences between DB and DM in the fed state.

SAŽETAK

Glavni ciljevi ovog rada bili su istražiti da li postoje razlike u apsorpciji između lipofilne slabe baze doksazosina (DB) i njegove mesilatne soli (DM), procijeniti sposobnost predviđanja tih razlika u ljudi temeljem *in vitro* podataka, te *in vivo* podataka dobivenih farmakokinetskom studijom na psima. Obzirom da baze i ne-hidrokloridne soli mogu prelaziti u hidrokloridne soli u kiselom mediju želuca, pored DB i DM pripremljena je, karakterizirana i *in vitro* ispitana i hidrokloridna sol doksazosina (DH).

Topljivost pripremljenih supstancija ispitana je *in vitro* u različitim medijima metodom zasićene otopine. Brzina oslobađanja ispitana je u jednostavnim vodenim puferima kao i biorelevantnim medijima koji simuliraju želučane i crijevne tekućine bez hrane i s hranom. Farmakokinetičke studije na psima napravljene su sa DB i DM 2 mg tabletama, sa i bez hrane, dok su rezultati studije na zdravim dobrovoljcima nakon primjene 2 mg DM tableta bez hrane bile raspoložive iz prijašnje Plivine studije. Analitička metoda za određivanje doksazosina u psećoj plazmi razvijena je i validirana koristeći pseću plazmu prikupljenu u studiji s hranom, s obzirom da se u tom slučaju očekuju niže koncentracije lijeka te veći broj interferirajućih spojeva u plazmi koji bi mogli narušavati specifičnost bioanalitičke metode.

Rezultati *in vitro* ispitivanja pokazuju da je topljivost DB i DM u humanom želučanom soku moguće bolje predvidjeti koristeći biorelevantni medij (FaSSGF-V2) nego razrijeđenu kiselinu $HCl_{pH1.8}$. Također je pokazano da je brzina oslobađanja doksazosina iz DB i DM tableta puno brža u $HCl_{pH1.6}$ nego u FaSSGF-V2. Nepotpuno oslobađanje aktivne supstancije iz DB tableta u FaSSGF-V2 ukazuje na prelazak DB u DH. Rezultati također pokazuju da su u odsustvu fizioloških solubilizatora, razlike u brzini oslobađanja doksazosina iz DB i DM tableta u tankom crijevu precijenjene.

Moguće razlike u apsorpciji između DB i DM procijenjene su mjerenjem rane izloženosti, tj. brzine apsorpcije 0–2 h nakon primjene lijeka. Direktna usporedba razlike u brzini apsorpcije između DB i DM kod ljudi i pasa nije moguća radi nedostatka podataka za DB na ljudima. Stoga je usporedba DB i DM kod ljudi pripremljena *in silico* modeliranjem. Kod pasa, s druge strane, nije moguće modeliranjem doći do krivulja koje bi opisivale postotak doksazosina u plazmi u ovisnosti o vremenu, radi nedostatka podataka nakon intravenske primjene, pa je stoga razlika u ranoj izloženosti između DB i DM kod pasa procijenjena koristeći parcijalne površine ispod krivulja apsorpcije (AUC_p).

Koristeći *in vitro* podatke, prosječnu bioraspoloživost nakon oralne primjene, enterohepatičku cirkulaciju doksazosina kod ljudi, te prethodno objavljene parametre *in silico* modeliranja, simuliran je kumulativni profil doksazosina u plazmi za DB i DM. Pojedinačni kumulativni profili doksazosina u plazmi nakon primjene jediničnih 2 mg doza DM tableta u 24 zdrava dobrovoljca, konstruirani su iz pripadajućih profila u plazmi.

Rezultati pokazuju da u slučaju DM tableta, *in vitro* podaci u biorelevantnim medijima bolje predviđaju ranu izloženost nego podaci u jednostavnim puferima.

Uzevši u obzir varijabilnost u ranoj izloženosti među pojedincima nakon primjene DM tableta te simulirane profile za DB i DM, dolazi se do zaključka da vrsta administrirane supstancije (DB ili DM) nema značajnog utjecaja na ranu izloženost kod ljudi. S druge strane, u studiji sa psima se pokazalo da je rana izloženost kod mesilatne soli bila značajno viša nego kod baze što upućuje na zaključak da psi nisu prikladan model za procjenu razlika u brzini apsorpcije između DB i DM kod primjene lijeka bez hrane, no mogli bi biti koristan model za predviđanje utjecaja hrane kao i razlika između DB i DM nakon primjene lijeka s hranom.

PUBLICATIONS OF DOCTORAL THESIS

Published scientific papers

1. Marijana Erceg, Marija Vertzoni, Helena Cerić, Miljenko Dumić, Biserka Cetina-Čižmek, Christos Reppas, 2011. *In vitro* vs. canine data for assessing early exposure of doxazosin base and its mesylate salt. European Journal of Pharmaceutics and Biopharmaceutics, *in press*.
2. Marijana Erceg, Mario Cindrić, Lidija Pozaić-Frketić, Marija Vertzoni, Biserka Cetina-Čizmek, Christos Reppas, 2010. A LC-MS/MS method for determination of low doxazosin concentrations in plasma after oral administration to dogs. Journal of Chromatographic Science 48: 114–119.

Posters presented on scientific meetings

1. *In vitro vs. canine data for assessing early exposure of doxazosin base and its mesylate salt*, poster presentation, AAPS 2011, Annual Meeting and Exposition, 2011, Washinghton DC, US.
2. *Dissolution behaviour of doxazosin base, mesylate salt and hydrochloride salt in biorelevant media,* poster presentation, Third Croatian congress of pharmacy with international participation, 2005, Cavtat, Croatia.

KEY WORDS

doxazosin base, doxazosin salts, absorption, early exposure, solubility, dissolution, dogs, humans

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1. INTRODUCTION

New drug molecules resulting form high-throughput screening are mainly large (high MW), lipophilic, low permeable and essentially less drug-like. These shifts have a significant negative impact on aqueous solubility, which represents a great challenge to drug product development due to affect on the bioavailability of a drug. The oral bioavailability of a drug is mainly a function of its solubility characteristics in gastrointestinal fluids, absorption into the systemic circulation and metabolic stability.

Therefore, during drug development huge efforts are invested in improving drug's solubility as well as finding appropriate environment *in vitro* for the solubility and dissolution evaluation, with the ultimate goal of increasing chances for successful clinical or bioequivalence study, and consequently reducing time and expenses of drug development.

There are numerous of ways for solubility enhancement, however, for ionisable drugs, preparation of salts is the most common and effective method for increasing solubility and dissolution rates. Changing a drug from its free base or acid to a salt form, changes its physicochemical properties, including solubility and dissolution rates, hygroscopicity, stability, impurity profiles, and crystal form (Paulekuhn et *al.*, 2007).

Although pharmaceutical salts theoretically exhibit higher dissolution rates, which is well known to be driving force for absorption, it still needs be to confirmed if this indeed affects absorption processes *in vivo*, in which cases and up to which extent.

Interestingly, literature review revealed that human bioequivalence studies comparing different salt forms of basic drugs with the free forms have been rather limited and none of them reported significant differences in bioavailability between different salt forms due to differences in their aqueous solubilities (Engel et *al*., 2000).

Obviously, the assumption that salts *in vivo* would exhibit higher dissolution and absorption rates based on *in vitro* data, should be taken cautiously. Such expectation must be based on thorough research regarding conditions and the biorelevance of *in vitro* experiments as well as physiological factors that influence the bioavailability of a drug.

If a dissolution test is used as a prognostic tool for *in vivo* behaviour, then the attention primarily has to be focused on the choice of dissolution medium. A need to develop dissolution test that would better predict the *in vivo* performance of drug was recognised long ago by Dressman et *al.* (1998). Since then, the compositions of biorelevant media have been improving by time, together with the awareness of physiological fluids compositions and processes that affect bioavailability. Today, *in vitro* data obtained with such thorough approach can predict *in vivo* behaviour with much more accuracy than ever before.

However, in order to obtain additional information about *in vivo* absorption characteristics, main absorption sites within the GI tract or absorption mechanism for the novel drug formulation, animals are still frequently used for the assessment of the *in vivo* performance of orally administered products at the preclinical level.

Dogs are most commonly used for evaluation of oral drug absorption, although it is recognized that there are many physiological differences between humans and dogs. For example, dogs have faster gastric emptying and often less acidic fasting intragastric pH compared with humans (De Zwart et *al*., 1999). Such differences may limit their usefulness, especially in the comparison of salt(s) with the free form of a base. In the literature, canine data have not been assessed for their usefulness in forecasting potential differences in such case.

Even without such absorption obstacles, prediction and evaluation of *in vivo* performance of week bases may be rather difficult. Due to their ionization properties, lipophilic bases dissolve easier in the acidic gastric environment than in the almost neutral pH of the upper small intestine but complete dissolution of the dose prior to reaching the small intestine may not always be possible.

Improvement of drug dissolution during gastric residence with the use of salt of a base is typically decided on the basis of dissolution data and/or equilibrium solubility data in hydrochloric acid solutions (Serajuddin, 2007), although the equilibrium solubility of weak bases is not exclusively dependent on hydrochloric acid concentration (Vertzoni et *al.*, 2007). If dissolution is more complete *in vitro* in simulated gastric fluid than in stomach, its importance on plasma levels may (depending on disposition characteristics) be underestimated and vice versa (Kortejärvi et *al*., 2007). Furthermore, if the dose is partly dissolved during gastric residence, problematic dissolution in the small intestine can lead to low and/or variable oral bioavailability, especially in cases where intralumenal concentrations control the overall absorption process.

Interestingly, the extent at which the environment in the small intestine needs to be simulated for evaluating differences in dissolution between a free base and its salts has not been addressed in the literature.

Therefore, a low soluble lipophilic week base doxazosin and its two salts, mesylate and hydrochloride, were chosen as the model drugs for the investigation of possible *in vitro* and *in vivo* advantages of salts in comparison with the free base.

2. THEORETICAL PART

2.1. Physiological factors influencing drug absorption

In vivo, drug dissolution rate and consequently absorption are influenced by numerous physicochemical factors as well as physiological factors. The most important physiological factors are gastrointestinal (GI) pH and transit time, and influence of content, volume and hydrodynamics of GI tract.

2.1.1. Gastrointestinal pH profile

The pH of gastrointestinal tract varies widely with location. Typical values in the fasted stomach are pH 1–2 while in the upper small intestine the pH usually lies between 5 and 6.5 (Hörter and Dressman, 1997).

2.1.1.1. Gastric pH

There are complex variations in pH between the fed and fasted state. Upon ingestion of a meal, the gastric pH increases because of buffering effects of food components. In response to food ingestion, however, gastric acid is secreted, and by 3–4 h after the meal intake, the fasted state pH is re-established (Figure 1).

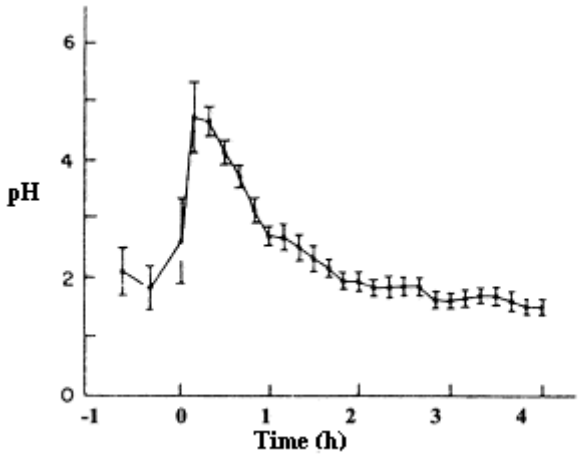


Figure 1. Gastric pH in the fasted state and after food intake (6,458 calories and 400 ml total volume) in 10 healthy volunteers (Hörter and Dressman, 1997)

The data from healthy human volunteers collected by Kalanzi et *al.*, 2006, revealed high variability of fasted stomach pH (range of individual pH values was 1.23–7.36). Median pH value was 2.4 twenty minutes after administration of water and stabilized to 1.7 at later time points.

Thirty minutes after food administration (Ensure Plus®) the median gastric pH was 6.4 and intersubject variability was low. This value is close to the pH value of Ensure Plus® (6.6).

Although intersubject variability increased with time, median pH values gradually decreased to reach 2.7 about 3 h and 30 min after the meal was given. The time required to restore the fasting pH levels depended mainly on the composition and the quantity of the meal.

Regarding weak bases, they will be less soluble in the stomach if given immediately after food intake because the gastric fluids are less acidic. This effect will however, be partly offset by the longer gastric emptying time in the fed state, which will afford more time for the drug to go into solution. It is important to note that the pH of the luminal fluids is also dependent on other factors like age, pathophysiological conditions such as achlorohydria etc.

2.1.1.2. pH in the small intestine

The small intestine pH at first decreases in response to a meal with the arrival of acidic chyme from the stomach but later the fasted state pH is re-established as a result of pancreatic bicarbonate output (Figure 2).

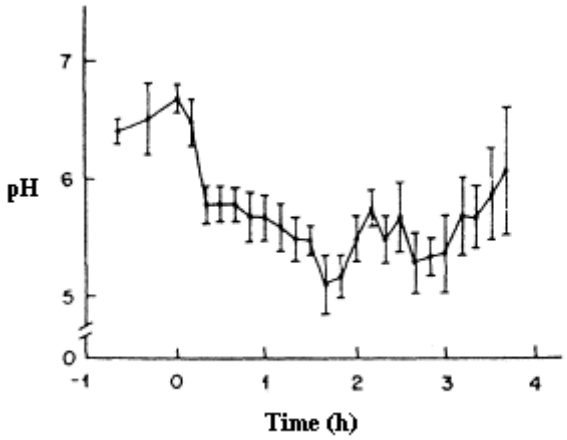


Figure 2. Duodenal pH in the fasted state and after food intake (6,458 calories and 400 ml total volume) in 10 healthy volunteers (Hörter and Dressman, 1997)

Kalanzi et *al.*, 2006, found out that, as in the fasting stomach, pH values in the fasted duodenum were also highly variable. The median pH was 6.2, which was in correlation with previously published data. In the fed state, data were less variable than in the fasting state. The median duodenal pH 30 min after meal administration was 6.6, somewhat higher than the fasting state value, but it fell slowly to 5.2 at 210 min after the administration of food (Ensure

Plus®). Although it was known earlier that in the fed state the pH decreases with time in the upper small intestine, these data suggested that it occurs faster than in the previous study.

2.1.2. GI transit time and hydrodynamics

Once a drug is given orally, the exact location and environment of the drug product within the GI tract is difficult to discern. GI motility tends to move the drug through the tract, so that the drug does not stay at the absorption site for long. Therefore, gastric emptying time and intestinal transit time are very important for drug release and absorption.

When given with liquid (water) or solid (with food) drugs may have very different gastric emptying times. It depends on the phase of the interdigestive migrating myoelectric complex (IMMC) and the amount of coadministered water; 200 ml of water generally caused a faster gastric emptying than 50 ml (Li et *al*., 2005).

In the fasted state, oral solution and solid dosage forms empty from stomach via pylorus to the intestine. Oral solutions follow first order kinetics and multiple unit formulations zero order kinetics. Single unit formulations are emptied as a rapid bolus from stomach to intestine as a result of "house-keeping wave". Different authors reported the following times for gastric emptying: for oral solution $t_{50\%}$ 12 min, single unit formulation in one case $t_{100\%}$ 32 min and in another 1 h, multiple unit formulations $t_{50\%}$ 66 min and $t_{90\%}$ 102 min. $T_{50\%}$, $t_{90\%}$ and $t_{100\%}$ were times when 50, 90 or 100 % of formulation was emptied from the stomach (Kortejärvi et *al*., 2007).

Intestinal propulsive movements will determine intestinal transit rate, and therefore the residence time of a drug in the intestine. Small intestinal transit is independent of dosage forms and it ranges from 3 to 4 hours (Li et *al*., 2005).

2.1.3. GI content and volume

Properties of gastric and intestinal content have been well characterized and reported in the literature. Even in the fasted state in humans, the *in vivo* dissolution medium is a complex and highly variable milieu consisting of various bile salts, electrolytes, proteins, cholesterol, and other lipids. The dominating ion in the stomach is chloride ion, with an average concentration of 102 mM, while the dominating ions in the jejunum are sodium and chloride ions (Li et *al.*, 2005).

Food influences absorption in many ways. Besides its impact on pH of gastric tract and gastric emptying time, the intake of food has two other main effects: first, food is a major source of lipids, which can then be digested to diglyceride and fatty acids; and second, food stimulates the secretion of bile salts, biliary lipids and pancreatic juice. All of these agents may solubilise drugs, thus enhancing absorption (Li et *al.*, 2005).

Beside this, the drug absorption process can be affected by many other factors, including calorie content of meal, nutrient composition, volume and temperature of the meal itself, fluid ingestion and interactions of the drug with food itself. Food can also increase blood flow to the liver (splanchnic blood flow) and therefore cause changes in first pass extraction (Lentz, 2008).

The influence of enzymes in the gastrointestinal tract is also significant. The primary enzyme found in gastric juice is pepsin. Lipases, amylases, and proteases are secreted from the pancreas into the small intestine in response to ingestion of food. These enzymes are responsible for most nutrient digestion.

Bacteria, which are mainly localized within the colonic region of the GI tract, also secrete enzymes that have been utilized when designing drugs or dosage forms to target the colon (Aulton and Cooper, 1988).

The volume of GI fluids have to be taken into account when dosing of drug substances orally because there could be a deficit in the volume available to dissolve a dose of a poorly soluble substance. The volume of fluids available in the gastrointestinal tract for drug dissolution is dependent upon the volume of coadministered fluids, secretions and water flux across the gut wall.

The fluid volume of the stomach in the fasted state is about 20–50 ml. The secretions of the paragastrointestinal organs (salivary gland, liver, and pancreas) are received by the first portion of the duodenum. Approximately 1.5 l of pancreatic juice and 600 ml of bile are secreted into the duodenum within 1 day. The sum of these secretions is about 5–6 l/day, and they are essential for the digestion of food (Dressman et *al.*, 1998). In addition, the intestine secretes about 1 l of water per day, mostly as a component of mucose.

With respect to media volume, for the successful prediction of *in vivo* drug behaviour, the *in vitro* setups should preferably be based on the physiology, but the practical limitations of the test apparatus also need to be considered. Based on the Jantratid and Dressman 2009, the fluid volume in the stomach would initially be around 300 ml in the fasted state and 500 ml or more in the fed state, while in the small intestine, volumes of 200 ml in the fasted and 1 l in the fed state appear to be reasonable.

2.2. Drug absorption after oral administration of immediate release dosage forms

As described in Figure 3, upon administration, solid oral dosage form first disintegrates into small particles. The process of disintegration does not imply complete dissolution of the dosage form; instead dissolution process occurs from the surface of all solids that are in contact with dissolution medium, including fine particles, granules/aggregates as well as solid dosage form itself.

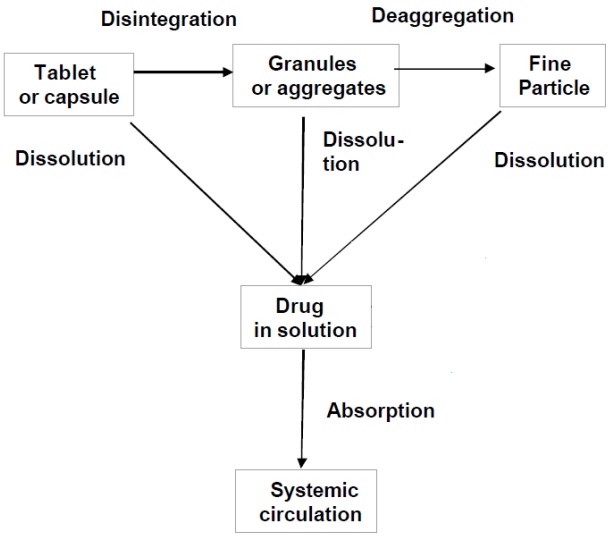


Figure 3. Dissolution process of orally administered drug

In order to have a chance to be adequately absorbed, drug needs to be rapidly dissolved in the aqueous environment of the gastrointestinal tract. Only dissolved drug can be absorbed across the intestinal walls and enter the portal vain.

This seemingly simple process is actually a significant challenge to the development of new oral drug candidates with undesirable biopharmaceutical properties.

As postulated by BCS classification, three major processes that are governing the absorption from solid dosage forms are dissolution rate and solubility, which determine how fast a drug reaches a maximum concentration in the luminal intestinal fluid, and intestinal permeability, which relates to the rate at which dissolved drug will cross the intestinal wall to reach the blood circulation.

Dissolved drugs are absorbed via a variety of transcellular and paracellular pathways across the intestinal tract. The paracellular pathway is defined as drug transport through the junctions between the cells, whereas the transcellular pathway is defined as drug transport

through both apical and basolateral cellular membranes, as well as through the internal aqueous environment of the cells themselves. Transcellular pathways also include both passive diffusion and carrier-mediated transport, whereas transport by the paracellular pathway is mainly passive (Figure 4).

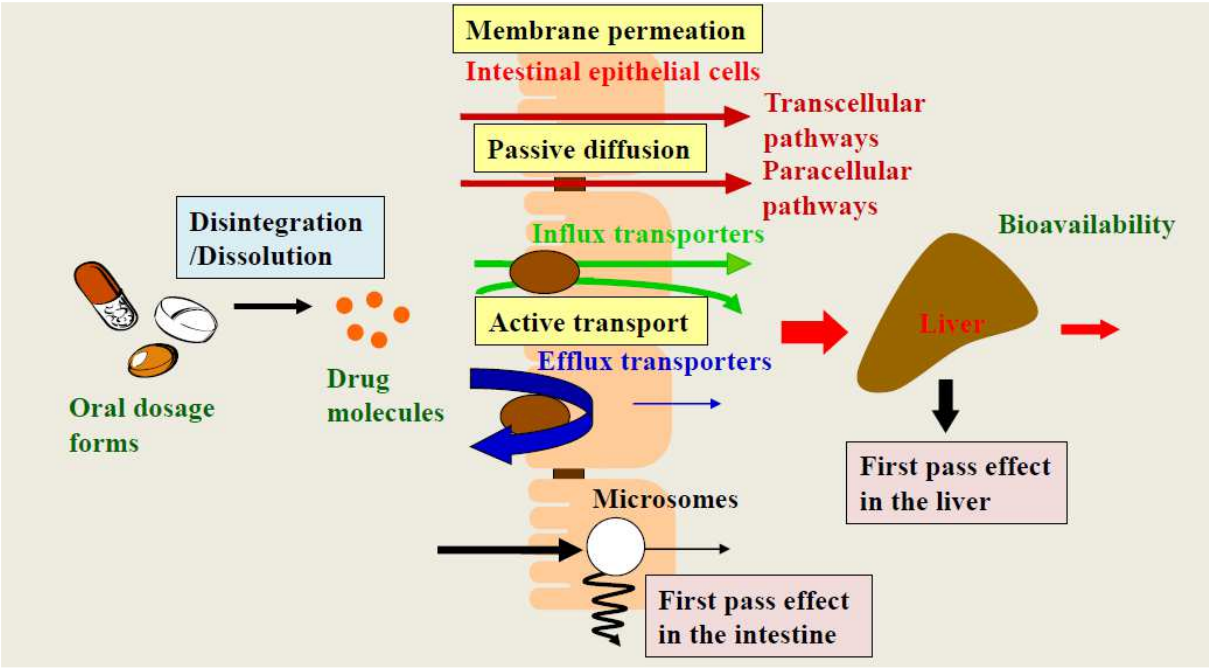


Figure 4. Drug absorption through the intestinal membranes (Sakuma and Yamashita, 2010)

Drugs are absorbed primarily in the small intestine, where the absorption area is very large (100 m^2 or an area equivalent to that of a tennis court). Absorption in the colon is generally limited, and can be highly variable because of the variability in undigested food, bacteria, and water content (Liu et *al*., 2009).

After drugs are absorbed into the intestinal cells, they may be subjected to first-pass metabolism by a variety of intestinal enzymes. Escaping intestinal metabolism does not guarantee bioavailability, however, since the drugs absorbed from the stomach and intestine (large and small), may be subjected to additional first-pass metabolism by the liver. Metabolites formed in the intestinal cells can be excreted back to the intestinal lumen, directly or indirectly, after they are taken up by the liver cells, and are then excreted via the bile ("enterohepatic recirculation") (Liu et *al*., 2009).

To conclude, drug absorption following oral administration of a solid dosage form should be distinguished from bioavailability because the latter can be decreased due the following reasons:

1) The drug is not delivered from its formulation over an appropriate time frame in solution form to those sites in the GI tract where it is well absorbed;
2) The drug is decomposed in GI tract or forms an nonabsorbable complex;
3) The drug is not transported efficiently accross the gut wall;
4) The drug is metabolized and/or eliminated on his way to the systemic circulation (Dressman et *al*., 1998).

Only the first reason is a subject of this thesis. The reasons for drug not to be delivered from formulation may lie in "deficiencies" of formulation, and/or limitations of active substance in terms of solubility and/or permeability.

The lack of ability of a drug to go into solution is in some cases more important to its overall rate of absorption than its ability to permeate the intestinal mucosa: for many drugs the onset of drug levels will be dictated by the time required for the dosage form to release its contents, and for the drug to dissolve. For such drugs, it can be said that dissolution is rate limiting step for absorption (Hörter and Dressman, 1997).

2.2.1. Drug solubility

Due to dramatic changes in the techniques applied in pharmaceutical discovery programs over the past 20 years, the physicochemical properties of development candidates have changed substantially.

Drug design based on combinatorial chemistry, high throughput screening and other drug discovery innovations, has in general led to more lipophilic compounds exhibiting low aqueous solubility (Lipinski, 2000). According to this author, approximately one-third of new compounds synthesized in medicinal chemistry laboratories have an aqueous solubility less than 10 µg/ml, another one-third have solubility from 10 to 100 µg/ml, and the solubility of the remaining third is > 100 µg/ml (Figure 5).

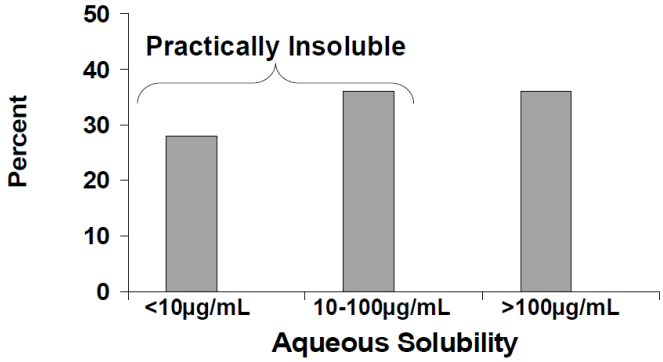


Figure 5. Solubility characteristics of current developed drug compounds

Such unfavourable biopharmaceutical properties of new drug candidates make development of final drug products extremely difficult.

According to pharmacopoeial criteria, in US Pharmacopoeia substances are classified as very slightly soluble when their nominal solubility is 0.1–1 mg/ml, while all of the substances having solubility below 0.1 mg/ml (100 µg/ml) are categorized as insoluble or practically insoluble substances.

If we calculate the volume of water necessary to dissolve for example 500 mg solid dosage form containing drug substance with aqueous solubility of 100, 10 or 1 µg/ml, we reach the numbers as high as 5, 50 and 500 litters of water!

2.2.2. BCS classification

A question that should be addressed here is: How soluble the drug substance should be to be considered "enough soluble" not to present obstacle for *in vivo* delivery of drug?

The answer depends mainly on the dose and permeability of drug. The classification that takes all three parameters into account was introduced in pharmaceutical industry by Amidon and co-workers in 1985, under the name of Biopharmaceutics Classification System (BCS).

The authors categorized drugs into four classes, depending on their solubility and permeability characteristics:

Class I: high permeability, high solubility

Class II: high permeability, low solubility

Class III: low permeability, high solubility

Class IV: low permeability, low solubility.

By definition, a drug is considered to be highly soluble if the highest single dose administered as immediate release formulation is soluble in < 250 ml of water over a pH range of 1–7.5 (FDA Guidance for Industry, Waiver of *In Vivo* Bioavailability and Bioequivalence Studies for Immediate-Release Solid Oral Dosage Forms Based on a Biopharmaceutics Classification System, 2000), or pH range from 1–6.8 (EMA Guideline on the investigation of bioequivalence, 2010). Since the solubility in pharmaceutical industry should always be related to the administered dose, this criterion is often defined through dose-solubility ratio (D/S) being 250 ml or less.

Dose/solubility ratio indicates the volume of liquid (in millilitres), that is required to dissolve the entire dose of the drug.

High permeability is defined with the extent of *in vivo* oral absorption of 90 % or greater according to FDA Guidance and greater than 85 % according to EMA Guideline.

2.2.3. Biowaiver

According to scientific principles of BCS, the *in vitro* differences in drug dissolution may be translated into the *in vivo* differences in drug absorption. If the *in vivo* dissolution is rapid in relation to gastric emptying, oral drug absorption is likely to be independent of dissolution, and therefore additional *in vivo* bioequivalence studies can, under well defined conditions, be waived (Yang and Yu, 2009).

To be a biowaiver, *in vitro* dissolution of BCS I drug product should be either very rapid (> 85 % within 15 min) or similarly rapid (85 % within 30 min) with the reference product at three different pH at the range 1–6.8. Furthermore, BCS I drug should have wide therapeutic window and liner pharmacokinetics. The excipients in formulation must be well known. Biowaiver may also be applicable for drug products containing BCS Class III substance but in that case dissolution of the test and the reference product should be very rapid (> 85 % within 15 min). According to EMA Guideline on the investigation of bioequivalence, 2010, the risks of an inappropriate biowaiver decision should be more critically reviewed (e.g. site-specific absorption, risk for transport protein interactions at the absorption site, excipient composition and therapeutic risks) for products containing BCS class III than for BCS class I drug substances.

Some publications suggest that also the acidic BCS Class II (Rinaki et *al.*, 2004) and Class III drugs (Blume and Schug 1999, Yu et *al.*, 2002, Cheng et *al*., 2004, Kortejärvi et *al*., 2005) can be biowaivers.

Interestingly, Kortejärvi et *al.*, 2007 systematically studied the influence of formulation types, physiology of GI tract and drug properties (dissolution, absorption and elimination) against current biowaiver criteria. In pharmacokinetic simulations the authors compared c_{max} (maximum concentration) and *AUC* (area under the plasma or serum concentration-time curve) of immediate release BCS I-IV drugs to oral solutions and found out that all BCS III drugs and slowly eliminating BCS I drugs are better biowaivers than BCS I drugs with rapid elimination. This topic is still a subject of a very extensive discussion.

2.3. *In vivo* studies for evaluating drug absorption

2.3.1. Pharmacokinetic evaluation

Bioavailability is measured by assessing the rate and the extent to which an active drug is absorbed from the drug product and becomes available at the site of action.

The extent of absorption is well estimated by the relative measures of *AUC*, but measures for rate of absorption in bioavailability and bioequivalence studies are more problematic.

Currently, the rate of drug absorption is evaluated by the peak concentration (c_{max}) and the time to peak (t_{max}) obtained from plasma/serum concentration-time profiles. The utilization of c_{max} and t_{max} as a measure of rate of absorption has been criticized, because they contain minimal information about the absorption rate and absorption process for the drug (Chen, 1992).

c_{max} is only an indirect measure of the rate of drug absorption and it has several drawbacks such as being influenced by the extent of absorption, insensitive to changes in rate of absorption, depends on the sampling schedule, etc. (Chen et *al.*, 2001).

However, others have argued that c_{max} is clinically relevant, as it reflects the highest drug exposure (e.g. concentration) in the body and may relate to patient's risk for toxicity and/or efficacy.

The use of t_{max} as a measure of absorption rate has also been debated. Mathematically, t_{max} is a function of both absorption and elimination rate constants. It is highly dependent on the sampling scheme, and it is therefore difficult to use it to detect differences between two products, especially when t_{max} values are less than 2 hours. In contrast, for slow-releasing dosage forms, plasma concentrations are maintained at a plateau for a long time and t_{max} does not reflect the rate of bioavailability.

Currently, the FDA guidance titled Bioavailability and Bioequivalence Studies for Orally Administered Products-General Considerations, 2003, recognizes that traditional measures (e.g., c_{max}, t_{max}, mean absorption time, mean residence time, and c_{max}/AUC) are limited in their ability to assess the rate of absorption. The Guidance recommends a change in focus from these direct and indirect measures of absorption rate (e.g., c_{max}, t_{max}, k_a) to measurements of systemic exposure, which include early exposure, peak exposure (c_{max}, t_{max}), and total exposure ($AUC_{0\text{-}t}$ and $AUC_{0\text{-}\infty}$). The new parameter, early exposure, would be

assessed based on a partial *AUC* in which the *AUC* is truncated at t_{max} for the reference compound.

The incremental area under the drug concentration-time curve representing 10 to 30 % of the total *AUC* might be more sensitive than either c_{max} or t_{max} in detecting input rate differences between formulations (Rosenbaum et *al.,* 1990).

To conclude, for orally administered immediate-release drug products, measures of peak and total exposure may be sufficient. However, in situations in which clinical safety and/or efficacy trials or pharmacokinetic-pharmacodynamic studies indicate that better control of drug absorption into systemic circulation may be warranted and the assessment of early exposure via the use of partial *AUC*s may be indicated.

2.3.2. Development and validation of bioanalytical method

Bioanalysis, employed for the quantitative determination of drugs and their metabolites in biological fluids, plays a significant role in the evaluation and interpretation of bioequivalence, pharmacokinetic (PK), and toxicokinetic studies. Selective and sensitive analytical methods for quantitative evaluation of drugs and their metabolites are critical for the successful conduct of pre-clinical and/or biopharmaceutics and clinical pharmacology studies (FDA Guidance for Industry, Bioanalytical Method Validation, 2001).

A bioanalytical method consists of two main components, sample preparation and detection of the component.

2.3.2.1. Sample preparation

Sample preparation is a technique used to clean up a sample before analysis and/or to concentrate a sample to improve its detection. Therefore, the main objectives of bioanalytical sample preparation are to dissolve the analyte in a suitable solvent, to remove as many interfering compounds as possible and to pre-concentrate the sample (Singh et *al.*, http://www.expresspharmaonline.com/20081231/research02.shtml).

Some of routinely used sample preparation methodologies are protein precipitation, liquid-liquid extraction and solid phase extraction.

Protein precipitation (PP)

PP is the least time-consuming sample preparation technique and has been widely used in bioanalytical methods.

It involves denaturation (loss of tertiary and secondary structures) of proteins present in biomatrix by external stress (such as a strong acid/base/heat or, most commonly, the use of an organic solvent such as acetonitrile/methanol) (Englard and Seifter, 1990).

Organic solvents, such as methanol, acetonitrile, acetone and ethanol, although having a relatively low efficiency in removing plasma proteins, have been widely used in bioanalysis because of their compatibility with high-performance liquid chromatography (HPLC) mobile phases (Singh et *al*., http://www.expresspharmaonline.com/20081231/research02.shtml).

Most of the bioanalytical methods employ addition of a minimum of three parts of organic solvent to one part biomatrix, following by vortexing and centrifugation. Centrifugation leads to formation of protein pellet and supernatant is separated for bioanalytical quantitation. As denaturation leads to active change in protein structure, the drug/metabolite/ biomarker bound to these proteins becomes freely soluble in the denaturation solvent. Prior to quantitation it is usually necessary to concentrate the sample by evaporating a supernatant to dryness and then reconstituating before analysis.

The whole process of PP, however, is time-consuming when handled manually for a large number of samples, especially drug discovery bioanalysis/ clinical bioanalysis. Considerable efforts have been made to make this process more efficient, less time-consuming and automatable for high-throughput bioanalysis (Kole et *al*., 2011).

Liquid-liquid extraction (LLE)

The principle of two immiscible liquids for extraction of analyte, commonly termed LLE/solvent extraction is one of the most commonly used sample preparation techniques in bioanalysis.

The analyte is isolated by partitioning between the organic phase and the aqueous phase. The analyte should be preferentially distributed in the organic phase under the chosen conditions. For effective LLE the analyte must be soluble in the extracting solvent, extracting solvent should have low viscosity to facilitate mixing with the sample matrix, a low boiling point to facilitate removal at the end of the extraction and a large surface area to ensure rapid equilibrium. This is achieved by thoroughly mixing using either mechanical or manual shaking or vortexing. Generally, selectivity is improved by choosing the least polar solvent in

which the analyte is soluble. (Singh et *al*., http://www.expresspharmaonline.com/20081231/research02.shtml, Kole et *al*., 2011).

Some of the limitations of LLE are low/variable recovery, the need for a large sample volume, poor selectivity and matrix effects in LC-MS methods (Zhou et *al*., 2005a, Capka and Carter, 2007). Recently, efforts have been made to overcome limitations associated with LLE.

Solid phase extraction (SPE)

In SPE the analyte is retained on the solid phase while the sample passes through, followed by elution of the analyte with an appropriate solvent. The SPE is typically carried out using a five-step process: condition, equilibrate, load, wash and elute. The solid phase sorbent is conditioned by passing a solvent, usually methanol, through the sorbent to wet the packing material and solvate the functional groups of the sorbent. The sorbent is then equilibrated with water or an aqueous buffer. Samples are diluted with aqueous solvent prior to loading to reduce viscosity and prevent the sorbent bed from becoming blocked. Aqueous and/or organic washes are used to remove interferences (Singh et *al*., http://www.expresspharmaonline.com/20081231/research02.shtml).

Although conventional SPE offers several advantages, it has its own limitations such as limited selectivity and/or sensitivity because many matrix constituents can also be adsorbed besides the target, leading to matrix effects in consecutive LC-MS/MS analysis (Rodriguez-Mozaz et *al*., 2007). In recent years many new approaches have been designed, developed and validated to overcome various limitations or to improve the performance of the SPE technique.

2.3.2.2. Analytical detection of the compound

In the last decade we have witnessed many technological breakthroughs in analytical methodology and instrumentation. Among these modern analytical techniques, liquid chromatography coupled with mass spectrometry is considered to be the benchmark for quantitative/qualitative bioanalysis, imparting specificity, sensitivity and speed (Saunders et *al*., 2009).

This analytical technique also suffers from limitations such as matrix effect, compromised selectivity and a fall in sensitivity of the analyte of interest in the processed biological matrix (Smeraglia et *al*., 2002).

Regardless of which analytical detection was chosen as the most appropriate for the anayte in question, before implementation for routine use, it must first be validated to demonstrate that it is suitable for its intended purpose (Kole et *al.*, 2011).

2.3.2.3. Bioanalytical method validation

Bioanalytical method validation includes the procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix, such as blood, plasma, serum, or urine, is reliable and reproducible for the intended use. The fundamental parameters for this validation include accuracy, precision, selectivity, sensitivity, reproducibility, and stability. Validation involves documenting, through the use of specific laboratory investigations, that the performance characteristics of the method are suitable and reliable for the intended analytical applications. The acceptability of analytical data corresponds directly to the criteria used to validate the method (FDA Guidance for Industry, Bioanalytical Method Validation, 2001).

Accuracy describes the closeness of the observed mean test results obtained with the bioanalytical method to the true (i.e., actual) concentration of the analyte. The accuracy of a particular method can be determined with replicate analysis of samples containing known amounts of the analyte.

Precision describes the closeness of the observed individual measures of an analyte when the method is applied repeatedly to multiple aliquots of single homogeneous determinations per concentration.

Selectivity is defined as the ability of an analytical method to differentiate and quantify an analyte in the presence of other components in the sample.

Substances in the biological matrix that may potentially interfere with the results include endogenous substances, metabolites, and degradation products.

Sensitivity refers to the ability of the bioanalytical method to measure and differentiate an analyte in the presence of components that may be expected to be present. For example, these components may include metabolites, degradants, and impurities. Reproducibility compares the precision of the analytical method between two different laboratories. Reproducibility can also represent the precision of the method obtained under the same operating conditions over a short period of time.

Typical method development and validation of a bioanalytical method such as a chemical assay includes determinations of selectivity, accuracy, precision, recovery,

calibration curve and stability of an analyte in the spiked samples (FDA Guidance for Industry, Bioanalytical Method Validation, 2001).

The acceptance criteria should be clearly established in a validation plan, prior to the initiation of the validation study.

2.4. Models for evaluating drug absorption of lipophilic compounds

Data obtained by *in vitro* dissolution testing can be combined with *in silico* physiologically based pharmacokinetic modelling, using model-dependent or model-independent approaches, for the prediction of *in vivo* dissolution and absorption and development of *in vivo in vitro* correlations (IVIVCs).

In model-dependent approaches the dissolution profile or the dissolution rate constant can be used in absorption modelling using softwares that simulate a complex system dynamically (i.e. Stella®) or physiologically based models of the gastrointestinal tract (i.e. GastroPlus™).

In model-independent approaches the amount dissolved/released over the time or the *in vitro* dissolution/release rate are directly compared with the amount absorbed over the time or the *in vivo* absorption rate (as calculated from deconvolution of the oral data-pharmacokinetic softwares, i.e. WinNonlin®, PCDCON®)(Fotaki and Vertzoni, 2010).

In this thesis, model-dependent approach was employed to predict the plasma profile of orally administered lipophilic drugs, based on *in vitro* dissolution profiles in simple and biorelevant media and two preconditions: that absolute bioavailability of drug is known and that its absorption is dissolution limited.

2.4.1. *In vitro* dissolution testing

2.4.1.1. Noyes-Whitney equation

According to the modified Noyes-Whitney equation, the dissolution rate is proportional to both solubility and surface area:

$$\frac{\mathrm{d}C}{\mathrm{d}t} = \frac{D \cdot A}{h}\left(C_{\mathrm{s}} - C_{\mathrm{b}}\right)$$

where:

d*C*/d*t* = dissolution rate,

D = diffusion coefficient,

A = the surface area of drug exposed to the dissolution media (effective surface area),

h = the thickness of the diffusion layer at the solid liquid interface,

C_s = aqueous drug solubility at the surface of the dissolving solid, saturated solubility

C_b = concentration of drug in the bulk aqueous phase.

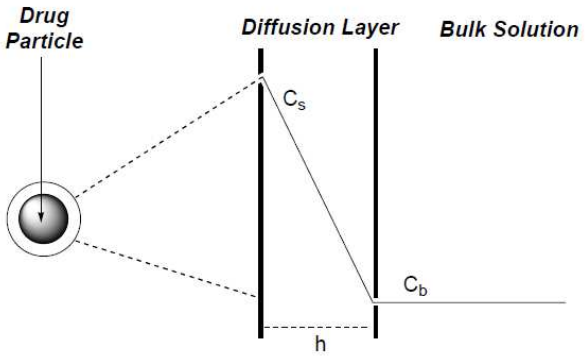


Figure 6. Dissolution of drug particles according to diffusion layer model

Initially, when the concentration of drug molecules is low in the solution, the number of molecules leaving the drug surface is high. As the solvent bulk starts becoming saturated with the drug molecules, the redeposition process starts to accelerate. Once sufficient drug molecules have populated the solvent bulk, the rate of molecules leaving becomes equal to the rate of redeposition (dynamic equilibrium). The concentration of the drug in the solvent at which this equilibrium is reached is defined as the thermodynamic solubility. The rate at which the equilibrium is achieved is the dissolution rate (Figure 6).

Thus, solubility is an equilibrium concept, while dissolution is a kinetic phenomenon. Both are dependent on the experimental conditions, including temperature (Rao et *al.*, 2009).

The dissolution rate of a solute in a solvent is directly proportional to its solubility, as well as surface area of drug exposed to the dissolution medium. This enables scientists to increase drug dissolution rate by manipulating these two factors, which is possible in a number of ways.

Solubility and surface area of pharmaceutical solid dosage forms are primarily influenced by physicochemical properties of the drugs, such as: particle size, wettability, molecular size, charge, lipophilicity and cristal structure. Conditions in the gastrointestinal tract, such as composition, volume and hydrodynamics of the contents in the lumen, which also influence drugs dissolution in GI tract, have already been described.

2.4.1.2. Dissolution methodology

Dissolution test *in vitro* measures the rate and extent of dissolution of the drug in an aqueous medium in the presence of excipients contained in the drug product.

The parameters of dissolution method depend on both, drug substance and drug product characteristics. The most critical part of defining dissolution method is definition of dissolution medium, in terms of composition and pH. Hydrodynamics of medium is assured by agitation apparatus (paddles or baskets) rotating at 50–100 rpm. The volume of dissolution medium usually varies between 500 and 900 ml. The temperature of medium is set to 37 ± 0.5 °C.

For regulatory purposes, an IR drug product is considered rapidly dissolving when no less than 85 % of the labeled amount of the drug substance is dissolved within 30 minutes, using baskets at 100 rpm or paddles at 50 rpm, in a volume of 900 ml or less in each of the following media:

- 0.1M HCl or simulated gastric fluid without enzymes,
- pH 4.5 buffer, and
- pH 6.8 buffer or simulated intestinal fluid without enzymes.

Otherwise, the drug product is considered to be slow-dissolving product.

However, the simple aqueous buffer solutions do not represent all aspects of physiological conditions in the gastrointestinal tract and usually offer, at best, *a posteriori* correlations with *in vivo* data (Jantratid et *al.*, 2008). If a dissolution test is to be used as a prognostic tool for *in vivo* behaviour, then much more attention has to be paid primarily towards the choice of dissolution medium.

2.4.1.3. Biorelevant dissolution media

A need to develop dissolution test that would better predict the *in vivo* performance of drug was recognised more than 10 years ago by Dressman et *al.*, 1998. The parameters that were recognized as the most important for design of predictive dissolution tests were composition, volume and hydrodynamics of the dissolution medium and the duration of the test. According to the authors, the choice of dissolution test conditions should be based on position of drug absorption in GI tract and dosing conditions (fasting or fed). The simulation of the *in vivo* hydrodinamics was recognized as the most problematic.

Although the problem of hydrodynamics simulation still remains a challenge, the composition of biorelevant media is continuously being improved as more physiological data become available.

The early papers (Dressman et *al*., 1998, Galia et *al*., 1999) suggested that the composition of **fasted state simulated gastric fluid** (FaSSGF) should include sodium lauryl sulphate to reduce the surface tension to the physiological values and that osmolality and buffer capacity should be low since the gastric secretions in the fasted state are at the baseline and the primary determinant of the volume would be fluid (water) coadministered with a dosage form.

Vertzoni et *al*., 2005, proposed to add physiological amounts of pepsin in composition of FaSSGF, and to change artificial surfactant sodium lauryl suphate with low amounts of bile salt sodium taurocholate and lecithin. The pH of such medium is 1.6, and surface tension is 42.6 mN/m. The composition of FaSSGF used in the experimental part of this thesis is presented in Table 1.

Table 1. Composition and physicochemical properties of FaSSGF (Vertzoni et *al*., 2005)

Composition	
Sodium taurocholate (μM)	80
Lecithin (μM)	20
Pepsin (mg/ml)	0.1
Sodium chloride (mM)	34.2
Hydrochloric acid qs	pH 1.6
Deionized water qs ad	1 l
pH	1.6
Osmolality (mOsm kg^{-1})	120.7±2.5
Buffer capacity (mmol l^{-1} ΔpH^{-1})	–

To make it more physiologically relevant, in terms of sodium chloride concentration (Lindahl et *al*., 1997) and osmolality (Kalantzi et *al*., 2006), FaSSGF used in this study contains 68 mM NaCl (Vertzoni et *al*., 2007) instead of 34 mM NaCl (Vertzoni et *al*., 2005) and will be referred to as FaSSGF-V2. The osmolality of such medium is 192 mOsm/kg.

For **fed state simulated gastric fluid (FeSSGF)** the luminal composition would be highly dependent on the composition of meal ingested, so homogenized form of meal used in the clinical studies and coadministered water would be the best starting point. However, due to difficulties in drug analysis, long life milk and Ensure® were considered also as an appropriate medium since they both have physicochemical properties that are similar to those

of the standard meal recommended by the FDA for the effects of food in bioavailability (BA) and bioequivalence (BE) studies (Klein et *al*., 2004).

In order to simulate composition changes of intragastric environment during digestion and emptying processes, two approaches were employed so far: the first one with periodical addition of aliquots of an acidic solution of pepsin into milk (Vertzoni et *al*., 2007), which was employed in this thesis, and the second one with "snapshot" media, each corresponding to a certain time frame after ingestion of meal. From these "snapshot" media, general media FeSSGF is recommended (Jantratid et *al*., 2008).

For intestinal media the presence of bile and higher pH should be assured. Bile salts and lecithin facilitate the wetting of solids and enhance solubilization of lipophylic drugs into mixed micelles (at concentrations above the critical micellar concentration). Sodium taurocholate is chosen as a representative bile salt because cholic acid is one of the most prevalent salts in human bile. Phosphate buffer is used in the **fasted state simulated intestinal fluid (FaSSIF)** as a substitute for the physiological bicarbonate buffer, to avoid instability in pH value due to the reaction with oxygen (Galia et *al*., 1998, Dressman et *al*., 1998).

To update the simulation of fasted state conditions in the upper small intestine, minor changes of FaSSIF were introduced by Jantratid et *al*., 2008. The amount of lecithin was decreased from 0.75 mM in FaSSIF to 0.2 mM in FaSSIF-V2. The osmolality is somewhat lower in FaSSIF-V2 than in FaSSIF, in accordance with *in vivo* data. The pH of 6.5 was maintained, with substitution of phosphate buffer with maleate buffer in FaSSIF-V2.

The **fed state simulated intestinal fluid (FeSSIF)** contains acetate buffer instead of phosphate in order to achieve the higher buffer capacity and osmolality while maintaining the lower pH value representative for fed state duodenal conditions.

The taurocholate and lecithin are presented in considerably higher concentration in this medium. Since fats and oils and their digestion products are also present after ingestion of food, they are often added in FeSSIF when testing lipophilic compounds, since monooleins, long chain fatty acids and/or triglycerids can modify the ability of drug to interact with micelles. Jantratid et *al*., 2008, presented "snapshoot" approach media also for FeSSIF, so early, middle and late compositions were proposed, and new general medium FeSSIF-V2 was recommended.

Even though the compositions of FaSSIF and FeSSIF as proposed by Galia et *al.*, 1998, represent simplification of the luminal composition, they have been shown to predict the *in vivo* dissolution processes and the absorption characteristics of poorly soluble, lipophilic week acids, week bases and non-ionazable compounds well, are they are widely used in pharmaceutical industry to evaluate new drugs and dosage forms (Vertzoni et *al.*, 2004).

Vertzoni et *al*., 2004, challenged the type of buffers used in FaSSIF and FeSSIF (Galia et *al*., 1998).

As mentioned previously, physiological buffer for FaSSIF would be bicarbonate buffer. However, such media would require continuous sparging with carbon dioxide to maintain the desired pH, buffer capacity, ionic strength and osmolality and it has some other practical obstacles for routine usage. For fed state, it is difficult to define the appropriate buffer system on a physiological basis, since at the pH of 5 it is highly unlikely that bicarbonate buffer alone could maintain the desired buffer capacity. It is assumed that buffer species generated by food ingestion (e.g. amino acids) would play important role in maintaining the pH. So, in this case too, practical issues led to use of non-physiological buffer species (McNamara et *al*., 2003, Vertzoni et *al*., 2004).

Therefore it was justified to test alternative buffer species since theoretically the type of buffer may affect the solubility of product, stability of the active compound and dissolution behaviour.

Vertzoni et *al.*, 2004, tested FaSSIF containing maleic anhydride ($FaSSIF_m$) and FeSSIF containing citrates ($FeSSIF_c$) and found out that in the case of tested week bases, the influence of anion buffer species is important only when pK_a of the dissolving compound is higher than about 5, because week bases with pK_a lower than 5 are not ionized in FaSSIF and FeSSIF, and also their solubility products are relatively low. Therefore, in such case it would be wise to run additional dissolution tests in FaSSIF and FeSSIF containing alternative buffer species.

The compositions of theese media are presented in Table 2.

Table 2. Physicochemical characteristics and composition (mM) of FaSSIF and FeSSIF and modified solutions $FaSSIF_m$ and $FeSSIF_c$, respectively (Vertzoni et *al*., 2004)

Fasted state simulated intestinal fluids				**Fed state simulated intestinal fluids**			
pH		6.5		pH		5.0	
Osmolality (mOsm kg^{-1})		270 ± 10		Osmolality (mOsmol kg^{-1})		635 ± 10	
Buffer capacity (mmol L^{-1} ΔpH^{-1})		12		Buffer capacity (mmol L^{-1} ΔpH^{-1})		76	
FaSSIF		FaSSIFm		FeSSIF		FeSSIFc	
Sodium taurocholate	3	Sodium taurocholate	3	Sodium taurocholate	15	Sodium taurocholate	15
Egg phosphatidylcholine	0.75	Egg phosphatidylcholine	0.75	Egg phosphatidylcholine	3.75	Egg phosphatidylcholine	3.75
Sodium dihydrogen phosphate	28.66	Maleic anhydride	25.01	Acetic acid	144	Citric acid	84
Sodium hydroxide	~13.8	Sodium hydroxide	~45	Sodium hydroxide	~101	Sodium hydroxide	~200
Sodium chloride	106	Sodium chloride	109	Sodium chloride	173	Sodium chloride	206

In the present study 5 mM glycerol-monooleate (GMO) has been added to FeSSIF to simulate the presence of food lipids.

The incentive for further update of FeSSIF composition was publishing of study results performed by Kalanzi et *al*., 2006, revealing that the conditions in healthy human volunteers in fasting and fed state differ in some ways from the compositions of the current simulating media. The differences were in terms of lower amount of bile salt observed *in vivo*, lipolisis products were found and the pH in the upper small intestine decreased slower than expected after ingestion of food.

In the last review of biorelevant media, prepared by Fotaki and Vertzoni 2010, tables with the ultimate media compositions are presented, as well as review of intraluminal hydrodynamics simulation and methods used for development of *in vivo in vitro* correlations.

Recently, a new biorelevant medium for simulation of fluids in the ascending colon was introduced (Vertzoni et *al*., 2010).

2.4.1.4. IVIVC and BCS

To establish IVIVC several factors have to be considered. *In vitro* dissolution test can only model the release and dissolution rates of the drug and it is only when these processes are rate limiting in the overall absorption that IVIVC can be established.

For BCS class I drugs the complete dose will be dissolved already in the stomach, and provided that the absorption in the stomach is negligible, the gastric emptying will be rate limiting, therefore ICIVC is not expected.

Class II drugs are expected to have dissolution-limited absorption, and IVIVC can be established using a well-designed *in vitro* dissolution test. But the IVIVC will not be likely for class II drugs if absorption is limited by the saturation solubility in the GI tract, rather than by

the dissolution rate. In this situation, the drug concentration in the gastrointestinal tract will be close to the saturation solubility, and changes in the dissolution rate will not affect the plasma concentration profile or the *in vivo* BA.

The absorption of class III drugs is limited by their intestinal permeability, and no IVIVC should be expected. However, when the drug dissolution becomes slower than the gastric emptying, a reduction of the extent of BA will be found at slower dissolution rates, since the time during which the drug is available for transport across the small intestinal barrier will then be reduced. The class IV drugs present significant problems for effective oral delivery. Very limited or no IVIVC is expected (Yang and Yu, 2009).

IVIVC, when successfully developed, can serve as surrogate of *in vivo* tests and may assist in supporting biowaivers. It can also support and validate the use of dissolution methods and specifications.

There are four different levels of IVIVC, depending on the degree of correlation between *in vivo* and *in vitro* data (A, B, C and multiple C correlations), but they will not be discussed in this thesis.

2.4.2. Animal models

Although it is recognized that there are many physiological differences between humans and animals, animals are still frequently used for the assessment of the *in vivo* performance of orally administered products at the preclinical level (e.g. Zhou et *al*., 2005).

Animal models are often used to obtain early pharmacokinetic knowledge of a pilot product's *in vivo* absorption characteristics, to identify the main absorption site within the GI tract, and to document preliminary absorption mechanisms for the novel drug formulation. In this way data collected from animals are often used to support selection of an optimal formulation, and guide the overall design of the final product's dosage form.

However, while planning animal studies researchers need to select the appropriate animal species that will yield optimal results and also to identify a reliable method of extrapolating the animal data so that they can adequately predict the drug's pharmacokinetic behavior in humans (Zhou and Seitz, 2009).

Preclinical evaluation of a new drug's pharmacokinetics should be conducted in an animal species with anatomical and physiological characteristics that are relevant to the research objectives which can be challenging.

It is assumed that anatomical and physiological differences are the main factors that cause discrepancy between humans and animals with respect to the absorption of xenobiotics. The extent to which the pharmacokinetics of a xenobiotic is affected by interspecies differences is depending on the physical/chemical characteristics of the compound (De Zwart et *al*., 1999). Animal models that are usually used for prediction of human GI absorption are rats, rabbits, monkeys, pigs and dogs.

Although sometimes they are used due to their availability and low cost, rats and rabbits are not very suitable model due to the obvious physical limitations of their diminutive GI tracts (Fotaki, 2009).

Pigs have also occasionally been used for oral bioavailability studies, but with limited overall success, although the morphology of the large intestine is similar to humans (De Zwart et *al*., 1999, Dressman and Yamada, 1991).

Monkeys may initially appear as most suitable for standard *in vivo* pharmacokinetic studies of solid oral dosage forms since they have generally similar GI morphology and adequate gut dimensions to that of humans. Their disadvantages are differences in clearance and metabolic activity in the small intestine, huge effect of food on the gastric pH, high costs, and difficulties in their handling (Fotaki et *al*., 2009).

2.4.2.1. Dogs as animal models for assessment of oral absorption of lipophilic compounds

Dogs have probably been used more extensively than any other animal models for *in vivo* evaluations of oral drug absorption.

Some physiological and anatomical characteristics in the GI tract of dogs are similar to those in humans. Typical gastric dimensions for mongrel dogs weighing 15–25 kg are particularly similar to those of humans, for example. In addition, dogs have physiological responses to feeding, and bile secretion profiles that are also generally similar to those of humans (Dressman and Yamada, 1991).

On the other hand, other physiologic features unique to the dog can affect pharmacokinetics, making extrapolations between canines and humans unreliable.

Some key differences are shown as following (De Zwart et *al*., 1999, Tibbitts, 2003, Dressman et *al*., 1986, Kararli, 1995; Kalantzi et *al*., 2006):

- Intestinal dimensions and GI transit times vary from dogs to humans. For example, the length of a dog's small intestine is only about half the length of a human's, and a

dog's colon is also generally shorter than a human colon. In a fasted state, for example, transit time for dogs is approximately half the time for humans. To account for this relatively accelerated transit time, *in vivo* dog models have occasionally been pretreated with drugs that can delay GI motility, and subsequently prolong intestinal residence time of the investigational oral dosage form (Yamakita et *al.*, 1995);

- Dogs have less acidic fasting intragastric pH and larger inter-individual variability in gastric pH than humans;
- Fasted dogs have a slightly higher (1–2 pH units) small intestinal pH than humans;
- Dogs may have lower osmolarity in the stomach and higher bile salts concentrations in the small intestine;
- The typical canine gastric emptying rate in a fed state is slower than that of humans;
- Dogs secrete bile salts at a higher rate in comparison with humans;
- Some cytochromes P450 (CYP450) isozymes that are unique for the dogs have been identified (e.g., 2B11, 2C21, 2D15, 3A12, 3A26).

Such differences from the human luminal environment may limit the usefulness of canine models especially in the comparison of salt(s) with the free form of a base for which sufficient gastric acidity is prerequisite for adequate dissolution and absorption *in vivo*.

With respect to higher intragastric pH of dogs, some authors reported pretreatment the dogs with pentagastrin (Zhou et *al*., 2005, Lui et *al*.,1986, Dressman et *al*., 1986, Knupp et *al*., 1993) to acidify the gastric content or famotidine for H2 blockade (Zhou et *al.*, 2005, Lin, 1995). In this way the pH dependent aspect of drug solubility and absorption was isolated and better prediction for human studies was possible.

Fotaki et *al*., 2005, found that for compounds with dissolution and permeability problems, dogs provide a poor estimate for absorption but seem to provide good predictions for compounds without dissolution and permeability problems housed in extended-release formulations.

Dressman et *al*., 1986 and Kalantzi et *al*., 2006 stated that for compounds on which administration of food has a pronounced effect on absorption, the differences in GI physiology and composition of intestinal fluids have to be taken into consideration.

In Khoo et *al*. study from 2001, dogs have been shown to be a good model for the prediction of absorption of lipophilic drugs that are absorbed through the lymph, and in the same year Paulson et *al*., 2001 observed that the food effect of celecoxib was threefold greater in dogs than in humans.

Chiou et *al*., 2000, found that the absorption data in dogs, based on 43 compounds including bases, acids, zwitterions, and neutral compounds, did not predict the human absorption very well.

Regarding the canine studies of weak basic drugs, Zhou et *al*., 2005 developed canine absorption model for studying pH effect on both dissolution *in vitro* and pharmacokinetics *in vivo*, using weak bases ketokonazole and dipyridamole as model drugs. These drugs exhibit pH dependent solubility profiles, and therefore *in vivo* study results could be misleading due to inconsistent gastric pH. Therefore the authors decided to perform absorption studies with dogs that have been pre-treated with pentagastrin (gastric pH 2–3), famotidine (gastric pH 4–7.5) and one group which has not pre-treated. The results showed marked differences in systemic exposure: for both drugs *AUC*s were much higher in pentagastrin group, while famotidine group didn't show statistically significant difference compared to control group, which may be due to large variability in the control group.

To conclude, the pharmacokinetic/absorption results obtained from the canine studies should be interpreted with caution.

2.5. Salts for enhancing intraluminal dissolution

There are many ways for solubility and dissolution enhancement in pharmaceutical industry. Some of the approaches are:

- Physical modifications: particle size reduction (e.g. micronization, nanosuspensions), modification of crystal habit, drug dispersion in carriers (e.g. solid dispersions), complexation, solubilization by surfactants (e.g. microemulsions);
- Chemical modifications: salt formation;
- Other methods: cocrystalisation, cosolvency, selective adsorption on insoluble carrier, functional polymer technology, nanotechnology approaches, etc.

Of all the mentioned approaches, preparation of salts is the most common and effective method of increasing solubility and dissolution rates of ionazable drugs (Mohanachandran et *al*., 2010, Rajesh Babu et *al*., 2010).

2.5.1. Pharmaceutical salts

Changing a drug from its free base or acid to a salt form is commonly done to improve its physicochemical properties, including solubility and dissolution rates, hygroscopicity, stability, impurity profiles and crystal habit (Paulekuhn, 2007). This is because the solubility of an organic molecule is frequently enhanced more by an ionized functional group than by any other single mean (Neau, 2000).

The modification of physicochemical properties, mainly solubility and dissolution rate, may lead to changes in biological effects such as pharmacodynamics and pharmacokinetics, including bioavailability and toxicity profile (Verbeeck et *al*., 2006). Therefore, an appropriate choice of the most desirable salt form is a critical step in the development process.

Over the last decades the process for salt selection has been well established resulting in a significant number of salts being registered and marketed. According to FDA Orange Book database, till the end of 2006, the 1356 chemically well-defined APIs (active pharmaceutical substances) comprised 659 (48.6 %) APIs in nonsalt forms, 523 (38.6 %) salts formed from basic compounds and 174 (12.8 %) salts formed from acidic molecules. Also, 37.9 % of APIs approved in the U.S.A. after 1981 for oral administration were salts formed from basic molecules (Paulekuhn et *al*., 2007).

Many examples can be found in the scientific literature showing that the water solubilitiy of alternative salts forms of the same active moiety can be quite different (Verbeeck et *al*., 2006).

The explanation for this lies in the process that controls dissolution; therefore, some basic theoretical considerations will be reviewed hereinafter. Week bases and their salts will be discussed in more details, since they are in focus of this thesis.

2.5.1.1. Aqueous solubility of weak bases and their salts

The solubility profile of a weakly basic compound in a range of aqueous solutions at different pH values looks similar to the one shown in Figure 7 (and the profile for a weakly acidic compound would be its mirror image).

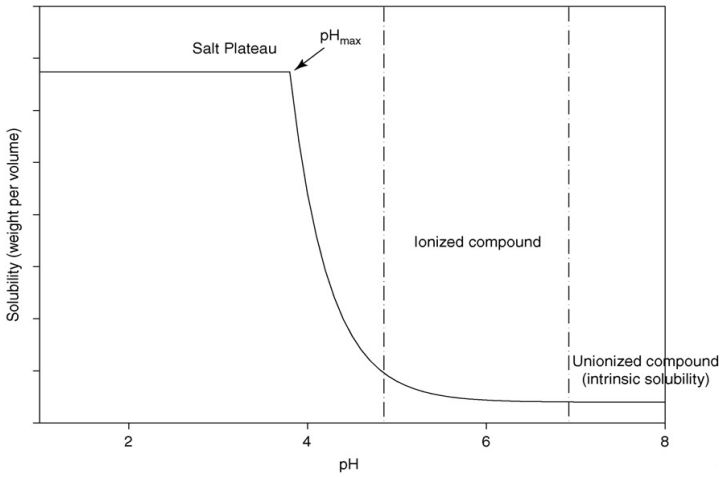


Figure 7. pH-solubility profile for a compound with a single, basic pK_a value of 5 (Bhattachar et *al.*, 2006)

The pH-solubility relationship of ionizable compounds is based on the Henderson-Hasselbach relationship, which relates the solubility of the completely 'unionized' compound (S_0, intrinsic solubility) to both the solubility measured at a given pH(S) and the pK_a of the compound. The Henderson-Hasselbach equation takes slightly different forms for acidic and basic compounds, which can be written as:

$$S = S_0\left[1+10^{(\mathrm{p}K_a-\mathrm{pH})}\right] \text{ for a monobasic compound}$$

$$S = S_0\left[1+10^{(\mathrm{pH}-\mathrm{p}K_a)}\right] \text{ for a monoacidic compound}$$

Thus, it can be seen that the solubility of an acid increases with pH at pH values higher than the pK_a. For basic drugs, the solubility value increases with decreasing pH at pH values lower than pK_a.

2.5.1.2. pH-dependent regions of solubility

It is clear from the above equations that pH has an enormous effect on the solubility of ionisable compounds. In general, the pH-solubility profile can be divided into four different regions according to the physical interactions that dominate (Figure 7):

1. The intrinsic solubility region (pH > 7 in Figure 7). This region is defined as the pH range in which the compound is completely unionized in solution and has the lowest solubility.

2. The ionizing portion of the curve and the region of the steepest slope. This region begins around the pK_a value (pH 4–5.5 in Figure 7). At the pK_a, there are equal concentrations of ionized and unionized forms of the compound in solution. Every pH unit change on either side of the pK_a will give a tenfold change in the amount of ionized drug in solution. Precipitate formed in this pH range can be in either the free form or the salt form, depending on the strength of the solid state interactions. In Figure 7 the pH-solubility profile for a base with a single pK_a is shown. The ionized portion of the curve is more complex for compounds with multiple ionization sites.

3. pH_{max}. This region corresponds to the pH that yields maximum solubility of the compound (pH 4 in Figure 7), where the ionizing portion of the curve meets the salt plateau on the pH-solubility profile. At this point, the equilibrium solid state will be a salt: that is, completely ionized drug associated with an oppositely charged counterion through coulombic interactions.

4. The salt plateau (pH < 4 in Figure 7). In this pH range, the salt solubility of the compound prevails. The solubility of the compound is almost constant: its value is dependent on the strength of solid-state interactions with the counterion forming the salt and it is given by the solubility product, K_{sp}, which is defined as the product of the concentrations of ion and counterion in solution:

$$\textit{Drug·salt} \xleftrightarrow{\textit{dissolution}} \textit{drug ion + salt counterion}$$

$$K_{sp} = [\textit{drug ion}][\textit{salt counterion}]$$

$$S = \sqrt{K_{sp}}$$

The K_{sp} value for a given salt of a compound is a constant value. Therefore, the drug concentration in saturated solutions is a function of the counterion concentration. As the counterion concentration in solution increases, the dissolved drug concentration decreases to maintain the K_{sp}. This is an important concept, especially for hydrochloride salts of poorly soluble compounds, because the active drug concentration that can be achieved is a function of the chloride concentration in the solvent or the gastrointestinal tract after an oral dosing (Bhattachar et *al.*, 2006).

The essential characteristic of salts that makes them so attractive in pharmaceutical applications is that the coulombic attraction between the drug molecule and counterion changes the potential energy landscape of the solid state and leads to stronger interactions

between the charged drug substance and polar aqueous solvents. This can result in enhanced dissolution rates and higher apparent solubility on physiologically relevant timescales, resulting in more effective drug delivery *in vivo* (Bhattachar et *al*., 2006).

The advantage of salts in terms of solubility and a higher dissolution rate can be interpreted through Noyes-Whitney equation too, since the salts have strong self-buffering capability at the diffusion layer, which increases the apparent solubility of the parent drug in that layer.

This is illustrated in Figure 8 where the hydrochloride salt dissolved faster at higher pH values, presumably due to the lower diffusion layer pH. A practical method of estimating pH at the surface of a dissolving solid is by measuring the pH of a saturated solution of the drug substance in the particular aqueous medium.

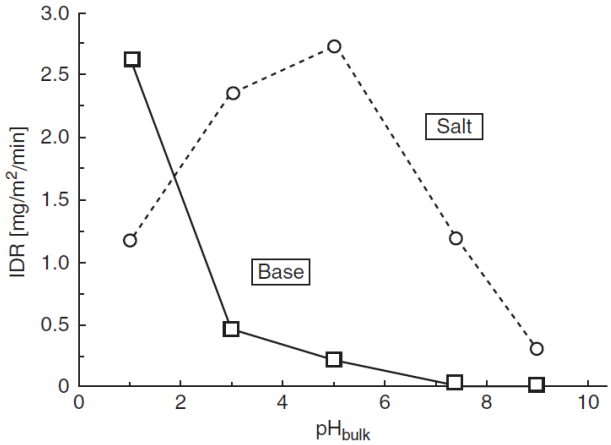


Figure 8. Intrinsic dissolution rate of a weak base, and its HCl salt, as a function of pH of the dissolution medium (Tong, 2009)

2.5.2. Advantages of salts *in vivo*?

Although pharmaceutical salts theoretically exhibit higher dissolution rate, which is well known to be driving force for absorption, it still needs be to confirmed if this indeed affects absorption processes *in vivo*, in which cases and up to which extent.

Interestingly, literature review revealed that human bioequivalence studies comparing salt forms of basic drugs have been rather limited and none of them reported significant differences in bioavailability between different salt forms due to differences in their aqueous solubilities (Engel et *al*., 2000).

Lin et *al*., 1972, for example, reported no enhancement in bioavailability when salts of a basic antihypertensive agent, 1-(2,3-dihydro-5-methoxybenzo[b]furan-2-ylmethyl)-4-(o-methoxyphenyl) piperazine, having significantly different intrinsic dissolution rates, were

compared. The intrinsic dissolution rates of monohydrochloride, dihydrochloride and disulphate salts and the free base were studied. Their hypotensive activity was studied in anesthetized dogs. The results of dog study indicated that hypotensive potencies of the three salts did not differ from each other although the intrinsic dissolution rates of the dihydrochloride and disulphate salts were 54 and 42 times greater, respectively, than that observed with the monohydrochloride salt. The free base failed to produce a significant hypotensive effect *in vivo* but this was attributed and correlated to the fact that *in vitro* dissolution rate of the free base was much lower than the rate of its salts.

Walmsley et *al*., 1986, also indicated that they did not observe a difference in the extent of bioavailability between oxalate and citrate salts of naftidrofuryl, while Jamuludin et *al.*, 1988, saw no significant differences in c_{max}, t_{max}, or *AUC* of quinine following oral administration of the hydrochloride, sulphate and ethylcarbonate salts to healthy volunteers.

For salts of weak acids there are some *in vivo* data published half a century ago indicating their potential superiority over the free acid in regard to the absorption characteristics.

For example, Furesz, 1958 and Nelson et *al*., 1962, demonstrated pronounced differences observed in rates and extents of absorption for novobiocin and tolbutamide, respectively, when compared to their sodium salts.

Lee et *al*., 1958, confirmed better absorption of potassium salt of penicillin V in comparison with free acid in experiments with dogs.

Holmdahl et *al*., 1959, in human study showed significantly improved absorption of sodium salt of iopanoic acid in comparison with the free acid.

The literature review reveals that correlation between *in vitro* and *in vivo* behaviour of drugs is far from obvious. Although *in vitro* salts exhibit higher dissolution rates, expectations that the same would happen *in vivo* must be based on thorough research regarding conditions and the biorelevance of i*n vitro* experiments as well as physiological factors that influence bioavailability of drug.

2.5.3. Chalenges for dissolution of salts in GI fluids

Due to their ionization properties, lipophilic poorly soluble weak bases administered in the fasted state are expected to be primarily dissolved during residence in stomach because

their solubility is expected to be higher in the fasting stomach than elsewhere in the GI tract (Fotaki and Vertzoni, 2010).

However, due to limited gastric residence times in the fasted state, complete dissolution of the dose prior to reaching the small intestine may not be possible.

According to Wilson et *al.*, 2010, retention times of formulations in the stomach are dependent on the size of the formulation and whether or not the formulation is taken with a meal. Incomplete dissolution of the dose of a lipophilic base in the fasted stomach is likely in cases where the subject is hypochlorohydric, the dosage form disintegrates slowly, and/or the compound is highly dosed.

Improvement of dissolution of the dose during gastric residence with the use of salt of a base is typically decided on the basis of dissolution data and/or equilibrium solubility data in hydrochloric acid solutions (Serajuddin, 2007).

However, equilibrium solubility of weak bases is not exclusively dependent on hydrochloric acid concentration (Vertzoni et *al*., 2007). Study with three lipophilic bases, performed by Vertzoni et *al*., 2007, showed that although combination of data in FaSSGF and $HCl_{pH1.6}$ seemed to be the most efficient way to estimate intragastric solubility, its accurate estimation remains problematic, due to difficulties in design of appropriate medium.

If dissolution is more complete *in vitro* than *in vivo*, its importance on plasma levels may (depending on disposition characteristics) be underestimated and *vice versa* (Kortejärvi et *al*. 2007).

Further on, the solubility of a salt decreases if common ions, such as Cl^- and Na^+, are present, and since the dissolution rate is proportional to the solubility in the diffusion layer at the surface of the solid, any impact of a common ion on solubility would also influence dissolution rate (Serajuddin, 2007).

Supersaturation in the intestinal fluid is an important property that can play a significant role in drug absorption. For compounds with poor intrinsic solubility in the intestinal fluid, solubility is often a limiting factor for absorption. For many of these compounds it may not be possible to enhance the saturation solubility to the extent required such that the whole dose is dissolved in the GI fluid. In this case, creating or maintaining supersaturation in the intestinal fluid can be an effective way to enhance absorption of these compounds. Depending on the properties of the salt and its corresponding base, the fate of the salt in the GI tract may vary significantly (Tong, 2009).

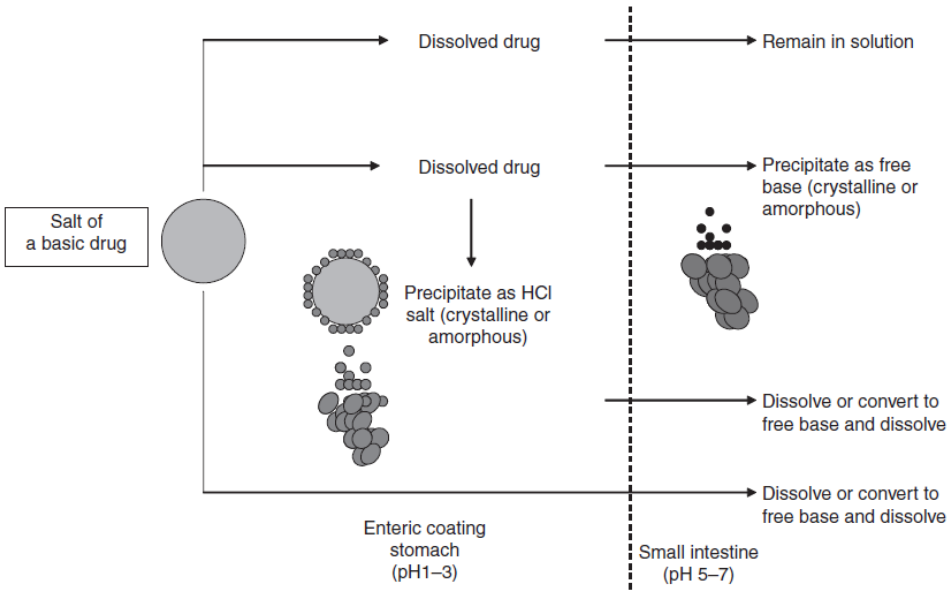


Figure 9. Dissolution processes of the salt of a basic drug in the gastric and intestinal fluids (Tong, 2009)

As illustrated in Figure 9, when the salt of the basic drug gets in the GI tract it may dissolve in the stomach and either remain in solution or precipitate out as the free base when it gets emptied into the intestine. It may also convert to the hydrochloride salt if the hydrochloride salt is less soluble, especially with the influence of the common ion effect. In this case, the dissolution in the intestine is actually the dissolution of the precipitated hydrochloride salt. To further complicate the situation, when salt conversion happens *in vivo,* the material can precipitate out as either crystalline or amorphous forms with different particle sizes.

Furthermore, if the dose is only partly dissolved during gastric residence, problematic dissolution in the small intestine can lead to low and/or variable oral bioavailability, especially in cases where intralumenal concentrations control the overall absorption process.

2.5.3.1. Effect of chloride ion on dissolution of free bases and their salts

There is an abundance of chloride ion in the gastrointestinal tract, in the gastric fluid as well as in the intestinal fluid. Lindahl et *al.*, 1997, reported that the chloride ion concentration in the intestine could be even higher than that in the stomach; values of 0.10 and 0.13 M in gastric and jejunal fluids, respectively, in the fasted state have been reported.

The question of influence of chloride ions on dissolution rate of free bases and their hydrochloride salts has often been addressed and well described in literature. Serajuddin and

Jarowski 1985, presented review of literature related to this topic. In reported papers the apparent dissolution rates and the solubilities of the bases in diluted HCl at pH 1–2 were higher than those of their respective hydrochloride salts. The dissolution rates of the bases increased with a decrease of pH and a corresponding increase in chloride concentration whereas the reverse was true for the hydrochloride salts. Basically, the common ion effect presents the shift in equilibrium that occurs when an ion already present in the equilibrium reaction is added (Le Chatelier's Principle).

The behavior of nonhydrochloride salts in the presence of chloride ions, and possible conversion of non HCl salt to HCl salt, has been studied more thoroughly in the last decade.

Lin et *al*., 1972, postulated that even if a non-HCl salt is used, it could convert into a HCl-salt in presence of chloride ion in the gastrointestinal fluid, and thus dissolution rates and bioavailability of hydrochloride and nonhydrochloride salts could be similar.

This finding was also described by Li et *al*., 2005, who confirmed conversion of haloperidol mesylate and phosphate salt to hydrochloride salt during dissolution test by FTIR. The common-ion effect, however, depended on the kinetics of the conversion of non-HCl salts to the HCl salt form. Although intrinsic dissolution rates of haloperidol mesylate and phosphate decreased in the presence of Cl^-, they were still higher than that of the hydrochloride salt. The role of the non-HCl to HCl salt conversion kinetics was particularly evident during powder dissolution. Because of high surface area, powders of haloperidol mesylate dissolved completely in 5 min at pH 2 (0.01 M HCl containing 0.1 M NaCl) before its dissolution rate could be affected by the conversion to the HCl salt form. It can be concluded that, if a nonhydrochloride salt form has sufficient aqueous solubility and a favorable apparent solubility product value, it could dissolve in dissolution medium before its complete conversion to the hydrochloride salt form occurs, and that the use of non hydrochloride salt could still be favourable.

In the paper from 2007, Serajuddin stated that in cases where besylate (benzenesulfonate) and bisulfate (hydrogen sulfate) salts were used, he did not experience salt conversion to the hydrochloride forms during the determination of solubility and dissolution rate in 0.1 M NaCl.

It is interesting to point out here that there has been strong historical trend in the pharmaceutical industry to market hydrochloride salts of amines, due to their low molecular weight and low toxicity (Engel et *al*., 2000).

However, several authors called for precaution on use of hydrochloride salts in pharmaceutical preparations, due to possible decrease of their solubility as a result of chloride ion common ion effect in the stomach (Miyazaki et *al*., 1981, Thomas and Rubino, 1996).

Engel et *al.*, 2000, reported that lately mesylate salts are becoming more common on the market; their portion in the FDA commercially marketed salts was just about 2 % in 1977, while in the last few years nearly 20 % of new chemical entities approved by FDA that had associated anionic salts were mesylate salts.

2.5.3.2. Precipitation in the small intestine

As mentioned previously, there is a possibility of precipitation of poorly soluble week bases as the drug moves from the favourable pH conditions of the stomach to a less favourable pH environment of the small intestine. As the pH nears or even exceeds the pK_a of the base, its solubility undergoes a sharp decrease. Therefore, after administration of the weak bases in the fasted state contents of the upper small intestine may be supersaturated with the base and the flux across the intestinal mucosa may be higher than expected from equilibrium solubility considerations (Psachoulias et *al*., 2011).

Kostewicz et *al*., 2004 addressed the question whether the drug stays in supersaturated solution or it precipitates and becomes no longer available for absorption. To answer the question the authors simulated the transfer from stomach into the intestine by using a transfer model in which a solution of the drug in simulated gastric fluid is continuously pumped into a simulated intestinal fluid and drug precipitation in the acceptor medium was examined. Three poorly soluble weakly basic drugs were investigated and for all three, extensive supersaturation was achieved in the acceptor medium. The precipitation of drugs occurred only under simulated fasted state conditions, while in fed state simulated conditions the bile components and the lower pH inhibited precipitation.

Psachoulias et *al*., 2011, evaluated the precipitation and the supersaturation of contents of the upper small intestine after administration of two lipophilic, highly permeable weak bases to healthy fasted adults. Precipitated fractions of the tested bases were not substantial, and estimating intestinal supersaturation in regard to free base was found to be inadequate as other phases may also precipitate.

2.5.3.3. The ion exchange interactions

The effect of ion exchange between ionized week bases and sodium ions from acidic disintegrants such as croscarmellose sodium (CCS) used in drug formulation, have been reported by several authors (Fransén et *al*., 2008, Hollenbeck, 1988, Narang et *al.*, 2010).

The interaction exists *in vitro* under conditions where the cationic form of the drug exists and can exchange with sodium ions associated with the insoluble dispersed croscarmellose (cross-linked sodium carboxymethyl cellulose).

Frensen et *al.*, 2008, tested three week bases having different hydrophilicity and found out that all three were binded to CSS to the same degree suggesting that the mechanism behind the binding was purely ion exchange.

Such interaction results in incomplete drug release as a function of pH. Dissolution was not affected by ionic strength changes. At physiological salt concentrations the interactions did not appear to be strong so the authors concluded that such interactions should not influence the *in vivo* bioavailability of the drugs.

2.5.4. Regulatory aspects of changes in salt form

According to EMA Guideline on the investigation of bioequivalence, 2010, medicinal products with different salts are pharmaceutical alternatives, meaning that they are considered to be the same active substance, unless they differ significantly in properties with regard to safety and/or efficacy.

When the test product contains a different salt than the reference medicinal product, their bioequivalence should be demonstrated in *in vivo* bioequivalence studies.

However, biowaiver (an exemption form bioequivalence study) may also be applicable even if test and reference medicinal product contain different salts provided that both belong to BCS-class I (high solubility and complete absorption), and fulfil other criteria necessary for biowaiver request, as has been described previously.

2.6. Doxazosin as model drug

The only registered and marketed form of doxazosin is doxazosin mesylate. The brand name is Cardura®, Pfizer.

Doxazosin mesylate is used in men to treat the symptoms of an enlarged prostate (benign prostatic hyperplasia or BPH), which include difficulty urinating, painful urination, and urinary frequency and urgency. It is also used alone or in combination with other medications to treat high blood pressure.

Doxazosin mesylate is a long acting, selective, alpha-1 antagonist. It reduces total peripheral resistance by selective postsynaptic alpha-1 blockade, without reducing cardiac output and consequently relieves the symptoms of BPH by relaxing the muscles of the bladder and prostate and lowers blood pressure by relaxing the blood vessels.

Doxazosin mesylate appears to maintain this antihypertensive effect over a 24-hour dosing interval. As well as other alpha-1 adrenoceptor antagonists, doxazosin also has favourable effects on the plasma lipid profile in terms of decreasing total cholesterol and triglycerides, and increasing high-density lipoprotein (HDL) cholesterol as well as the HDL/total ratio. Pharmacokinetic profile of doxazosin is characterized by rapid absorption and long estimated elimination half-life.

After oral administration of therapeutic doses, peak plasma levels of doxazosin mesylate tablets occur at about 2–3 hours. Bioavailability is approximately 65 %, reflecting first pass metabolism of doxazosin by the liver.

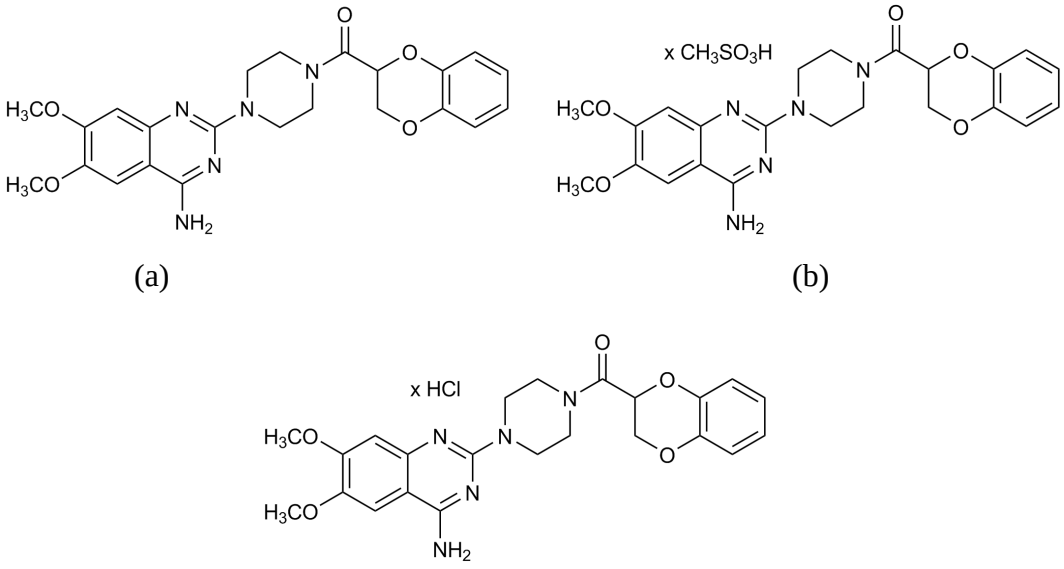
The effect of food on the pharmacokinetics of doxazosin mesylate tablets was examined in a crossover study with twelve hypertensive subjects. The presence of food delayed absorption by about 1 hour. Reductions of 18 % in mean maximum plasma concentration and 12 % in the area under the concentration-time curve occurred when doxazosin mesylate tablets was administered with food. Neither of these differences was statistically or clinically significant.

Plasma elimination of doxazosin is biphasic, with a terminal elimination half-life of about 22 hours. Steady-state studies in hypertensive patients given doxazosin doses of 2–16 mg once daily showed linear kinetics and dose proportionality. Enterohepatic recycling is suggested by secondary peak of plasma doxazosin concentrations.

It is extensively metabolized, with majority of administered dose excreted in faeces. Plasma concentrations are increased in a linear dose-dependent manner. (Product monograph Cardura®, Pfizer, Babamoto and Hirokawa, 1992, Elliot et *al.*,1987, Carlson et *al.,* 1986).

The empirical formulas for doxazosin base, doxazosin mesylate and doxazosin hydrochloride are $C_{23}H_{25}N_5O_5$, $C_{23}H_{25}N_5O_5 \cdot CH_4O_3S$ and $C_{23}H_{25}N_5O_5 \cdot HCl$, respectively. Their molecular weights are 451.48 (DB), 547.6 (DM) and 487.94 (DH).

Structures of doxazosin base, doxazosin mesylate and doxazosin hydrochloride are presented in Figure 10.



(a) (b)

(c)

Figure 10. Structures of doxazosin base (a), doxazosin mesylate (b) and doxazosin hydrochloride (c)

Doxazosin is a highly permeable compound, with a pK_a of 6.93 at 25 ^oC (Product monograph Cardura®, Pfizer), and a log *P* between 2.1 and 2.8 (http://www.drugbank.ca/drugs/DB00590; http://69.20.123.154/services/bcs/results.cfm, accessed 27 December 2010).

Doxazosin mesylate is freely soluble in dimethylsulfoxide, soluble in dimethylformamide, slightly soluble in methanol, ethanol, and water (0.8 % at 25 °C), and very slightly soluble in acetone and methylene chloride.

3. GOALS OF THESIS

Although it is well known that pharmaceutical salts usually exhibit higher dissolution rate *in vitro*, surprisingly, for week bases and their salts this is not supported by results of human or animal bioequivalence studies in literature (Engel et *al*., 2000).
On the other hand, for salts of week acids, there have been some published data from half a century ago, which indicate their potential superiority over the free acids in regard to the absorption characteristics.

Since weak bases are most frequently administered as salts, the primary motivation for the present thesis was to investigate potential superiority of salts of weak bases over their free form. In order to investigate this, *in vitro* studies as well as pharmacokinetic studies on dogs in fasted and fed state have been performed.

Two salts of doxazosin were chosen for investigation: mesylate and hydrochloride salt. Mesylate salt was chosen because it is the only marketed salt of doxazosin, and because pharmacokinetic *in vivo* data on humans were already available. The hydrochloride salt was chosen to be tested only *in vitro*, and collected data would be used to support the conclusions brought for base and mesylate. Specifically, hydrochloric salt of doxazosin was studied *in vitro* to evaluate potential conversion of bases and/or non-HCl salts to their HCl salts in stomach (Serajuddin et *al*., 2007).

In general, three main goals of the thesis had been established.

The first goal was to perform *in vitro* solubility and dissolution characterization in media with different pH and compositions, in order to evaluate the importance of simulating the lumenal composition for predicting differences in early exposure between a salt and the free form of a lipophilic weak base doxazosin in the fasting state.
The examples from the literature indicated that improvement of dissolution of the dose during gastric residence with the use of salt of a base is typically decided on the basis of dissolution data and/or equilibrium solubility data in hydrochloric acid solutions (Serajuddin, 2007). However, equilibrium solubility of weak bases is not exclusively dependent on hydrochloric acid concentration (Vertzoni et *al*., 2007). If dissolution is more complete *in vitro* than *in vivo*, its importance on plasma levels may (depending on disposition characteristics) be underestimated and vice versa (Kortejärvi et *al*., 2007). Furthermore, if the dose is partly dissolved during gastric residence, problematic dissolution in the small intestine can lead to low and/or variable oral bioavailability, especially in cases where intralumenal concentrations control the overall absorption process. The extent at which the environment in the small intestine needs to be simulated for evaluating differences in dissolution between a free base and its salts has not been addressed in the literature.

The second goal was to evaluate the usefulness of the canine model in forecasting differences in early exposure between the free form and a salt of a base in the fasting state.
As explained earlier, dogs have faster gastric emptying and often less acidic fasting intragastric pH compared with humans (De Zwart et *al*., 1999). Also, they may have lower osmolarity in the stomach and higher bile salts concentrations in the small intestine (Kalantzi et *al*., 2006). Such differences from the human luminal environment may limit their usefulness in the comparison of salt(s) with the free form of a base.
So far, canine studies have not been reported in literature to address the potential differences between bases and their salts.

The third objective of this study was to evaluate the food effect in order to determine the influence on doxazosin absorption, to roughly evaluate the usefulness of the canine model for prediction of food effect, based on human food effect data for DM and to evaluate the usefulness of biorelevant data in predicting food effects in humans.
Therefore additional experiments would be performed in fed state simulating conditions *in vitro* and also in fed study on dogs.
If the existing bioanalytical methods would be found inappropriate for the canine study samples (due to lower concentration in canine samples, unsatisfying selectivity, lower plasma volume available for sample preparation, lower recovery etc.), the new specific and sensitive bioanalytical method should also be developed.

This and other specific goals will be numbered hereinafter.

3.1. Specific goals

The specific goals of this thesis were the following:

- To prepare DB, DM and DH drug substances and to characterize them in order to confirm their identity and physicochemical properties;
- To prepare DB, DM and DH tablets, each containing 2 mg of doxazosin calculated on the free base;
- To evaluate solubility of drug substances and dissolution behaviour of drug products in different media, which would later on enable proper evaluation of results of pharmacokinetic studies and bringing conclusions about the relevance of each media;
- To evaluate potential conversion of DB and/or DM to DH in acidic conditions;
- To perform *in vivo* pharmacokinetic study on dogs, in the fasted and fed state, using DB and DM tablets;
- To develop sensitive analytical method for determination of low concentrations of doxazosin in canine plasma;
- To use already existing results of pharmacokinetic study in humans with DM tablets and compare them with results of DM tablets in dogs, in fasted state. In this way the usefulness of canine data for evaluating differences in early exposures of DM over the free DB would be assessed. For this purpose, indirect comparison would be employed. Using absorption simulation modelling based on solubility and dissolution data in gastric and intestinal media (simple vs. biorelevant media), cumulative % of doxazosin in plasma for DB and DM would be simulated and compared to individual cumulative % of doxazosin in plasma for DM in human study. The degree of correlation with the individual values for DM in humans would enable conclusions on
    - advantage of salt vs. the free base,
    - the usefulness of canine data for evaluating advantages of salts in terms of early exposure,
    - advantage of using the biorelevant media in *in vitro* experiments in comparison with the simple hydrochloric and buffer media;
- To evaluate the food effect on doxazosin absorption, to roughly evaluate the usefulness of the canine model for prediction of food effect, based on human food

effect data for DM and to evaluate the usefulness of biorelevant data in predicting food effects in humans.

4. MATERIALS AND METHODS

4.1. Materials

Sodium taurocholate, min 95 % pure (lot no. 033K5306) and 99.5 % pure (lot no. 2003040161) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and Prodotti Chimici e Alimentary S.p.a. (Basaluzzo, Italy), respectively. Egg-phosphatidylcholine min. 98 % pure (lot no. 105019-1/129 and 105026-1/62) was donated by Lipoid GmbH, (Ludwigshafen, Germany). Potassium dihydrogen phosphate, sodium hydroxide, potassium chloride, sodium chloride, phosphoric acid, hydrochloric acid and trichloromethane, all analytical grade, were purchased from Panreac Quimica SA (Barcelona, Spain). Maleic anhydride was purchased from Sigma-Aldrich (Steinheim, Germany). Acetic acid (glacial) and dimethyl sulfoxide (DMSO) of analytical grade and ammonium acetate, ethanol, acetonitrile and methanol, all HPLC grade were purchased from E. Merck (Darmstadt, Germany).

Prazosin hydrochloride (purity > 99 %) was used as internal standard in LC-MS/MS method and it was purchased from Sigma (Sigma Aldrich Chemie, GmbH, Germany). Tetramethylsilane was used as internal standard for ^{1}H NMR analyses and it was purchased from Fluka Chemie GmbH (Buchs, Switzerland),

Pepsin from porcine stomach mucosa (Cat.No. P-7125, lot 11K1242, 66 units/mg solid, 1.031 units/mg prot.) was purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), and distilled monoglyceride (95 % monoglycerides, 4 % diglycerides, 89 % oleic acid, lot no 173403-2202/76, Rylo MG 19, Pharma,) was obtained from Danisco (Denmark). Maleic anhydride, min 99 % pure, purchased from Fluka Chemie GmbH (Buchs, Switzerland), and citric acid monohydrate, p.a., min. 99.5–101 % pure, purchased from Riedel-de Haen (Seelze, Germany).

The source of the long life milk, 3.5 % fat, was Landesgenossenschaft Ennstal Molkereibetriebe (Steinach, Austria).

The water of HPLC grade was obtained from a Labconco® water pro ps. System (Kansas City, Missouri/USA) or from Q-system (Millipore, Milford, MA). Syringe filters were regenerated cellulose 0.45 µm (Titan®, Scientific resources INC, Eatown, NJ/USA) or 0.2 µm Gelman acrodisc CR PTFE syringe filters (Gelman Science, Ann Arbor, MI).

Syringes for sampling procedure were Fortuna Optima® Syringe (Fischer Labortechnik, Frankfurt/Main, Germany).

4.2. Methods

4.2.1. Preparation and characterization of DB, DM and DH

Doxazosin base (DB), doxazosin mesylate (DM) and doxazosin hydrochloride (DH) powders were prepared by Chemical department in PLIVA (Zagreb, Croatia). Briefly, DB was prepared by the reaction of 2-chloro-6,7-dimethoxyquinazolin-4-amine and (2,3-dihydrobenzo[b]-[1,4]dioxin-2-yl)(piperazin-1-yl)methanone, according to established procedures (Campbell et *al.*, 1987; Chou et *al.*, 2001). DM was prepared by the reaction of doxazosin base with methanesulphonic acid. DH was prepared by the reaction of doxazosin base with HCl. The three phases were assayed for purity by a validated HPLC method and the results were 97.2 % for DB, 101.2 % for DM and 98.0 % for DH.

DB, DM and DH were characterized with nuclear magnetic resonance (^{1}H NMR), X-ray powder diffraction (XRPD), Fourier transforms infrared spectroscopy (FTIR), dynamic vapour sorption (DVS), differential scanning calorimetry (DSC), and scanning electronic microscopy (SEM).

^{1}H NMR spectra were recorded on Varian Gemini 300 spectrometer in DMSO at room temperature using tetramethylsilane as internal standard.

The XRPD patterns were recorded using a Phillips X'pert Pro powder diffractometer at 40 mA, 45 kV and with monochromatised CuKα radiation (λ = 1.54056 Å). The samples were scanned at room temperature in continuous scan mode over the range 3–40˚ with step size of 0.01671 2θ. Data were analyzed using software package Xpert Plus, version 1.3e.

FTIR spectra were recorded using Perkin Elmer Spectrum GX (Perkin Elmer, UK). The samples were prepared by the potassium bromide disc method and scanned over the range 4000–370 cm^{-1}. For each sample, 16 scans were collected at a 4 cm^{-1} resolution.

DVS data were recorded on DVS1 instrument (Surface Measurement Systems, UK) in 10 steps (dm/dt 0.003) in relative humidity range 0–90 % at 25 ^oC.

DSC thermograms of DB, DM and DH were recorded on Perkin Elmer Pyris 1 (Perkin Elmer, UK). The instrument was calibrated with indium and zinc prior to analyzing the samples. Accurately weighed samples (2–5 mg) were placed in sealed aluminium pans, and scanned at the heating rate of 10 °C min^{-1} over the temperature range 30–300 °C under dry nitrogen (35 ml/min).

SEM analysis was carried out using JSM 5800 (JEOL, Tokyo, Japan) microscope. The samples were previously gold sputtered using Edwards S150 sputter coater under argon atmosphere to render them electrically conductive. Images were analyzed using software package Link ISIS, Series 300, Version 3.35.

4.2.2. Solubility measurements of DB, DM and DH

Equilibrium solubility of DB, DM and DH was measured in triplicate using the shake-flask method in human gastric fluid (HGF), in canine gastric fluid (CGF), in fasted state simulating gastric fluid (FaSSGF-V2), in USP pH 1.2 buffer solution (The United States Pharmacopeia, 2011), in pH 1.8, 2.6, and 3.0 HCl solution. The preparation of solubility media will be described hereinafter.

Human gastric fluids (HGF) were collected from the stomach of fasted healthy adults, after receiving appropriate approvals from the Scientific and the Executive Committee of the Red Cross Hospital of Athens (AP 8203) as described previously (Kalantzi et *al.*, 2006a). Individual human gastric fluids were kept frozen at –70 °C until used. Assuming that during a bioavailability study the particles of an immediate release dosage form will empty from the stomach together with the co-administered water and in order to aspirate fluids that reflect the average gastric composition, fluids were aspirated between 20 and 40 minutes post administration of 250 ml of water. On the day of solubility measurement, individual fluids from 5 subjects were brought to room temperature and pooled so that from each individual a total of two samples were obtained (one aspirated at 20 min and one at 40 min) and each sample had a volume of approximately 3 ml. Following to centrifugation at a low speed to remove possible solid particles, the pooled sample (~ 30 ml, human gastric fluid, HGF) was used immediately for the solubility measurements. The pH of HGF was 1.8.

Canine gastric fluids (CGF) were collected from four healthy female mongrel dogs (4-years old, 28–32 kg) that were accommodated in an animal facility operating according to the European Union regulations for the maintenance and experimentation on animals and it has been approved by the Veterinary Directorate of the Municipality of Athens (EL 25 BIO 08). Dogs were fasted from the afternoon prior to the experimental day. As in humans (Kalantzi et *al.*, 2006a), the objective was to collect canine gastric fluids at various times, after water administration. Knowing that gastric emptying rates in fasted dogs state are faster than in fasted humans (De Zwart et *al.*, 2005), on the experimental day, each dog was administered 400 ml of water using a sterile disposable tube (Levin #14). Assuming that during a

bioavailability study the particles of an immediate release dosage form will empty from the stomach together with the co-administered water and in order to aspirate contents that reflect the average gastric composition, 10 min. after the administration of 400 ml water the tube was again inserted into the dog's stomach, and about 10 ml of gastric contents were aspirated from each dog. No medication was given to the dogs prior to or during the aspiration period. Aspirates were kept at –70 ^{o}C. On the day of the solubility measurement, the 4 samples were brought to room temperature and pooled. The pooled sample (~ 40 ml, canine gastric fluid, CGF), after centrifuging at a low speed, was used immediately for the solubility measurements. The pH of CGF was 2.6.

FaSSGF-V2 was prepared as follows: 4.0 g sodium chloride and 1.0606 g of pepsin (70000 IU) are dissolved in about 900 ml of HCl 20 mM. The pH is then adjusted to 1.6 with HCl conc. Sufficient HCl 20 mM is added in order to produce a final volume of 1000 ml (blank FaSSGF). 450 ml of blank FaSSGF is used to dissolve 0.0430 g of sodium taurocholate. 3.167 ml of 5 mg/ml lecithin in chloroform solution is added. The chloroform is then evaporated on a Rotavap under vacuum using a water bath adjusted to 40 °C. After cooling to room temperature, the volume is adjusted to 1000 ml with blank FaSSGF. To make it more physiologically relevant in terms of sodium chloride concentration (Lindahl et *al.*, 1997) and osmolality (Kalantzi et *al.*, 2006a), FaSSGF-V2 contains 68 mM sodium chloride (Vertzoni et *al.*, 2007), instead of 34 mM NaCl (FaSSGF, Vertzoni et *al.*, 2005).

USP pH 1.2 buffer contained 50 mM KCl (USP 33/NF 28, 2011), while aqueous media with different pHs (pH 1.8, 2.6 and 3.0) were prepared by diluting concentrated hydrochloric acid in 1000 ml.

The solubility medium (5 or 10 ml) and the drug phase in excess (150 mg) were transferred into Erlenmeyer flasks (ca. 25 ml). Flasks were covered with parafilm and put in a shaking water bath (37 °C). All samples were prepared in triplicate.

Based on the equilibration time measured in FaSSGF-V2, 6 h was considered adequate for the determination of equilibrium solubility of DB, DM, and DH in all media. At equilibrium, the pH in each flask was measured and samples were filtered through regenerated cellulose 0.45 μm filters (Titan®, Scientific resources INC, Eatown, NJ/USA), discarding the first millilitre, and assayed by HPLC directly or appropriately diluted with FaSSGF-V2 or HCl solution. The parameters of the validated HPLC method used for assay determination are described later on. Peak areas were evaluated against standard curves that were prepared on the experimental day.

Differences between solubility data in two different media were evaluated with unpaired *t*-test at the 0.05 level.

4.2.3. Preparation of DB, DM and DH tablets

Uncoated immediate release tablets of DB, DM and DH were prepared by using a combination of commonly used excipients (lactose monohydrate, croscarmellose sodium, sodium lauryl sulphate, colloidal anhydrous silica, starch, and magnesium stearate) and a direct compression method. Each tablet contains 2 mg of doxazosin in a form of doxazosin mesylate, hydrochloride or base.

After the compression of tablets, samples were tested for their average weight, weight variation, hardness and disintegration.

4.2.4. Dissolution studies of DB, DM and DH tablets

Dissolution experiments were run in triplicate at 37.0 ± 0.5 °C using the USP II Apparatus (Distek® dissolution tester, model 2100B, North Brunswick, NJ, USA and Varian; VanKel, model 7010, Cary, NC) with the paddle rotating at 100 rpm.

Experiments were performed in 500 ml of the following media: pH 1.6 and pH 2.6 HCl solution, FaSSGF-V2, pH 5.0 acetate buffer (144 mM acetates), pH 5.0 citrate buffer (84 mM citrates), pH 6.5 phosphate buffer (29 mM phosphates), pH 6.5 maleate buffer (25 mM maleates), FaSSIF, FaSSIF containing maleates ($FaSSIF_m$) (Vertzoni et *al.*, 2004), FeSSIF (Dressman et *al.*, 2000, Galia et *al.*, 1998), FeSSIF containing citrates (Kalantzi et *al.*, 2006) and milk digested with pepsin (Macheras et *al.*, 1987, Klein et *al.*, 2004, Fotaki et *al.*, 2005). Dissolution media were prepared freshly on the day of each experiment.

Due to complexity of media preparation and experimental procedure, dissolution studies in digested milk will be described separately at the end of this chapter. FaSSGF-V2 and diluted hydrochloric media with different pH were prepared as described in Solubility section.

Dissolution media used in dissolution experiments were prepared as follows:

Phosphate buffer pH 6.5

3.9 g KH_2PO_4 and 6.2 g NaCl was dissolved in cca 750 ml of water. pH was adjusted to 6.5 with cca 138 ml of 0.1 M NaOH (prepared by dissolving 4 g in 1000 ml water). The solution was made to volume of 1000 ml.

This buffer is used also in preparation of FaSSIF.

Maleate buffer pH 6.5

2.4512 g maleic anhydride and 6.3 g NaCl was dissolved in cca 750 ml of water. pH was adjusted to 6.5 with cca 225 ml of 0.2 M NaOH (prepared by dissolving 8 g in 1000 ml water). The solution was made to volume of 1000 ml.

This buffer is used also in preparation of $FaSSIF_m$.

Acetate buffer pH 5.0

144 ml acetic acid and 10.1 g NaCl was dissolved in cca 750 ml of water. pH was adjusted to 5.0 with cca 101 ml of 0.1 M NaOH (prepared by dissolving 4 g in 1000 ml water). The solution was made to volume of 1000 ml.

This buffer is used also in preparation of FeSSIF.

Citrate buffer pH 5.0

420 ml 0.2 M citric acid (prepared by dissolving 40.2 g of citric acid monohydrate in 1000 ml of water) and 12.07 g NaCl was dissolved in cca 750 ml of water. pH was adjusted to 5.0 with cca 200 ml of 1 M NaOH (prepared by dissolving 40 g in 1000 ml water). The solution was made to volume of 1000 ml.

This buffer is used also in preparation of $FeSSIF_c$.

Fasted State Simulated Intestinal Fluid with phosphate buffer (FaSSIF)

FaSSIF containing 3 mM sodium taurocholate and 0.75 mM egg lecithin, with a pH of 6.5 and an osmolality of about 270 mOsm/kg, is prepared as follows:

3.9 g of KH_2PO_4 and 6.2 g of NaCl are added to approximately 750 ml of water, HPLC grade. The pH is then adjusted to exactly pH 6.5 using approximately 138.5 ml of NaOH 0.1 M. Sufficient water HPLC grade is added in order to produce a final volume of one litre (blank FaSSIF).

450 ml of blank FaSSIF is used to dissolve 1.65 g of sodium taurocholate (> 97 % pure). 5.908 ml of 100 mg/ml lecithin in chloroform solution are added. The chloroform is then

evaporated on a Rotavap under vacuum using a water bath adjusted to 40 °C. After cooling to room temperature, the volume is adjusted to 1000 ml with blank FaSSIF (Galia et *al.*, 1998).

Fasted State Simulated Intestinal Fluid with maleate buffer (FaSSIF$_m$)

FaSSIF$_m$ containing 3 mM sodium taurocholate and 0.75 mM egg lecithin, with a pH of 6.5 and an osmolality of about 270 mOsm/kg, is prepared as follows:

2.45 g of $C_4H_2O_3$ (maleic anhydride) and 6.3 g of NaCl are added to approximately 750 ml of water, HPLC grade.

The pH is then adjusted to exactly pH 6.5 using approximately 225 ml of NaOH 0.2 M. Water (HPLC grade) is added in order to produce a final volume of one litre (blank FaSSIF).

450 ml of blank FaSSIF is used to dissolve 1.65 g of sodium taurocholate (> 97 % pure). 5.908 ml of 100 mg/ml lecithin in chloroform solution are added. The chloroform is then evaporated on a Rotavap under vacuum using a water bath adjusted to 40 °C. After cooling to room temperature, the volume is adjusted to 1000 ml with blank FaSSIF$_m$.

Fed State Simulated Intestinal Fluid with acetate buffer and glycerol-monooleate (FeSSIF)

FeSSIF containing 15 mM sodium taurocholate, 5 mM glycerol-monooleate, and 3.75 mM egg lecithin, with a pH of 5.0 and an osmolality of about 635 mOsm/kg, is prepared as follows:

144 ml CH_3COOH and 10.1 g of NaCl are added to approximately 750 ml of water, HPLC grade. The pH is then adjusted to exactly pH 5.0 by using approximately 101 ml of NaOH 0.1 M. Sufficient water HPLC grade is added in order to produce a final volume of 1l (blank FeSSIF).

450 ml of blank FeSSIF is used to dissolve 8.25 g of sodium taurocholate (> 97 % pure). 29.54 ml of 100 mg/ml lecithin in chloroform solution is added, as well as 10 ml of glycerol-monooleate (GMO), prepared by dissolving 1.8382 g of GMO in 10 ml of chloroform. The chloroform is then evaporated on a Rotavap under vacuum using a water bath adjusted to 40 °C. After cooling to room temperature, the volume is adjusted to 1 l with blank FeSSIF.

Fed State Simulated Intestinal Fluid with citric acid and glycerol-monooleate (FeSSIF$_c$)

FeSSIF containing 15 mM sodium taurocholate, 5 mM glycerol-monooleate, and 3.75 mM egg lecithin, with a pH of 5.0 and an osmolality of about 635 mOsm/kg, is prepared as follows:

420 ml of $C_6H_8O_7 \cdot H_20$ (citric acid monohydrate) 0.2 M and 12.07 g of NaCl are added to approximately 750 ml of water, HPLC grade. The pH is then adjusted to exactly pH 5.0 by using approximately 200 ml of NaOH 0.1 M. Sufficient water HPLC grade is added in order to produce a final volume of 1l (blank FeSSIF).
450 ml of blank FeSSIF is used to dissolve 8.25 g of sodium taurocholate (> 97 % pure). 29.54 ml of 100 mg/ml lecithin in chloroform solution is added, as well as 10 ml of glycerol-monooleate (GMO), prepared by dissolving 1.8382 g of GMO in 10 ml of chloroform. The chloroform is then evaporated on a Rotavap under vacuum using a water bath adjusted to 40 °C. After cooling to room temperature, the volume is adjusted to 1 l with blank FeSSIF.

In all dissolution experiments samples were withdrawn using a 5 ml Fortuna Optima® syringe (Fischer Labortechnik, Frankfurt/Main, Germany) fitted with stainless tubing to facilitate representative sampling with sample replacement. Samples were filtered through Titan® membrane filters (regenerated cellulose, 0.45 μm, Scientific Resources INC, Eatown, NJ/USA), discarding the first 1 ml. Absorption of the active substance on the filters was found to be negligible.

Standard curves were prepared every experimental day and consisted of five points. Stock solution was prepared by dissolving DB, DM or DH drug powder in methanol and proper dilution with relevant dissolution media in order to cover a concentration range for percentage of released drug from tablets.

The determination of assay in samples from dissolution studies was performed using a validated HPLC method.

Dissolution studies in milk digested with pepsin

The methodology for experiments in milk was adopted from Fotaki et *al.*, 2005. Pepsin solution was prepared by dissolving 1.742 g of pepsin (66 IU/mg) in 100 ml of HCl 1.83 M. Prior to the performance of experiments in milk digested with pepsin, the influence of pepsin concentration to the partitioning of drug between aqueous and lipid part of milk was assessed by using various concentrations of pepsin corresponding to values present in the dissolution vessel during the test.

100 μg/ml stock solution of doxazosin (base, mesylate salt or hydrochloride salt) was prepared in methanol. 24.1, 23.9, 23.7, 23.5, 23.3 and 23.1 ml of milk (3.5 %) was transferred

in six 25 ml Erlenmeyer flasks (ca. 25 ml). In each flask 500 µl of stock solution of doxazosin was added and flasks were placed into a shaking bath thermostated at 37 °C. At the specific time points after temperature equilibration, 200 µl of pepsin solution was added in each flask, according to the scheme in Table 3.

Table 3. Schedule for addition of pepsin solution and removal of flasks from the shaking bath

Flask No.	**Time (min)**						
	0	15	30	45	60	75	90
1	+	+	—	—	—	—	—
2	+	+	+	—	—	—	—
3	+	+	+	+	—	—	—
4	+	+	+	+	+	—	—
5	+	+	+	+	+	+	—
6	+	+	+	+	+	+	+

A flask was removed from the shaking bath upon completion of pepsin addition. After vortexing, 5 ml were transferred into a test tube and centrifuged (10 min, 4000 rpm, 5 °C). 500 µl of supernatant was then transferred into tubes containing 1000 µl of acetonitrile, vortexed and centrifuged again, under the same conditions. Clear supernatant was injected directly into the HPLC system.

Areas were evaluated against standard curves prepared every experimental day, which consisted of six points, in concentration range from 0.25 to 4 µg/ml.

Standard curves were prepared as described below.

23.538, 23.475, 23.350, 23.100, 22.850 and 22.600 ml of milk (3.5 %) and 62.5, 125, 250, 500, 750 and 1000 µl of standard stock solution prepared in methanol (100 µg/ml), were transferred in six 25 ml Erlenmeyer flasks, respectively. Flasks were placed into a shaking bath thermostated at 37 °C.

At time points 0, 15, 30, 45, 60, 75 and 90 min, 200 µl of pepsin solution was added in each flask. After 90 min, all flasks were taken out and handled in the same way as described previously.

The conditions of dissolution experiments in digested milk were the same as for other media (experiments were run in triplicate at 37.0 ± 0.5 °C using the USP II Apparatus at 100

rpm). However, sampling procedure for milk digested with pepsin was much more complex: at sampling times 5, 10, 15, 20, 30, 60, 90, 120, 150 and 180 minutes, 4 ml of sample was withdrawn and the volume was replaced with the same volume of blank dissolution medium. The blank dissolution medium (milk 3.5 % fat initially) was kept in separate vessel at the temperature 37 ± 0.5 °C with the paddle rotating at 100 rpm (exactly the same period with the actual dissolution vessel) and 4 ml of a pepsin solution (4600 IU of pepsin in 4 ml HCl 1.83 M) was added at times 0, 15, 30, 45, 60, 75 and 90 minutes after the beginning of the experiment. At sampling times 15, 30, 45, 60, 75 and 90 minutes the drawn sample from dissolution vessel was replaced with 4 ml of pepsin solution. With this procedure the volume of the dissolution medium was constant at 500 ml during the entire duration of experiment.

Samples were withdrawn using a 5 ml Fortuna Optima® syringe (Fischer Labortechnik, Frankfurt/Main, Germany) fitted with stainless tubing to facilitate representative sampling with sample replacement.

After withdrawal from dissolution vessels, samples were centrifuged at 4000 rpm at 5 °C for 10 minutes. 1000 μl of acetonitrile was added to 500 μl of supernatant and vortexed. Sample was centrifuged at 4000 rpm at 5 °C for 10 minutes, and a sample from the supernatant was injected directly into HPLC system.

To quantify dissolution data at different time points of dissolution test, due to the different extraction properties of the drug in various concentrations of pepsin (please refer to the section *Results and discussion*) it was decided to prepare six different calibration curves, with the concentrations of pepsin equivalent to the amount present in the release experiments at 10, 15, 30, 60, 75 and at 90 minutes for DB and DM and at 30 and 75 min for DH.

In six Erlenmeyer flasks appropriate amount of milk was transferred, so that volume of milk, pepsin and standard solution made 25 ml.

Stock solution of doxazosin (100 μg/ml) was added in six different concentrations to cover the range from 0.25 to 4 μg/ml. Finally, pepsin solution was added in each flask, in the following amount:

- 200 μl to the flasks for calibration curve corresponding to 10 min of dissolution testing
- 400 μl to the flasks for calibration curve corresponding to 15 min of dissolution testing
- 600 μl to the flasks for calibration curve corresponding to 30 min of dissolution testing
- 1000 μl to the flasks for calibration curve corresponding to 60 min of dissolution testing
- 1200 μl to the flasks for calibration curve corresponding to 75 min of dissolution testing
- 1400 μl to the flasks for calibration curve corresponding to 90 min of dissolution testing

Samples were thoroughly mixed and approx. 5 ml of each sample was transferred into test tubes and centrifuged (10 min, 4000 rpm, 5 °C). 500 µl of supernatant was then transferred into tubes containing 1000 µl of acetonitrile, vortexed and centrifuged again, under the same conditions. Clear supernatant was injected directly into the HPLC system.

In dissolution experiments the difference factor, $f_{1,area}$ was used for testing the difference of cumulative % dissolved vs. time profiles according to a previously proposed methodology (Vertzoni et *al.*, 2003). Since the coefficient of variation of data points was in almost all cases less than 20 %, the mean data sets were compared. The limit for identifying differences between the test and reference set of data was set to $f_{1,area} > 0.15$ (Vertzoni et *al.*, 2003). In cases where more than 85 % of drug was dissolved in less than 15 minutes, the profiles were considered similar without further mathematical evaluation.

4.2.5. HPLC method for determination of doxazosin assay in samples from solubility studies and dissolution test

High performance liquid chromatography method (HPLC) was used for quantitative determination of doxazosin in samples from solubility studies and dissolution test.

The HPLC system consisted of a Spectra System pump P1000, a SpectraSystem detector UV1000, a controller Spectra System SN4000, a Spectra System autosampler AS3000 and the Chromquest software (ThermoQuest Inc., San Joe, USA). Merck Superspher 100 RP-18 endcapped (250 x 4 mm, 5 µm) column with LiChrospher® 100 RP-18 (5 µm) guard column was used.

The mobile phase consisted of methanol, acetonitrile and 50 mM phosphate buffer pH 3.0 (40:15:45, *v/v/v*) and the flow rate was 1 ml/min. The injection volume was 10 µl, column temperature was set to 40 °C and the retention time of doxazosin was about 7 minutes. The detection wavelength was 246 nm.

The method was previously validated in Pliva and validation data are not shown in this thesis. The results of validation experiments satisfied required criteria regarding selectivity, linearity, accuracy, precision, intermediate precision, solution stability and robustness.

4.2.6. Simulated data after administration of DB and DM tablets to adults

Simulated cumulative doxazosin in plasma vs. time profiles after single-dose administrations of DB and DM tablets were constructed (Stella® 9.0.2 software, isee systems, Inc., USA) by using the *in vitro* dissolution data and previously described procedures (Nicolaides et *al.*, 2001, Rausl et *al.*, 2006) after taking into account the *in vitro* solubility data, the average bioavailability after oral administration of doxazosin (Vincent et *al.*, 1983), and the enterohepatic circulation of doxazosin (Cardura®, Summary of product characteristics). Briefly, dissolution of the appropriate phase in stomach (taking into account the conversion of DB to DH) was assumed to occur until gastric contents are saturated or the entire dose is dissolved, based on *in vitro* dissolution data. If *in vitro* dissolution under gastric conditions was rapid, then a 250 ml solution with concentration equal to the maximum concentration observed in *in vitro* experiments was assumed to have been administered. Absorption by the gastric mucosa was assumed to be negligible, gastric emptying of solids and liquid (250 ml at administration time) occurred with the same rate constant, 2.8 h^{-1}, according to population values (Vertzoni et *al*., 2005, Nicolaides et *al*., 2001), and absorption from the small intestine occurred without any limitation. In all cases, dissolution was assumed to occur according to a dissolution rate constant that had been estimated from the *in vitro* data based on the Noyes-Whitney theory for dissolution (Vertzoni et *al*., 2005; Nicolaides et *al*., 2001). Characterization of the enterohepatic circulation of doxazosin was possible by fitting a previously described model (Rausl et *al*., 2006) to intravenous doxazosin data (Elliott et *al*., 1987) and assuming that the gallbladder emptied into the duodenum at 4, 10, and 24 h post-dosing (according to the feeding schedule of the volunteers in the actual *in vivo* study). The cumulative doxazosin profile in plasma until 2 hours after administration was used for evaluating early exposure.

4.2.7. Development and validation of new LC-MS/MS method for determination of doxazosin assay in canine plasma

A LC-MS/MS method for determination of low doxazosin concentrations in plasma after oral administration to dogs is described in Erceg et *al*., 2010.

The liquid chromatograph (Agilent 1100; Agilent Technologies, Inc., Palo Alto, CA) was coupled to a mass spectrometer with a turbo electrospray ion source (Qtrap; Applied Biosystems, Foster City, CA) and an electrospray ionization (ESI) interface. An Analyst

software 1.3.1 (Applied Biosystems) was used for LC-MS/MS control and signal acquisition. LC system was equipped with Agilent 1100 gradient pump, Thermo autosampler, column oven and diode array detector.

A Hettich centrifuge Universal 32R (Tuttlingen, Germany) was utilized to centrifuge plasma samples. Vacuum evaporator used in sample extraction procedure was Concentrator Eppendorf 5301, (Eppendorf, Hamburg, Germany).

Separation was achieved on an XTerra MS C18 column (150 mm × 2.1 mm, 3.5 µm particle size) equipped with a XTerra MS C18 guard column (20 mm × 2.1 mm, 3.5 µm particle size), both from Waters (USA). The gradient mobile phase was composed of acetonitrile: 2 mM ammonium acetate (10:90 *v/v*) as mobile phase A and acetonitril : 2 mM ammonium acetate (90:10 *v/v*) as the mobile phase B. % mobile phase A at times 0, 1, 8, 10 and 15 min was 90, 90, 30, 90, 90, respectively. The flow rate was 400 µl/min and the injection volume was 100 µl. The column temperature was maintained at 35 °C and the autosampler cooler was maintained at 10 °C. The elution time for prazosin and doxazosin was approximately 8 and 10 min, respectively. The HPLC effluent was sprayed directly into the mass spectrometer at 400 µl/min flow rate. The Q-TRAP mass spectrometer was operated with turbo ion-spray interface in positive ion mode at unit resolution. Doxazosin and prazosin (IS) were detected by selected reaction monitoring (SRM) in the multi reaction monitoring mode using the following settings: transitions *m/z* 452.3→344.4 and 384.3→247.2 with dwell time 200 msec. Ion source and other instrument parameters were optimized for the transition and the following settings were used: 30 psi curtain gas, 300 °C temperature, 30 psi nebuliser gas, 60 psi heater gas, 4 eV nebulizer current, 60 V declustering potential, 12 V entrance potential, 40 eV collision energy, and collision cell exit 4 eV.

For the isolation of doxazosin from plasma samples protein precipitation was applied and optimized. Since liquid-liquid extraction is still the most widely used technique for extraction of doxazosin from spiked plasma samples, in our study it was tested for comparative purposes.

Various solvents (methanol, ethanol and combination of methanol and acetonitrile) and conditions (4 °C and –20 °C) were evaluated for protein precipitation.

Liquid-liquid extraction was performed with 500 µl of plasma by alkalinization with 1M NaOH, followed by extraction with 30 % dichloromethane in hexane. The upper organic layer was evaporated to dryness and the remaining dry residue was dissolved in mobile phase

and injected into the LC-MS/MS system. Recovery was calculated by comparing the peak areas obtained from plasma samples with those obtained by direct injection of the working standard solutions of doxazosin. Extraction was performed so that the entire volume of supernatant was obtained and subsequently evaporated to dryness. In both cases internal standard was added into the solution. Method validation procedures were based on relevant guidelines (Green et *al.*, 1996, Shah et *al.*, 2000).

4.2.7.1. Calibration curves

Stock solutions of doxazosin base, doxazosin mesylate and prazosin hydrochloride (100 µg/ml) were prepared by dissolving 10 mg of each compound in 100 ml of methanol. All solutions were prepared freshly every day. Concentration of the working solution of IS was 60 ng/ml. All dilutions to volume were performed with water.

Usual doxazosin calibration curves in plasma were constructed in the concentration range of 1–20 ng/ml as follows: 100 µl of blank canine plasma were transferred in a centrifuge tube containing 50 µl of doxazosin working solutions (2.5–48 ng/ml in water). After vortexing, 20 µl of working solution of IS (60 ng/ml in water) was added. Further preparation procedure was according to the same manner as described in section *Sample preparation procedure*.

Regression equations were obtained through unweighted least square linear regression analysis with a regression equation $y = ax + b$, where y was the peak area ratio of doxazosin to IS and x was doxazosin concentration in ng/ml.

4.2.7.2. Precision, recovery and accuracy

Quality control (QC) standards for determination of accuracy and precision of the method were independently prepared at 2 ng/ml, 5 ng/ml, and 10 ng/ml concentrations in the same manner as the calibration standards. QC standards represent the matrix of the samples with known amounts of the analyte, used for validation purposes and to validate the test run. For recovery, accuracy and intra-day precision QC standards were prepared and analyzed in triplicate. Inter-day precision was also tested using QC standards in triplicate.

4.2.7.3. Stability

To evaluate doxazosin stability in canine plasma, drug free plasma samples were spiked with analytes at 5 ng/ml and 10 ng/ml. To test the short- and long-term stability of

doxazosin, quality control standards of 5 ng/ml and 10 ng/ml were prepared in duplicate and kept at ambient temperature (25 ± 2 °C) and at –20 °C for 24 h and 60 days, respectively.

4.2.7.4. Limits of detection and quantification and carryover effects

For the determination of limits of detection (*LOD*) and quantification (*LOQ*) calibration curves were prepared in the range of 0.5–12 ng/ml. The *LOD* and *LOQ* values were defined as follows (Miller and Miller, 1984):

$$LOD = \frac{3.3 s_{y/x}}{b} \quad \text{and} \quad LOQ = \frac{10 s_{y/x}}{b}$$

where b is the slope and $s_{y/x}$ is the residual standard deviation of the regression line, calculated using working standards. QC standard for determination of carry over was prepared at the concentration of 20 ng/ml.

4.2.7.5. Sample preparation procedure

150 µl of plasma samples were transferred in a centrifuge tube and 20 µl of working solution of IS (60 ng/ml) were added. After vortexing for 30 s, 400 µl of precipitation solvent (methanol: acetonitrile 50:50, *v/v*) were added. The new mixture was vortexed for 30 s and stored for at least 12 hours in a freezer at –20 °C. Then, the samples were centrifuged for 15 minutes at 12000 rpm. The supernatant was filtered using 0.2 µm Gelman acrodisc CR PTFE syringe filters (Gelman Science, Ann Arbor, MI) and evaporated to dryness using vacuum evaporator at 40 °C. Dry residue was then redissolved in 120 µl of mobile phase and the solution was vortexed for 1 min. Finally, 100 µl were injected into the LC-MS/MS system.

4.2.8. Data after administration of DM tablets to adults in the fasting state

After receiving all required approvals and following ethical principles from Declaration of Helsinki, actual plasma data following to single administration of DM tablets to 24 healthy adults were collected. Each volunteer was administered one DM tablet with 240 ml of water after a 10-hour fast. Blood samples were drawn prior to dosing and at 0.5, 1, 1.5, 2, 2.33, 2.67, 3, 3.5, 4, 5, 6, 8, 10, 12, 16, 24, 36, 48, and 72 h post dosing. Plasma samples were assayed for their doxazosin content using a validated HPLC method that involved

liquid-liquid extraction and fluorescence detection (unpublished, developed in house at PLIVA). A two-compartment model with first-order absorption was fitted to each individual concentration vs. time profile (WinNonlin® 5.2, Pharsight Corporation, Mountain View, CA, USA). The correlation coefficient for the linear correlation between an observed individual data set and the calculated by the best fitted line data set ranged from 0.93 to 0.997. Individual cumulative doxazosin in plasma vs. time plots were then constructed by using the estimated apparent absorption rate constant and the average bioavailability coefficient of doxazosin in the literature (0.65, Vincent et *al.*, 1983). These data were used for validating the simulated cumulative doxazosin in plasma vs. time plot after DM administration and evaluating the importance of using biorelevant *in vitro* data in such simulation. Profiles in plasma until 2 h after administration were used for evaluation of early exposure.

4.2.9. Data after administration of DB and DM tablets to dogs in the fasting state

Canine studies were performed in four healthy female mongrel dogs (4-years old, 28–32 kg) that were accommodated in an animal facility operating according to the European Union regulations for the maintenance and experimentation on animals and it has been approved by the Veterinary Directorate of the Municipality of Athens (EL 25 BIO 08). After 16 h fasting from food but not water, each dog was administered one DB tablet or one DM tablet with 250 ml of water via an orogastric tube. Blood samples were collected by means of an indwelling catheter positioned in a suitable foreleg vein. Five to eight hours after drug administration each dog consumed a standard meal (150 g pellets and 250 ml tap water). Twelve hours after dosing the catheter was removed and the dog returned to her cage, where she was allowed to eat and drink *ad libitum*. Samples after 12 h were collected by individual venipuncture. Blood samples were centrifuged and plasma was stored at –20 °C in brown glass vials. The LC-MS/MS analytical method that was used for the determination of doxazosin in plasma has been published recently, by Erceg et *al*., 2010.

After each administration, total exposure was estimated by the total area under the plasma profile, $AUC_{0\text{-}24\ h}$. Since canine plasma vs. time after intravenous administration are not available in literature and modelling of enterohepatic circulation in dogs is problematic due to inconsistent emptying of canine gallbladder (Kalantzi et *al*., 2006), cumulative doxazosin in plasma profiles could not be constructed and early exposure was estimated by the partial area under the plasma profiles, $AUC_{p,base}$ i.e. from $t = 0$ up to time, t, at which the first peak on plasma profile, after the administration of DB to the specific dog, was observed.

Areas were estimated with the trapezoidal rule and statistically significant differences between DB and DM administrations were evaluated with the paired *t*-test at the 0.05 level.

4.2.10. Data after administration of DB and DM tablets to dogs in the fed state

In the fed state study, the same dogs and the same procedure was used as described previously in the fasted state study, except for the dosing part. In this study, each dog was fasted for 16 h from food but not water before administration. After that each dog was administered one DB tablet or DM tablet (both containing 2 mg doxazosin) with 500 ml of cow's milk (3.5 % fat) via an orogastric tube. Eight hours after drug administration each dog was offered a standard meal (150 g pellets and 250 ml tap water). Twelve hours after dosing the catheter was removed and the dog returned to her cage, where she was allowed to eat and drink *ad libitum*.

Pharmacokinetic parameters including c_{max}, t_{max} and $AUC_{p,base}$ (partial area under the plasma concentration *vs*. time curve from t = 0 up to the first peak of the profile after administration of the base (Chen et *al.*, 2001)) were calculated using Prism Software (GraphPad Prism 3.02. Software, Inc., San Diego, CA).

Apparent terminal half life was calculated by log-linear regression of the terminal segment of the plasma concentration-time curve (0.693/λz) where λz is the apparent elimination rate constant.

5. RESULTS AND DISCUSSION

5.1. Characterization of DB, DM, and DH

In order to confirm the structures and physicochemical characteristics of the free base and two salts, mesylate and hydrochloride, several techniques were employed. Nuclear magnetic resonance (^{1}H NMR) and fourier transforms infrared spectroscopy (FTIR) were used to confirm the structures of the tested specimen, X-ray powder diffraction (XRPD) to confirm polymorphic form, dynamic vapour sorption (DVS) to evaluate the hydroscopicity of the substances, differential scanning calorimetry (DSC) to show their thermal behaviour, and scanning electronic microscopy (SEM) to evaluate differences in size and morphology of drug particles.

Proton NMR (^{1}H NMR) is the application of nuclear magnetic resonance in NMR spectroscopy with respect to ^{1}H nuclei within the molecules of a substance, in order to determine the structure of its molecules. Proton NMR spectra of most organic compounds are characterized by chemical shifts and by spin-spin coupling between protons (Silverstein et. *al.*, 1991).

^{1}H NMR spectrum of DB shows signals corresponding to both quinazoline and piperazine-benzodioxane moiety of molecule. In ^{1}H NMR spectrum of DM, two additional signals appear, corresponding to the protonated quinazoline cation and methanesulphonyl counter anion. ^{1}H NMR spectrum of DH shows signal corresponding to the protonated quinazoline amine, designating the substance as a salt.

Fourier Transform-Infrared Spectroscopy (FTIR) is an analytical technique used to identify organic materials, which measures the absorption of infrared radiation of the sample material versus wavelength. When a material is irradiated with infrared radiation, absorbed IR radiation usually excites molecules into a higher vibration state. The wavelength of light absorbed by a particular molecule is a function of the energy difference between the at-rest and excited vibration states. The wavelengths that are absorbed by the sample are characteristic of its molecular structure (Materials Evaluation and Engineering, Inc., 2010).

FTIR spectrum of DB shows sharp peaks at 3477, 3346 and 3248 cm^{-1}, corresponding to aromatic NH_2 group. In FTIR spectrum of doxazosin mesylate, the same group appears as broader bands at 3348 and 3182 cm^{-1}. In FTIR spectra of DB and DM, bands of amide carbonyl groups appear at 1656 ± 1 cm^{-1}, while bands at 1635 ± 1 cm^{-1} are in good

accordance with conjugated C=N groups. Several stretchings in the range of 1441–1593 cm^{-1} can be found in FTIR spectra of both compounds, corresponding to aromatic C-C and C=N groups. A very sharp peak at 1043 cm^{-1} can be seen only in FTIR spectrum of doxazosin mesylate, which is in good accordance with its $R\text{-}SO_3^-$ counter anion.

X-ray powder diffraction is used for the analysis of polymorphism in crystalline solids. The general principle behind XRPD is that a narrow beam of X-rays is passed through the sample. The wavelength of the X-rays is on the order of the distance between the molecules in the crystal lattice, and the sample therefore acts like a diffraction grating. The diffraction occurs at angles corresponding to the Bragg equation ($n\lambda = 2d \sin \theta$). This law relates the wavelength of electromagnetic radiation to the diffraction angle and the lattice spacing in a crystalline sample. These diffracted X-rays are then detected, processed and counted.

The X-ray diffractograms of DB, DM and DH are shown in Figure 11. X-ray diffractograms of DB and DH could not be found in literature, while DM exists in a number of different crystalline forms (Sohn and Lee 2005). The X-ray diffractogram of DM is identical to crystal form III standard reference pattern (Grafe and Morsdorf, 1996) (Figure 11).

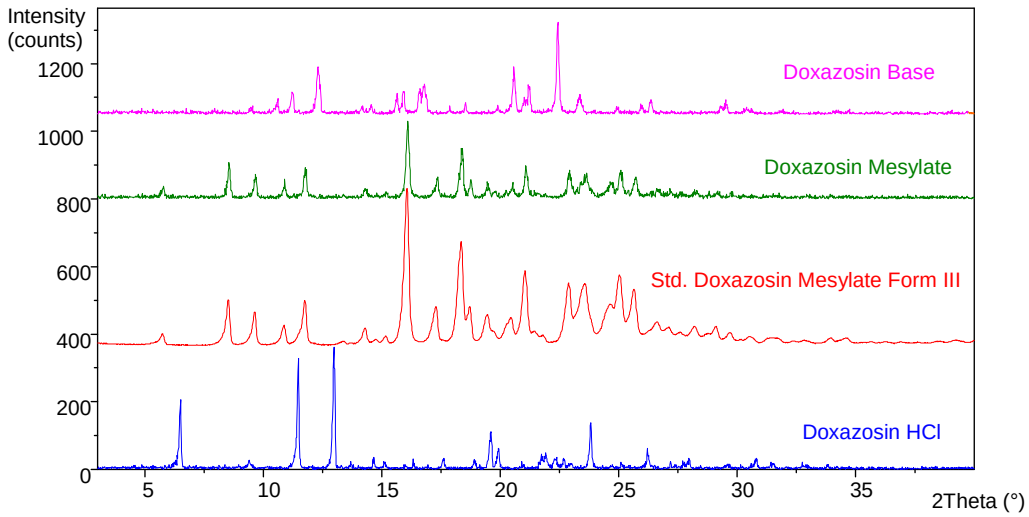


Figure 11. X-ray powder diffraction pattern of DB, DM, standard DM and DH

Dynamic vapour sorption (DVS) is a gravimetric technique that measures sorption and desorption of a solvent onto the sample. In a DVS experiment this is accomplished by exposing a sample to a series of step changes in relative humidity and monitoring the mass change as a function of time. The sample mass must be allowed to reach gravimetric

equilibrium at each step change in humidity before progressing to the next humidity level. Then, the equilibrium mass values at each relative humidity step are used to generate the isotherm. Isotherms are typically divided into two components: *sorption* for increasing humidity steps and *desorption* for decreasing humidity steps. DVS data of the three tested substances, DB, DM and DH, show that all three are non-hygroscopic, with similar adsorption and desorption cycles.

Differential scanning calorimetry (DSC) is a thermoanalytical technique in which the difference in the amount of heat required to increase the temperature of a sample and reference is measured as a function of temperature. Both the sample and the reference are maintained at nearly the same temperature throughout the experiment. The basic principle underlying this technique is that when the sample undergoes a physical transformation such as phase transitions, it will need more or less heat than the reference to maintain both at the same temperature, depending on whether the process is exothermic or endothermic.
DSC thermograms of all three substances show a single sharp endothermic peak corresponding to the melting of each substance (Table 4).

Table 4. DSC data

Sample name	**Onset / °C**	**ΔH / J g^{-1}**
DB	255.8	121.3
DM	278.4	113.2
DH	288.1	201.2

The obtained DSC data indicate that DH is thermally the most stable solid, while DB is thermally the least stable.

Scanning electron microscopy (SEM) is a method for high-resolution imaging of surfaces. The SEM uses electrons for imaging, the same as a light microscope uses visible light. The advantages of SEM over light microscopy include much higher magnification (>100 000 x) and greater depth of field up to 100 times that of light microscopy.
SEM pictures show that particles of DB and DM have plate like regular shape, and size less than 10 µm, with DB particles being smaller than DM.
DH particles have also plate like regular shape but their size is about 50 µm.

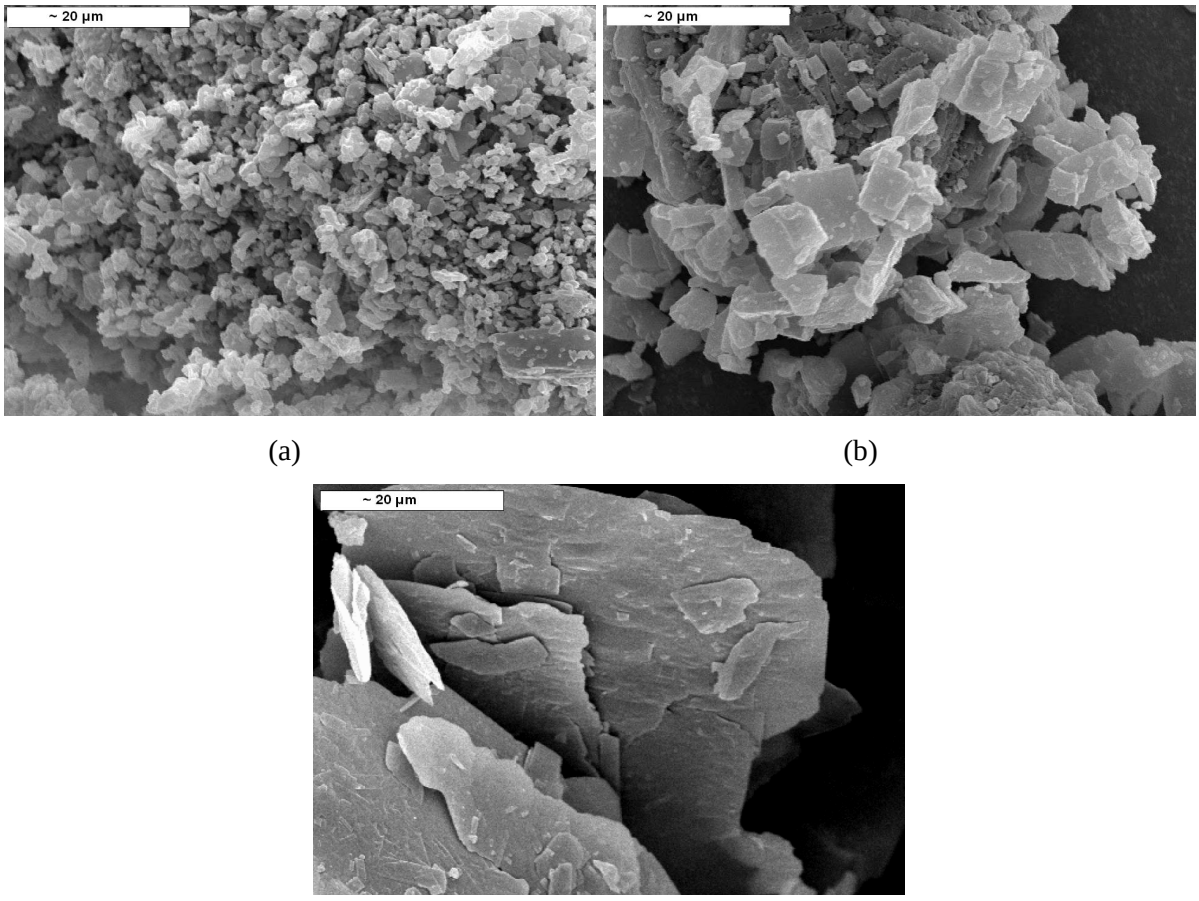


Figure 12. SEM micrograph (2000 x magnification, scale bar 20 µm) of DB (a), DM (b) and DH (c) powder

The physicochemical techniques used for characterization of doxazosin base and its salts, mesylate and hydrochloride, confirmed their identity and crystalline form. The water uptake properties of all three substances were found to be rather similar, while some differences in thermal behaviour were observed, as expected for different salts and base.

A certain differences were noticed also in terms of particle size and morphology of the three substances, so that should be kept in mind during evaluation of properties and behaviour of drug products, especially in pH environment where its solubility will be challenged.

5.2. BCS classification of doxazosin

According to new EMA Guideline on the investigation of bioequivalence, 2010, the drug substance is considered highly soluble if the highest single dose administered as immediate release formulation is completely dissolved in 250 ml of buffers within the range of pH 1–6.8 at 37 ± 1 °C.

According to the same Guideline, the complete absorption is considered to be established where measured extent of absorption is ≥ 85 %, and complete absorption is generally related to high permeability.

Since there was no literature data available for BCS classification of doxazosin or it salts that were based on 8 mg as the highest single dose administered, BCS class was determined experimentally by evaluating if a complete 8 mg dose of DB, DM and DH, respectively, was dissolved in 250 ml of pH 1.2, 4.5 and 6.8 at 37 ± 1 °C. By visual inspection it was observed that the 8 mg of each of the drug substances were completely dissolved in pH 1.2 and 4.5, but not in pH 6.8.

Since doxazosin was reported to be highly permeable compound (Product monograph Cardura®, Pfizer), it can be concluded that doxazosin belongs to BCS Class II.

5.3. Physical characterization of DB, DM and DH tablets

Uncoated immediate release tablets of DB, DM and DH containing 2 mg of DB per tablet, were prepared as described in *Materials and methods* section. After the compression of tablets, samples were tested for their average weight, hardness and disintegration. The results (average values) are presented in Table 5.

Table 5. Physical characteristics of DB, DM and DH 2 mg tablets

Tablet	Mass (g)	Hardness (kP)	Disintegration time (s)
DB	0.1219	5.81	140
DM	0.1199	6.53	117
DH	0.1198	6.24	147

There was no significant difference in the tested parameters.

5.4. Solubility and dissolution studies in the fasted state

Solubility and dissolution properties of DB, DM and DH are expected to be crucial for *in vitro* behaviour as well as *in vivo* performance of the drug product.

Doxazosin, as a lipophilic weak base, has better solubility in acidic pH environment than in any other pH environment. Knowing that drug product was formulated as immediate release tablets, and that it has relatively short t_{max} (2–3 hours), it is expected that the drug product is primarily dissolved during residence in stomach, especially after administration in fasted state, when the pH is the lowest.

Equilibrium solubility and dissolution of weak bases is usually determined in simple hydrochloric acid solutions, although it is known that media composition, as well as pH may both affect solubility of lipophilic weak bases. As explained previously, the components of biorelevant media may additionally solubilise the active substance and increase its wettability, so it is important to evaluate both, pH and media composition.

Solubility and dissolution data in acidic media are expected also to reveal whether a drug's solubility and availability on the site of absorption is further challenged with effects such as common ion effect or conversion to hydrochloride salt. Moreover, the influence of ionic strength of media would also be evaluated, as well as the influence of buffer anion component on solubility of drug.

All of these effects will be first evaluated in fasted state, and then in fed state environment too.

As poorly soluble week base moves from the favourable pH conditions in the stomach to a less favourable pH environment in the small intestine, its solubility undergoes a sharp decrease. This may cause supersaturation and precipitation of drug, which of course, makes it unavailable for absorption. Kostewicz et *al.*, 2004, reported that the precipitation of studied weakly basic drugs occurred only under simulated fasted state conditions, while in fed state simulated conditions the bile components and the lower pH inhibited precipitation.

In case of doxazosin, it was decided to evaluate dissolution behaviour in the environment of the upper small intestine only for DB and DM, since the solubility and dissolution characteristics of DH were only studied in acidic environment in order to evaluate potential conversion of DB and DM into DH and to evaluate the common ion effect.

The specific goals of *in vitro* solubility and dissolution studies were multiple:

- To study the solubility of DB, DM, and DH more closely in acidic pH range, in simple buffer media as well as biorelevant media, since both the pH and composition are expected to be important for their *in vivo* behaviour;
- To evaluate possible conversion of DB and DM to DH, based on solubility and dissolution data in acidic pH range;
- To check the influence of common ions on solubility of salts, primarily the influence of chloride ions on solubility of DH;
- To check the influence of ionic strength of solubility/dissolution media on solubility of DB, DM and DH;
- To evaluate the influence of media composition on dissolution profiles of DB, DM and DH in gastric environment (simple acidic media vs. FaSSGF);
- To evaluate the influence of media composition on dissolution profiles of DB, DM and DH in gastric environment in fasting and fed state (simple buffers vs. FaSSGF/ FeSSGF);
- To evaluate the influence of media composition on dissolution profiles of DB, DM and DH in upper small intestine environment in fasting and fed state (simple buffers vs. FaSSIF/FeSSIF);
- To evaluate the influence of different buffer anion species on dissolution of DB, DM and DH in the upper small intestine environment.

5.4.1. Evaluation of DB, DM and DH in the gastric environment in the fasting state

Solubility of all three substances was determined in media having different pH and compositions. The goal was to compare the solubility in simple hydrochloric media with other complex media, having the same or different pH, and different properties such as buffer capacity, osmolality and ionic strength.

Solubility of DH was determined in a reduced manner, in order to check whether DB or DM converts to DH in acidic media.

Concentrations in solubility measurements were evaluated using standard curves prepared on the experimental day (n = 5, 0.5–4 µg/ml in FaSSGF and n = 6, 1–10 µg/ml in hydrochloric acid)

Analytical parameters for the standard curves are given in Table 6.

Table 6. Analytical parameters for the standard curves of doxazosin base/salts in FaSSGF and hydrochloric acid used in solubility experiments

Medium	Substance	r^2	Slope ± SD	Intercept ± SD
HGF (pH 1.8)	Standard curves in FaSSGF-V2 (pH 1.6) were used			
FaSSGF-V2 (pH 1.6)	DB	0.9996	314977024 ± 3724489	–24226 ± 9161
		0.9999	326177390 ± 2060796	–14943 ± 5069
		0.9991	324430012 ± 5570695	–13102 ± 1702
	DM	0.9998	298488829 ± 2446608	–10807 ± 6018
USP buffer (pH 1.2)	DM	0.9999	116.0796 ± 0.676176	0.0091 ± 0.0078
HCl (pH 1.8)	DB	0.9996	311116142 ± 3108617	–34933 ± 1866
HCl (pH 2.6)	DB	0.9990	317780137 ± 5015720	–129891 ± 3441
HCl (pH 3.0)	DB	0.9992	311336142 ± 3243917	–15933 ± 2166

Solubility results are presented in Table 7.

Table 7. Mean ± SD (n = 3) solubility (μg/ml) of doxazosin base (DB), doxazosin mesylate (DM) and doxazosin hydrochloride (DH) in human gastric fluid (HGF) and in various simulated gastric fluids*

Medium	DB	DM	DH
HGF pH 1.8	0.256 ± 0.049 (pH_{eq} 2.7)	0.200 ± 0.014 (pH_{eq} 1.8)	N/A
CGF pH 2.6	0.247 ± 0.013 (pH_{eq} 3.7)	0.609 ± 0.006 (pH_{eq} 2.6)	N/A
FaSSGF-V2 pH 1.6	0.235 ± 0.016 (pH_{eq} 2.3)	0.141 ± 0.013 (pH_{eq} 1.6)	0.021 ± 0.002 (pH_{eq} 1.6)
USP pH 1.2	0.535 ± 0.013 (pH_{eq} 1.2)	0.254 ± 0.008 (pH_{eq} 1.2)	0.040 ± 0.008 (pH_{eq} 1.2)
HCl pH 1.8	2.763 ± 0.599 (pH_{eq} 2.6)	1.511 ± 0.419 (pH_{eq} 1.8)	0.099 ±0.006 (pH_{eq} 1.8)
HCl pH 2.6	0.751 ±0.014 (pH_{eq} 3.7)	3.364 ± 0.187 (pH_{eq} 2.7)	0.501 ± 0.018 (pH_{eq} 2.6)
HCl pH 3.0	0.521 ± 0.054 (pH_{eq} 4.1)	N/A	N/A

* pH_{eq} is pH at equilibrium; N/A means not available.

Solubility of DB in HGF is significantly lower than solubility in pH 1.8 HCl solution, despite the fact that both initial and equilibrium pH, pHeq, are similar (Table 7), due to the much higher ionic strength of HGF (Vertzoni et *al.*, 2007, Lindahl et *al.*, 1997). This may further be confirmed by the fact that solubility data of DB in FaSSGF-V2 are similar with solubility data in HGF at approximately similar pH_{eq} values (Table 7). DB solubility in pH 1.2 USP buffer is significantly higher than the solubility of DH (Table 7). This difference should be attributed to common ion effects (e.g., Streng et *al.*, 1984), especially if conversion of DB to DH occurs (please see below).

The amount of DM in excess did not affect pH_{eq} values (Serajuddin, 2007), as in all media pHeq was similar with the initial pH (Table 7).

Solubility of DM is higher than DH in HCl solutions, which is in correlation with its lower melting point and the lack of common ion effect. Due to differences in ionic strength between pH 1.8 HCl and FaSSGF-V2, solubility of DM in FaSSGF-V2 is much closer to solubility in HGF than solubility in pH 1.8 HCl (Table 7).

Further on, dissolution experiments with DB, DM and DH were performed in HCl pH 1.6 and FaSSGF (pH 1.6) in order to distinguish between the contribution of pH and other components of biorelevant media on solubility and dissolution rate of the tested compounds. Concentrations in dissolution experiments were evaluated using standard curves prepared on the experimental day (n = 5, 0.5–4 μg/ml in both media)

Analytical parameters for the standard curves are given in Table 8.

Table 8. Analytical parameters for the standard curves of doxazosin base and salts in FaSSGF-V2

Medium	**Substance**	r^2	**Slope ± SD**	**Intercept ± SD**
FaSSGF-V2	DB	0.9996	134066463 ± 162426	−7661 ± 343
	DM	0.9997	130342402 ± 177554	−1975 ± 342
	DH	0.9997	138596401 ± 1410851	−11488 ± 468
HCl pH 1.6	DM	0.9992	138126.8347 ± 2256.7264	4.709 ± 5.585

Dissolution profiles are presented in Figure 13.

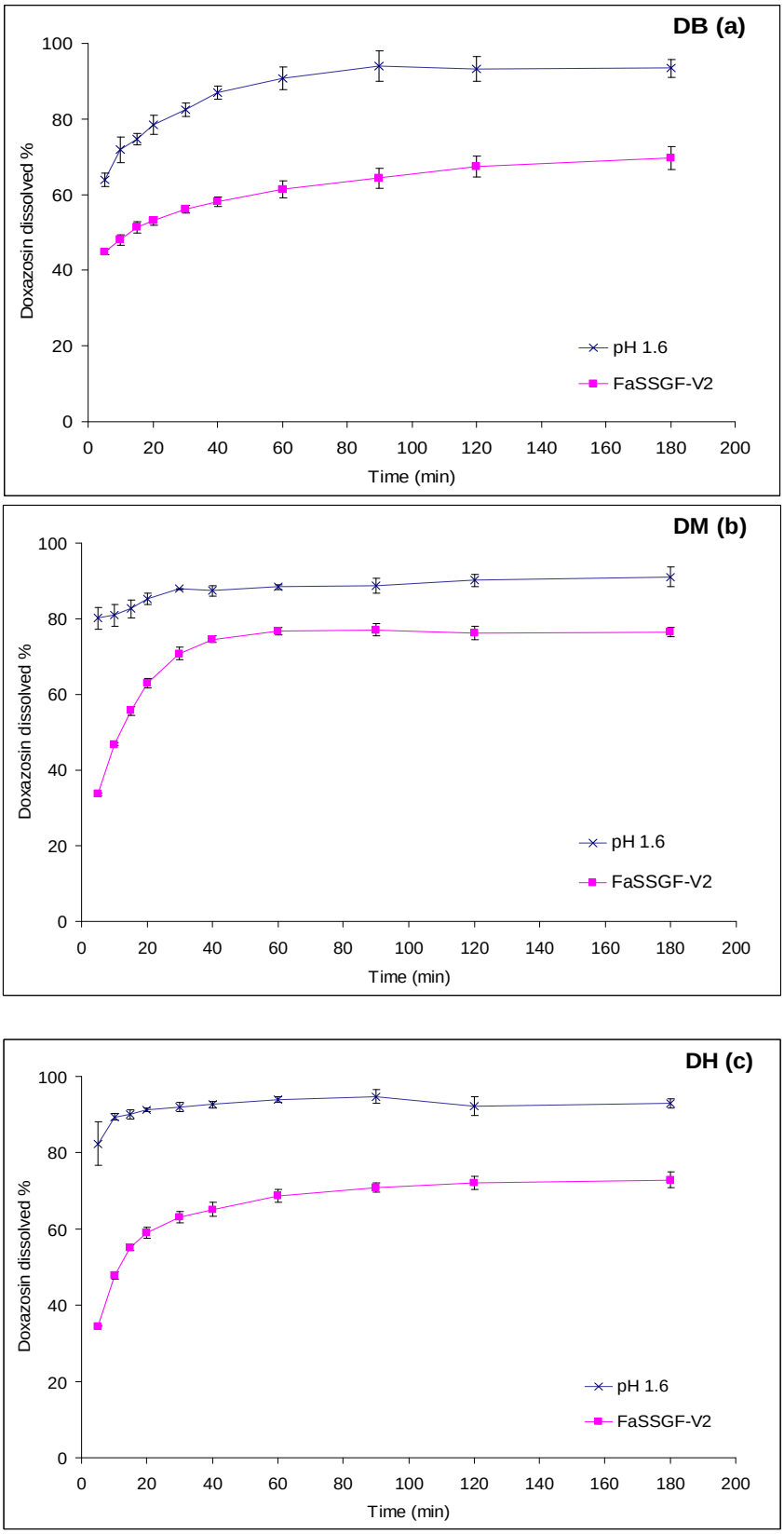


Figure 13. Mean ± SD (n = 3) % dissolved DB tablets (a), DM tablets (b) and DH tablets (c), in pH 1.6 HCl solution and in FaSSGF-V2

Dissolution of DM, DH, and with a less extent DB tablets, is rapid in $HCl_{pH1.6}$ (Figure 13). However, in contrast with equilibrium solubility data (Table 7) dissolution of the dose is slightly less than complete (Figure 13). As previously observed with other ionized weak bases (N. Fransén et *al.*, 2008, Hollenbeck et *al.*, 1988, Narang et *al.*, 2010), the cationic form of doxazosin could exchange with sodium ions associated with insoluble croscarmellose (the disintegrant in the tablets). For all three phases, dissolution of the dose in FaSSGF-V2 was slower than in $HCl_{pH1.6}$, which is in line with the lower solubility results of the phases in FaSSGF-V2. In addition, dissolution was less complete than in $HCl_{pH1.6}$ solution. Since the increased ionic strength does not affect the interaction of protonated compounds with croscarmellose sodium (N. Fransén et *al*., 2008, Hollenbeck et *al*., 1988, Narang et *al*., 2010), an interaction between protonated doxazosin and taurocholate with subsequent formation of insoluble salt(s) can be postulated. Such interaction indeed would have a significant impact on % dissolution profile, due to the low dose of doxazosin.

The difference between the dissolution profiles of DB and DM tablets is significant ($f_{1,area}$ = 0.22), mostly due to the more complete dissolution of DM tablets. For DB and DH tablets, dissolution in FaSSGF-V2 is incomplete, pH remains unaltered until completion of the process, and dissolution profiles do not differ significantly ($f_{1,area}$ = 0.11). Based on solubility data of DB in pH 1.2 USP buffer and in FaSSGF-V2 (please note that pH_{eq} of FaSSGF-V2 is significantly increased), solubility of DB in FaSSGF-V2 (pH 1.6) is expected to be about ten times higher than solubility of DH in FaSSGF-V2 (Table 7). The similar dissolution profiles of DB and DH in FaSSGF-V2, therefore, suggest conversion of DB to DH during dissolution of the dose. The slightly slower dissolution of DH tablets at early time points is in agreement with its bigger particle size (Figure 12).

Additionaly, dissolution under conditions simulating the canine gastric pH (pH 2.6) was performed (Figure 14) and the results showed that it is rapid for all three tested compounds, as it was in $HCl_{pH1.6}$ (Figure 13).

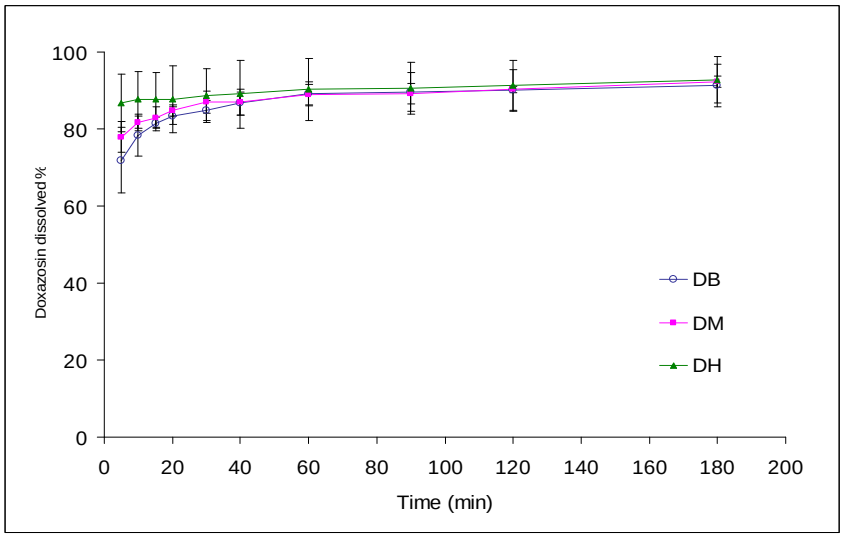


Figure 14. Mean ± SD (*n* = 3) % dissolved DB tablets (a), DM tablets (b) and DH tablets (c), in pH 2.6 HCl solution

5.4.2. Evaluation of DB and DM in the environment of the upper small intestine in the fasting state

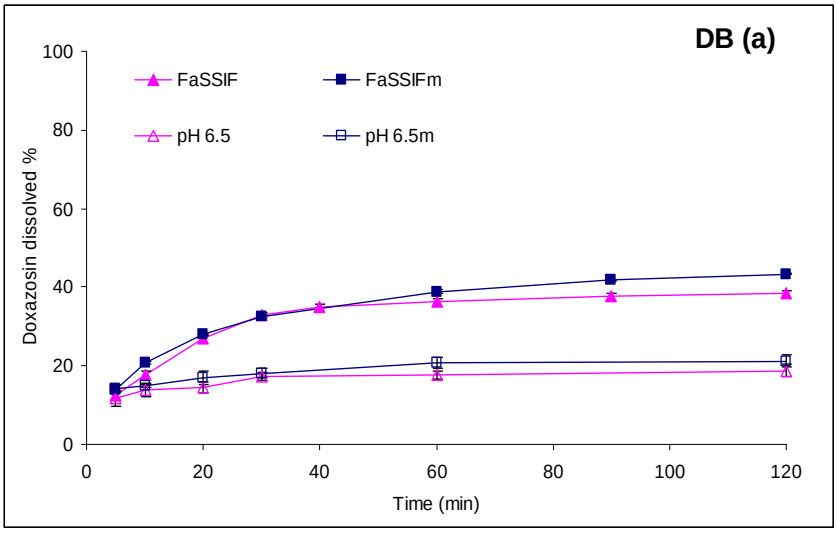
Dissolution studies of DB and DM were performed in fasted and fed state environment, in simple buffers having pH 6.5 and 5.0 and in biorelevant media FaSSIF and FeSSIF.

As said previously, regarding the choice of physiological buffer in FaSSIF and FeSSIF, practical issues led to use of non-physiological buffer species. Therefore alternative buffer species were tested, since theoretically the type of buffer may affect the solubility product and stability of the active compound and its dissolution behaviour. In case of doxazosin, phosphate and maleate buffers were tested in FaSSIF, and acetate and citrate buffers in FeSSIF. Analytical parameters for the standard curves (*n* = 5, 0.5–4 ng/ml) in pH 6.5 and FaSSIF are given in Table 9.

Table 9. Analytical parameters for the standard curves in pH 6.5 and FaSSIF

Medium	Substance	r^2	Slope ± SD	Intercept ± SD
pH 6.5	DM	0.9975	129073 ± 3721	10 ± 2
pH 6.5_m	DM	0.9995	138171 ± 1814	−3 ± 2
FaSSIF	DB	0.9993	120648280 ± 181780	−14649 ± 483
	DM	0.9995	100235416 ± 146892	−12232 ± 371
	DH	0.9997	97593714 ± 102438	−15382 ± 251
$FaSSIF_m$	DB	0.9995	86274500 ± 163825	−9984 ± 263
	DM	0.9998	93715939 ± 697875	−9034 ± 1717

Dissolution profiles of DB and DM tablets in media simulating the environment in the upper small intestine in fasting state are presented in Figure 15.



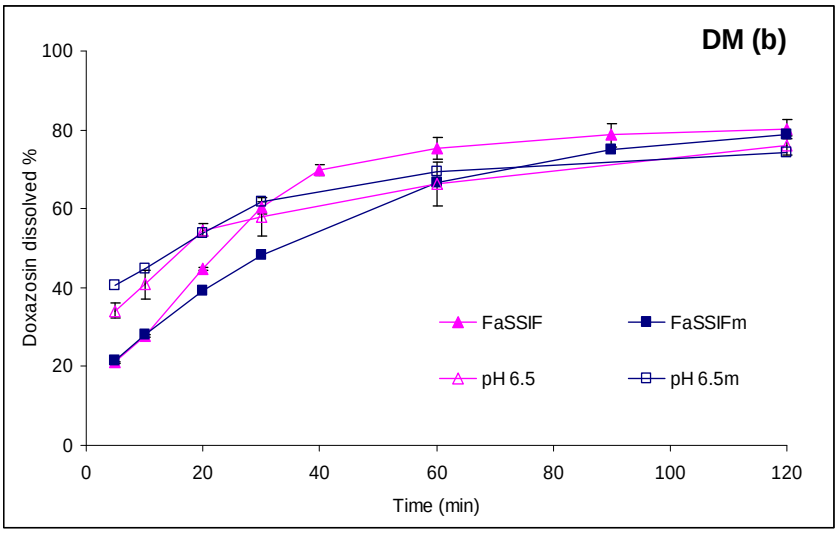


Figure 15. Mean ± SD (n = 3) % dissolved DB tablets (a) and DM tablets (b) in FaSSIF, FaSSIF$_m$, pH 6.5 phosphate buffer (pH 6.5), and pH 6.5 maleate buffer (pH 6.5$_m$)

Dissolution of DB tablets in media simulating the environment in the upper small intestine in fasting state was not complete (Figure 15a).

Based on the plateau levels (last experimental point in relevant curve in Figure 15a, solubility of DB in phosphate and maleate buffer is 1.0 and 0.9 µg/ml, respectively, whereas in FaSSIF and FaSSIFm it is 1.6 and 1.9 µg/ml, respectively. These data indicate that, unlike with other weakly alkaline compounds with similar pK_as and in similar media prepared with crude materials (Vertzoni et *al.*, 2004), the effect of the anion of the buffer system on doxazosin solubility is minimal.

Dissolution of DM tablets was more complete and unaffected by the presence of solubilizing agents (Figure 15b). As a result, the difference between DM and DB in FaSSIF ($f_{1,area}$ = 0.42) and in FaSSIF$_m$ ($f_{1,area}$ = 0.36) is smaller than the difference between DM and DB in phosphate buffer ($f_{1,area}$ = 1.44) and in maleates ($f_{1,area}$ = 1.10), i.e., accurate simulation of the environment in the small intestine increases solubility with subsequently smaller differences in dissolution profiles between DB and DM.

Based on the plateau level in pH 6.5 buffer (Figure 15a), DB is according to BCS, low solubile compound (the dose to solubility ratio is 2036 ml). Similarly, DM is also a low solubile compound (based on the plateau level in pH 6.5 buffer, dose to solubility ratio is 612 ml, Figure 15b), as indicated in literature (Yamashita and Tachiki, 2009).

5.5. Solubility and dissolution studies in the fed state

Solubility and dissolution studies in the fed state are part of overall assessment of food effect on absorption of doxazosin. Dissolution behaviour of the tested substances will be evaluated in the gastric and small intestinal environment in the fed state.

5.5.1. Evaluation of DB, DM and DH in the gastric environment in the fed state

Milk has been proposed to mimic gastric conditions after meal intake since the ratio of carbohydrates, proteins and fat is similar to that of a typical meal administered in bioavailability study (Macheras and Reppas, 1987, Klein et *al*., 2004).

As described in *Materials and methods* chapter, the influence of pepsin to the partitioning of doxazosin between aqueous and lipid part of milk was evaluated prior to dissolution experiments in milk.

Analytical parameters for the standard curves are given in Table 10.

Table 10. Analytical parameters for the standard curves of doxazosin base and salts in milk digested with pepsin

Medium	**Substance**	r^2	**Slope ± SD**	**Intercept ± SD**
Milk digested with pepsin	DB	0.9960	26630609 ± 848755	1922 ± 108
	DM	0.9995	25622956 ± 283650	787 ± 68
	DH	0.9988	29513866 ± 504949	1036 ± 135

The results of doxazosin partitioning are presented in Figure 16.

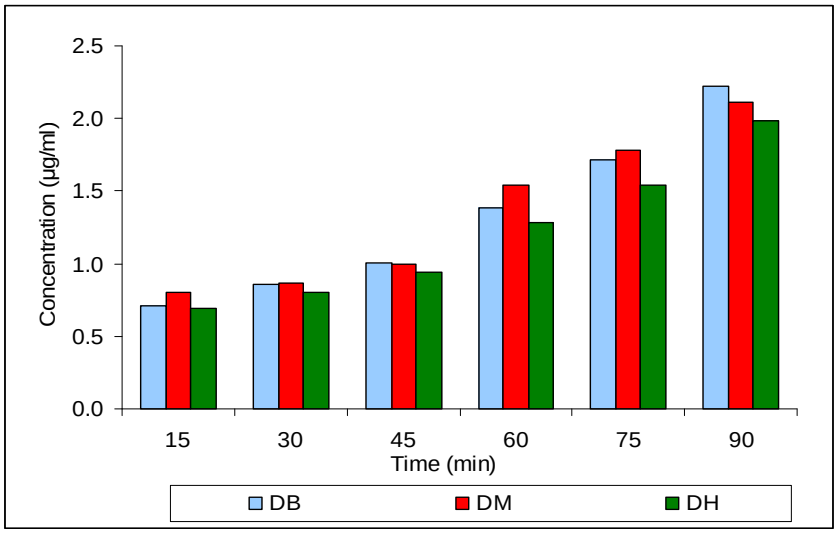


Figure 16. The influence of pepsin concentration to extraction rate of drug to aqueous part of milk (supernatant)

The results indicated that the amount of doxazosin in the supernatant increases with the addition of pepsin solution into a whole milk and therefore quantification in dissolution experiment at different time points should be based on different standard curves.

In order to quantify dissolution data at different time points of dissolution test, it was necessary to prepare six different calibration curves, with the concentrations of pepsin equivalent to the amount present in the release experiments at 10, 15, 30, 60, 75 and at 90 minutes.

Preparation of standard curves was described in Materials and methods chapter. Analytical parameters for the standard curves are given in Table 11.

Table 11. Analytical parameters for the standard curves of doxazosin base/salts in milk with different concentrations of pepsin

Standard curve for	Substance	r^2	Slope ± SD	Intercept ± SD
10 min	DB	0.9996	8857702 ± 99129	237 ± 168
	DM	0.9979	8254828 ± 188126	1922 ± 423
15 min	DB	0.9979	10296351 ± 238775	–434 ± 537
	DM	0.9992	10969746 ± 152542	–212 ± 343
30 min	DB	0.9971	12143713 ± 325050	–1700 ± 731
	DM	0.9971	11326967 ± 303946	–202 ± 683
	DH	0.9991	11354767 ± 173049	–660 ± 389
60 min	DB	0.9964	17882183 ± 536855	–1899 ± 1207
	DM	0.9970	17934711 ± 489785	–985 ± 1101
75 min	DB	0.9998	24729131 ± 166584	–628 ± 374
	DM	0.9983	23112528 ± 482356	–936 ± 1084
	DH	0.9988	24019257 ± 420993	–20 ± 946
90 min	DB	0.9998	27424373 ± 169937	–710 ± 382
	DM	0.9987	29086066 ± 520814	–1273 ± 1171

The slopes of standard curves at the same time points were statistically evaluated by using *t*-test (Glantz S.A., Primer of biostatistics). Please refer to Table 12.

Table 12. Estimated *t* values after the application of a *t*-test for the comparison of slopes of standard curves in milk digested with pepsin with various amounts of pepsin. The critical value of *t* for 8 degrees of freedom at the 99 % level is 3.36 (Glantz S.A., Primer of biostatistics)

Pepsin concentration corresponding to	Estimated *t* values (DB vs. DM)	Estimated *t* values (DB vs. DH)
10 min of dissolution testing	2.84	–
15 min of dissolution testing	2.38	–
30 min of dissolution testing	1.84	2.14
60 min of dissolution testing	0.07	–
75 min of dissolution testing	3.17	1.57
90 min of dissolution testing	3.03	–

Based on Table 12, at a given time of dissolution testing the slope of the standard curve does not vary with the form of doxazosin (base-hydrochloride-mesylate).

Therefore, it was decided that on days of analysis, standard curves at 30 and 75 minutes would be constructed, and % dissolved at other time points would be calculated according to the ratio of slopes between the standard curves.

Analytical parameters for the standard curves prepared on every experimental day are given in Table 13.

Table 13. Analytical parameters for the standard curves of doxazosin base/salts in milk at different time points

Standard curve for samples at:	**Substance/Time (min)**	r^2	**Slope±SD**	**Intercept ± SD**
Milk digested with pepsin	DB/30	0.9996	8857702 ± 99129	237 ± 168
	DB/75	0.9973	24872621 ± 74397	–3063 ± 1828
	DM/30	0.9993	10152987 ± 132810	–603 ± 299
	DM/75	0.9991	19784573 ± 292694	–1656 ± 658
	DH/30	0.9991	11354767 ± 173049	–660 ± 389
	DH/75	0.9988	24019257 ± 420993	–20 ± 946

Dissolution profiles of DB, DM and DH in milk digested with pepsin are presented in Figure 17.

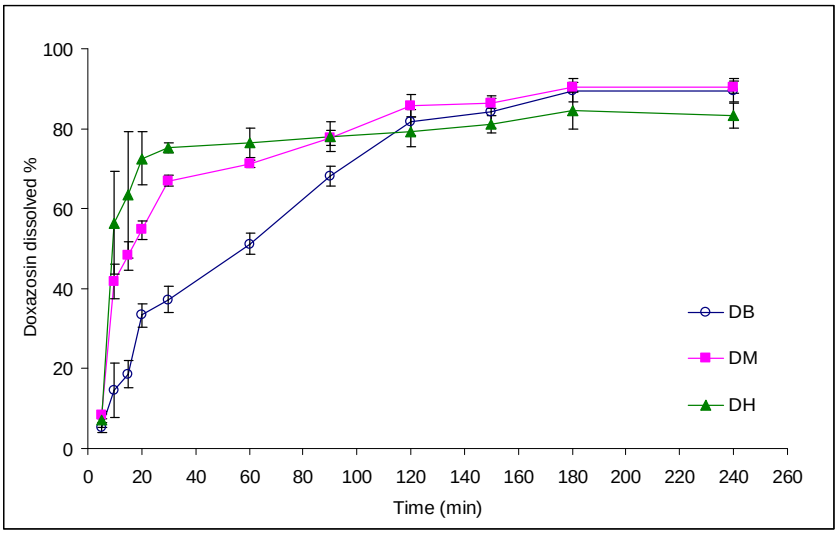


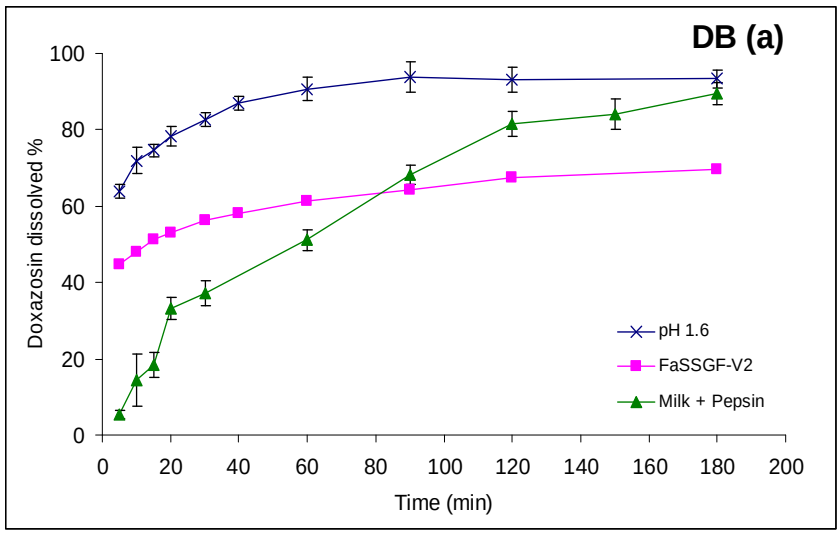
Figure 17. Mean ± SD (n = 3) % dissolved DB tablets, DM tablets and DH tablets in milk with pepsin

Dissolution of the dose is slightly less than complete in milk with pepsin. Since the final pH in milk digested with pepsin is little more than about 2 (Dressman et *al.*, 2007) similar bahaviour in acidic media as previously described can be expected.

Interestingly, lipophilic components of milk do not significantly influence dissolution profiles of DM and DH compared to FaSSGF-V2 (Figure 18). However, in case of DB, dissolution rate in milk digested with pepsin is significantly lower than in FaSSGF-V2 in the first hour, but after that reaches more complete dissolution. This might be due to the slower diffusion of the micelle-bound drug to the bulk (Macheras and Reppas, 1987).

Macheras et *al*. (1989) have demonstrated that chlorothiazide and hydrochlorothiazide are well solubilized by casein micelles in milk, whereas more lipophilic compounds, such as indomethacin and diazepam, are additionally solubilized into the milk fat (Macheras et *al.*, 1990).

The results in simple hydrochloric acid media (pH 1.6) for all three compounds are significantly faster than in FaSSGF-V2 or milk with pepsin (Figure 18).



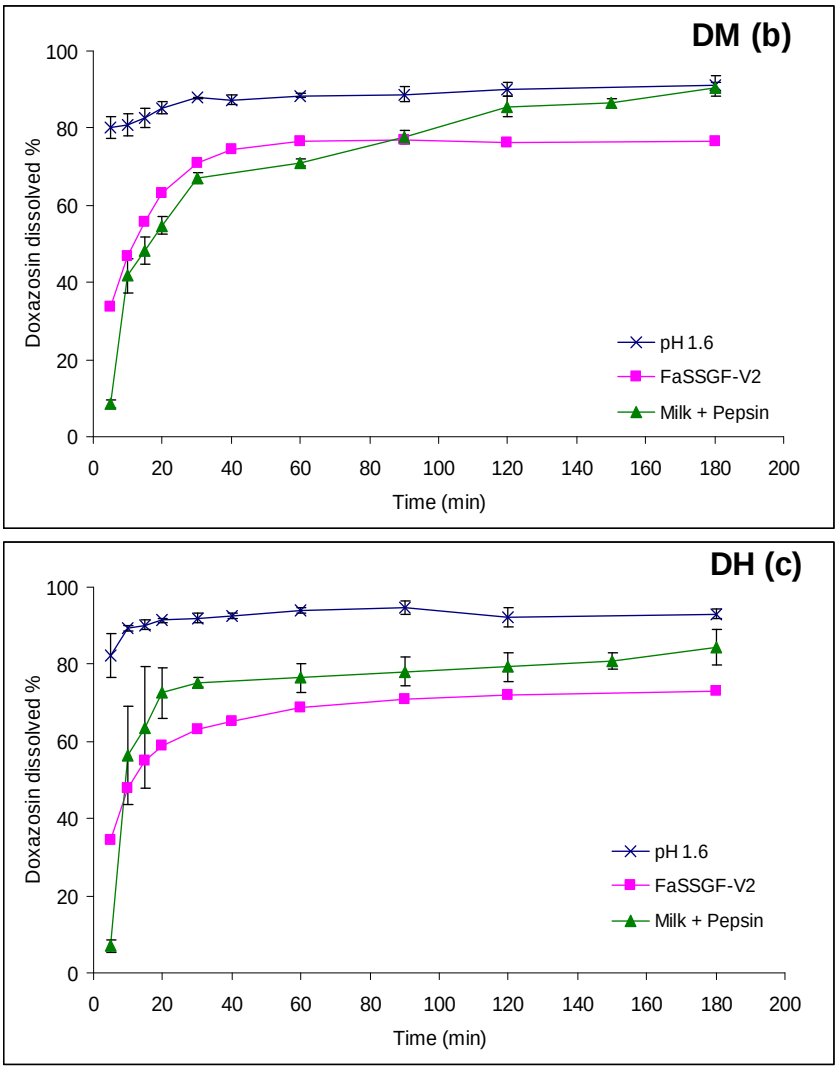


Figure 18. Mean ± SD (n = 3) % dissolved DB tablets (a), DM tablets (b) and DH tablets (c), in pH 1.6 HCl solution, in FaSSGF-V2 and milk with pepsin

Regarding the expected food effect, based on *in vitro* biorelevant data it could be assumed that the differences in dissolution in fasted and fed stomach for neither DB nor DM would be important due to a small dose and a longer gastric residence time in the fed state.

5.5.2. Evaluation of DB and DM in the environment of the upper small intestine in the fed state

Analytical parameters for the standard curves (n = 5, 0.5–4 ng/ml) in pH 5.0 and FeSSIF are given in Table 14.

Table 14. Analytical parameters for the standard curves in pH 6.5 and FaSSIF

Medium	Substance	r^2	Slope ± SD	Intercept ± SD
pH 5.0	DM	0.9995	138825 ± 1788	–3 ± 4
pH 5.0_c	DM	0.9973	129983 ± 3914	7 ± 10
FeSSIF	DB	0.9994	114482037 ± 1622048	2858 ± 3990
	DM	0.9983	118560580 ± 2822190	3257 ± 6942
	DH	0.9998	125348732 ± 932616	–3175 ± 2294
FeSSIF $_c$	DB	0.9995	123880061 ± 1611398	705 ± 3964
	DM	0.9986	296328500 ± 6490439	13544 ± 15964

Dissolution profiles of DB and DM tablets in media simulating the environment in the upper small intestine is presented in Figure 19.

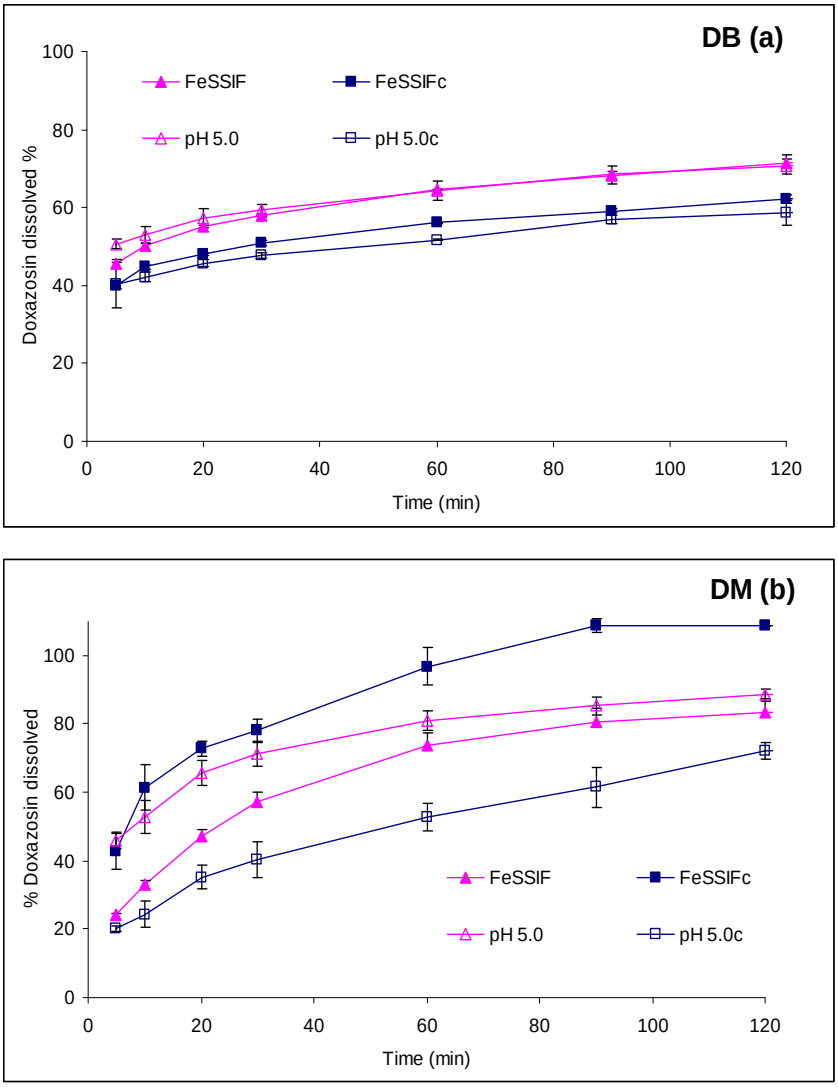


Figure 19. Mean ± SD (n = 3) % dissolved DB tablets (a) and DM tablets (b) in FeSSIF, FeSSIF$_c$, pH 5.0 acetate buffer (pH 5.0), and pH 5.0 citrate buffer (pH 5.0_c)

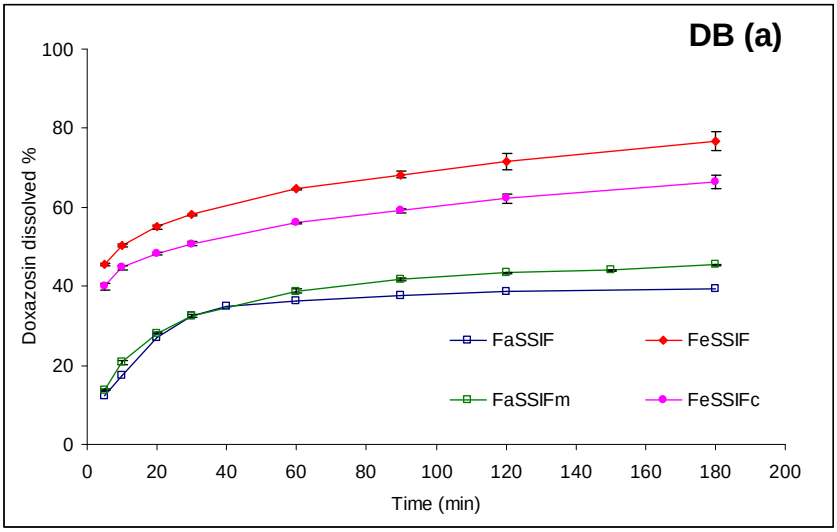
Dissolution of DB tablets in media simulating the environment in the upper small intestine in the fed state was not complete (Figure 19a).

Based on the plateau levels in dissolution experiments presented in Figure 19, the effect of the anion of the buffer system on doxazosin solubility is minimal for DB, but it is significant for DM (the difference between pH 5.0 with acetate and citrate buffer and FeSSIF and FeSSIFc is $f_{1,area} = 0.43$ in both cases).

This could be attributed to the differences in solubility of the salts that exist in pH of FeSSIF (Vertzoni et *al.*, 2004).

Dissolution of DM tablets is complete only in FeSSIFc (Figure 19b), probably due to the same reasons as explained earlier. As in the case of fasting state conditions dissolution of DM tablets in the environment of the small intestine is also more efficient than dissolution of DB.

The comparison of dissolution behaviour of DB and DM in the fed and in the fasted state simulating intestinal media is given in the Figure 20.



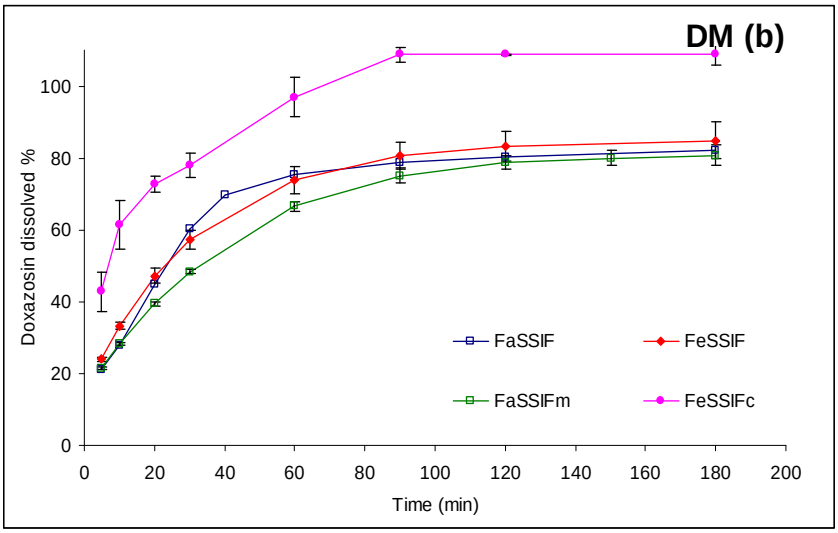


Figure 20. Mean ± SD (n = 3) % dissolved DB tablets (a) and DM tablets (b) in FaSSIF, $FaSSIF_m$, FeSSIF and $FeSSIF_c$

In biorelevant dissolution media representing intestinal fluid with and without food the behaviour of DB and DM was different. Dissolution of DB was significantly higher in FeSSIF than in FaSSIF, irrespectively of the buffer anion used in experiments. For DM the results are not so straight forward: the extent of dissolution is significantly higher in FeSSIF prepared with citrate buffer than in FaSSIF with either phosphate or maleate buffer. On the other hand, the difference between FeSSIF and FaSSIF is not visible if FeSSIF with acetate buffer is used.

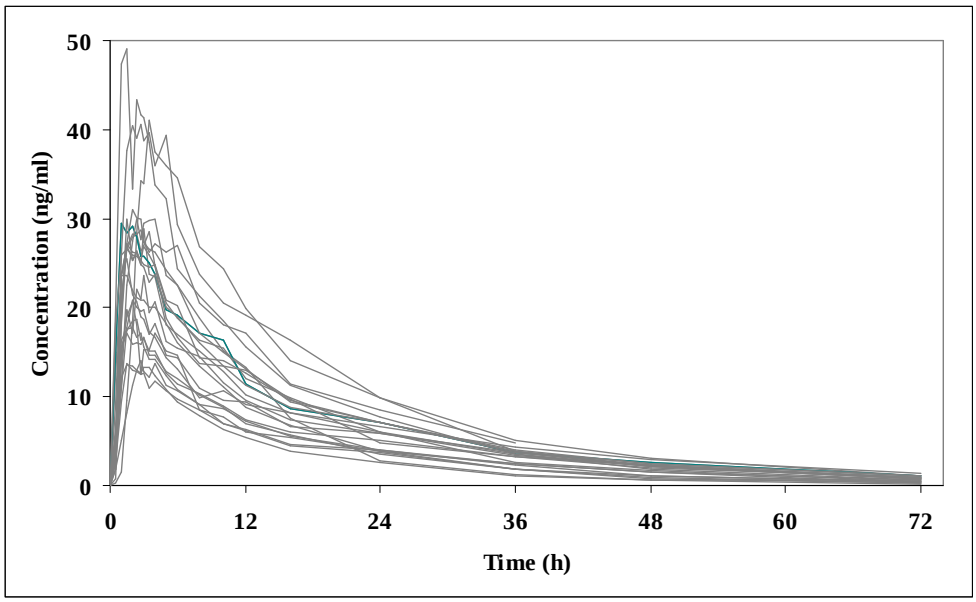
The other two objectives that were supposed to be evaluated in the fed state, the food effect after absorption of doxazosin and the evaluation of canine model for prediction for food effects in humans, will be commented after the presentation of fed state *in vivo* results.

5.6. Assessment of early exposure after the administration of DB and DM tablets by using *in vitro* data in the fasted state

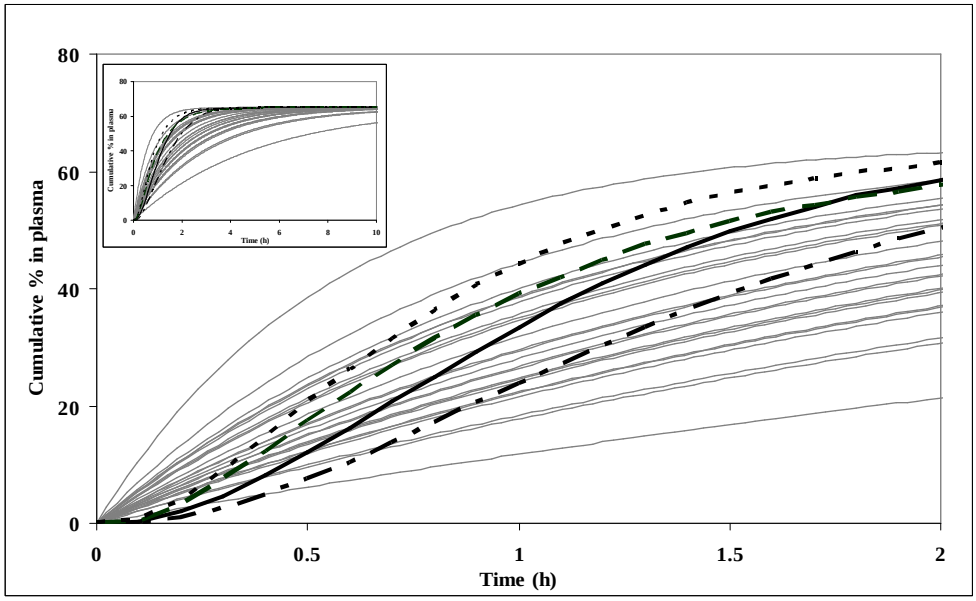
The objective of this part of thesis was to evaluate the usefulness of biorelevant *in vitro* data and of canine data in forecasting early exposure after administration of DB and DM.

Figure 21a shows the actual individual plasma concentration vs. time profiles, after single administrations of DM tablets to 24 healthy adults. In all profiles secondary peaks were observed, in agreement with the enterohepatic circulation of doxazosin (Cardura®, Summary of product characteristics). As with plasma concentration profiles, individual cumulative

doxazosin profiles in plasma after single administrations of DM tablets were highly variable (Figure 21b).



(a)



(b)

Figure 21. (a) Individual doxazosin concentration in plasma vs. time profiles (n = 24), after single dose administrations of DM tablets to healthy adults (b) Partial (and complete, in insert) individual cumulative % doxazosin in plasma vs. time profiles (n = 24, grey lines), simulated cumulative % doxazosin in plasma vs. time profiles after administration of one DB tablet that were constructed by using dissolution data in $HCl_{pH1.6}$ and in phosphate buffer pH 6.8 (__ __) and by using dissolution data in FaSSGF-V2 and FaSSIF (__ . __), and simulated cumulative % doxazosin in plasma vs. time profiles after administration of one DM tablet that were constructed by using dissolution data in $HCl_{pH1.6}$ and in phosphate buffer pH 6.8 (…….) and by using dissolution data in FaSSGF-V2 and FaSSIF (_____)

Regardless of the type of *in vitro* data used for generating the simulated cumulative input profile of DM, the early phase, 0–0.5 h post-dosing of DM tablets, is underestimated by the simulated profiles (Figure 21b). This was found to be related to the methodology applied for estimating the kinetics of excretion into the bile.

To specify, it was tested whether the underprediction of cumulative % doxazosin in plasma vs. time during the first half hour after administration of DM is due to inaccurate estimation of gastric emptying rates (gastric emptying rate constant in the fasted state has been set to 2.8 h^{-1}) so the simulated cumulative % doxazosin in plasma vs. time was also done with gastric emptying rates of 3.5 h^{-1} and 4 h^{-1}).

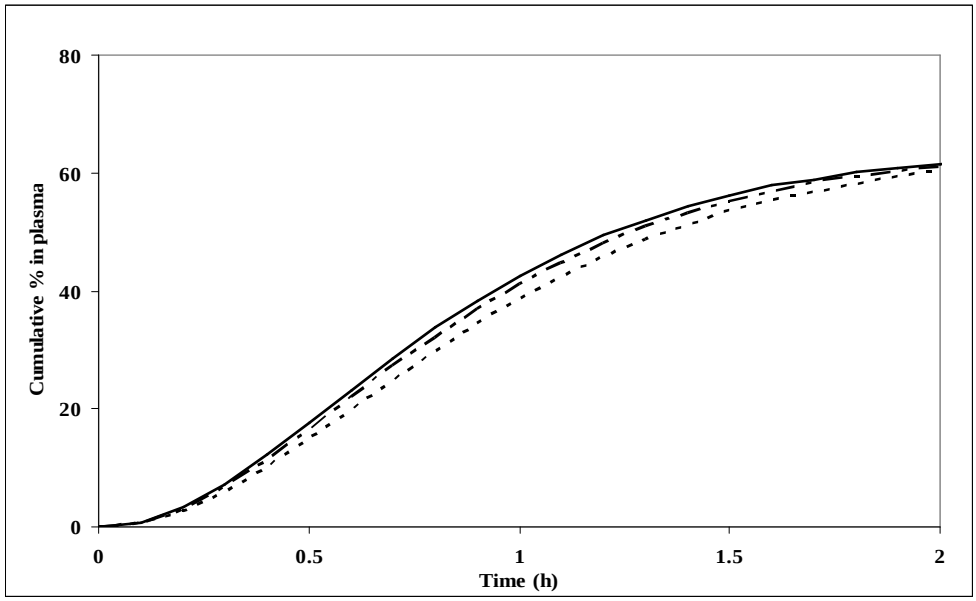


Figure 22. Simulated cumulative in plasma doxazosin profiles after DM administration by using in vitro data in pure aqueous buffers, where (…….) is gastric emptying rate constant 2.8 h^{-1}, (__ . __) is gastric emptying rate constant 3.5 h^{-1} and (_____) gastric emptying rate constant 4 h^{-1}

The results showed in Figure 22 that the possible underprediction of cumulative % doxazosin in plasma vs. time during the first half hour after administration of DM is not due to underestimation of gastric emptying rates.

After that, the importance of enterohepatic circulation on the initial input phase was evaluated. Based on the Figure 23 below, it can be concluded that the inaccurate estimation of the kinetics of excretion into bile is probably the main reason for the underprediction of the early input phase (0–0.5 h post dosing) after DM administration.

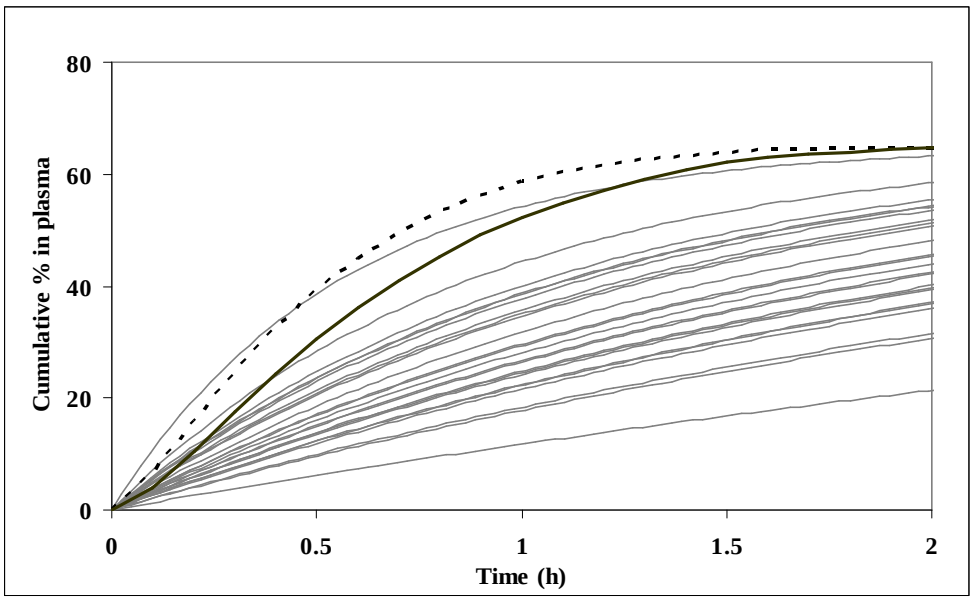


Figure 23. Individual actual cumulative in plasma doxazosin profiles after DM administration (grey lines) and simulated profiles without taking into consideration the enterohepatic circulation of doxazosin and by using in vitro data in pure aqueous buffers (…….) and data in biorelevant media (_____)

Simulation of enterohepatic circulation involved the use of mean intravenous plasma concentration vs. time data that were estimated from a limited number of individual profiles (Elliott et *al*., 1987). Also, simulated profiles of DM at late times after administration (times > 3 h) seem to over predict the average actual profile (insert of Figure 21b). This can be related to variability of gallbladder emptying patterns.

Compared with *in vitro* data collected in simple aqueous buffers, biorelevant *in vitro* data led to better evaluation of the average input profile of DM, during the 0.5–2 hours post-dosing (Figure 21b).

The results showed that simulated cumulative % in plasma vs. time profiles during the first 2 h after administration constructed by using *in vitro* data in simple aqueous buffers or in biorelevant media suggests that the administered phase, DB or DM, does not have a significant impact on early exposure (Figure 21b).

5.7. Development and validation of new LC-MS/MS method for determination of doxazosin assay in canine plasma

For determination of doxazosin in human plasma several analytical methods have been reported, mainly chromatographic methods coupled with fluorescence (Cowlishaw and Sharman, 1985, Fouda et *al*., 1988, Jackman et *al*., 1991, Sripalakit et *al*., 2005, Sripalakit et *al*., 2006, Kim et *al*., 2006, Kwon et *al*., 2007) UV (Wei et *al*., 2007), MS (Ma et *al*., 2007) or MS/MS detection (Al-Dirbashi et *al*., 2006, Ji et *al*., 2008). Sample treatment involves primarily liquid-liquid extraction of doxazosin from plasma (Fouda et *al*., 1988, Jackman et *al*., 1991, Sripalakit et *al*., 2006, Kim et *al*., 2006, Kwon et *al.*, 2007, Ma et *al.*, 2007, Al-Dirbashi et *al.*, 2006, Ji et *al.*, 2008), although offline and online solid-phase extraction (Jackman et *al.*, 1991, Wei et *al.*, 2007) as well as protein precipitation (Sripalakit et *al.*, 2005) have also been applied.

Most of the developed methods have been applied to human pharmacokinetic studies, after oral administrations of 4–10 mg doses of doxazosin tablets (Jackman et *al*., 1991, Kim et *al*., 2006, Kwon et *al*., 2007, Wei et *al*., 2007, Ma et *al*., 2007, Ji et *al*., 2008).

The objective of this study was to develop a method that would be suitable for determination of low concentrations of doxazosin in canine plasma, i.e. after oral administration of a 2 mg dose in the fed state. Although literature data suggest that oral bioavailability and maximum plasma concentration (c_{max}) are not affected significantly by dosing conditions in humans (Conway et *al*., 1993), the lower absorption rates in the fed state (as a consequence of the slower gastric emptying rates) (P. Macheras et *al*., 1995) frequently lead to lower plasma concentrations during absorption in the fed state. Also, the number of interfering compounds in plasma samples is expected to be bigger in the fed state.

5.7.1. Optimization of sample treatment

Several sample treatment procedures were tested. Liquid-liquid extraction was not adopted, primarily because it resulted in low recovery of analyte from plasma samples (Table 15).

Table 15. Doxazosin (20 ng/ml) and of prazosin (internal standard, IS, 30 ng/ml) % recovery from canine plasma using various sample treatment procedures

Sample treatment procedures	**Recovery Doxazosin (%)**	**Recovery IS (%)**
Liquid-liquid extraction (with 30 % dichloromethane in hexane after alkalinization with 1M NaOH)	43	37
Double precipitation with methanol and storage at 4 ^{o}C for 12 h	12	18
Double precipitation with ethanol and storage at 4 ^{o}C for 12 h	16	16
Double precipitation with methanol:acetonitrile (50:50, *v/v*) and storage at 4 ^{o}C for 12 h	22	38
Precipitation with methanol:acetonitrile (50:50, *v/v*) and storage –20 ^{o}C for 12 h	91	98

In addition, it required significantly larger volumes of plasma and increased time of analysis.

A previously described precipitation method (Sripalakit et *al.*, 2005) which involved the use of methanol was found not to be appropriate for removal of proteins from our samples. Supernatant of samples collected in the fed state was not clear and recoveries of doxazosin and prazosin were low (Table 15).

In contrast, when plasma sample was diluted with a mixture of methanol/acetonitrile (50:50, *v/v*), vortexed for 30 seconds, stored for 12 hours in a freezer at –20 °C and subsequently treated as described in the next section (*Analysis of plasma samples)*, proteins were efficiently precipitated, i.e. supernatant was clear and suitable for further chromatographic analysis, whereas recovery of spiked doxazosin and prazosin was higher than 90 % (Table 15).

It should be noted that protein precipitation has recently been reported to be inefficient for accurately measuring doxazosin in pharmacokinetic studies (Sripalakit et *al.*, 2006). However, in the relevant study, although storing conditions of the plasma samples drawn from the PK study are mentioned, storing conditions of *blank* plasma sample are not specified.

5.7.2. Method validation

5.7.2.1. Selectivity

Typical chromatograms of blank canine plasma and canine plasma samples from the pharmacokinetic study are shown in Figure 24.

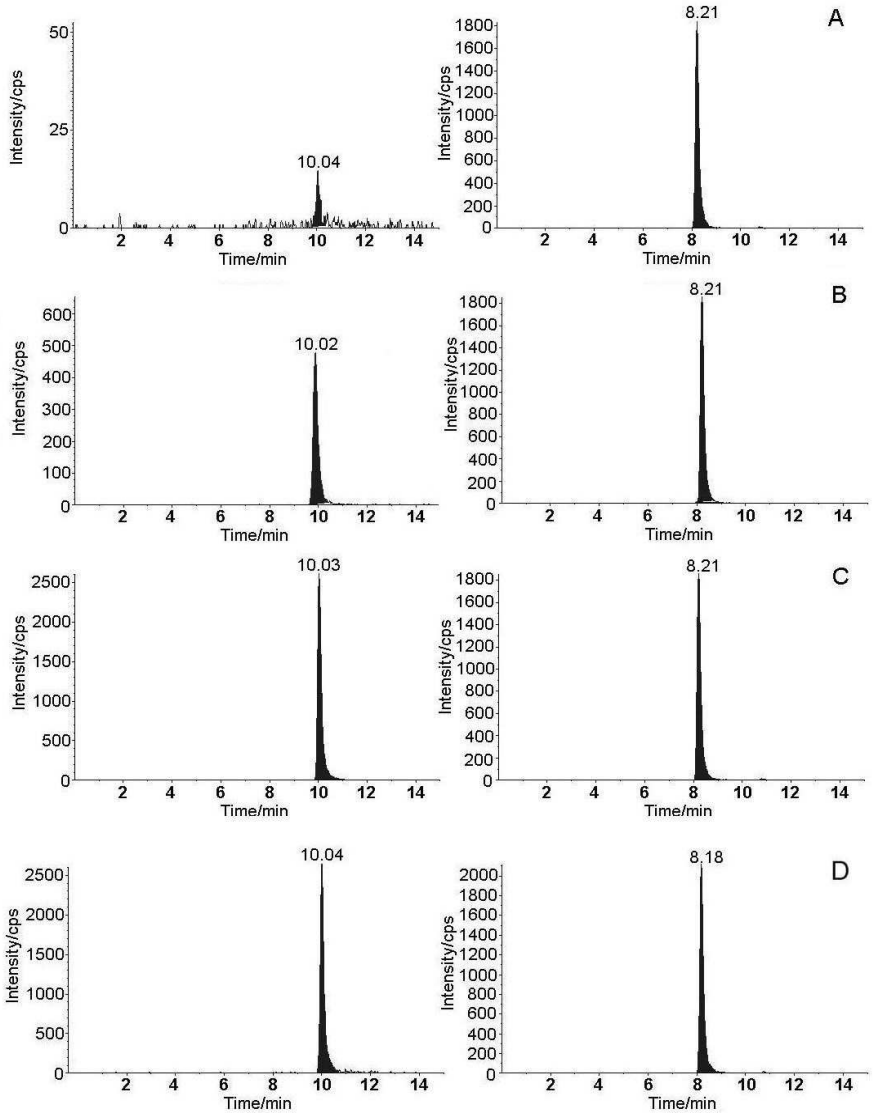


Figure 24. Chromatograms of (A) canine plasma spiked with 10 ng/ml prazosin, (B) canine plasma spiked with 2 ng/ml doxazosin and 10 ng/ml of IS (concentration of doxazosin is the lowest above *LOQ*), (C) canine plasma spiked with 10 ng/ml doxazosin and 10 ng/ml of IS and (D) plasma sample collected 5 h after administration of one doxazosin mesylate tablet (2 mg doxazosin in tablet)

Prazosin and doxazosin were eluted at approximately 8 and 10 min, respectively, with a total run time of 15 min, which is within range of other published methods (Carlson et *al*., 1986, Cowlishaw et *al*., 1985, Fouda et *al*., 1988, Jackman et *al*., 1991, Sripalakit et *al*.,

2005, Sripalakit et *al*., 2006, Kim et *al*., 2006, Kwon et *al*., 2007, Wei et *al*., 2007, Ma et *al*., 2007, Al-Dirbashi 2006 et *al*., Ji et *al*., 2008). Product ion mass spectra of doxazosin and prazosin have already been published (Ji et *al*., 2008, Erve et *al*., 2007).

A good separation of doxazosin and prazosin was obtained whereas no interfering peaks were found at the retention time of doxazosin and/or prazosin (Figure 24).

5.7.2.2. Calibration curves

Linear calibration curves for doxazosin were obtained throughout the concentration range studied (1–20 ng/ml) over the three consecutive days. The number of points in each calibration curve was six. Linearity criteria imposed correlation coefficient $r \geq 0.99$. Regression analysis was performed for the ratios of peak area of doxazosin to that of the IS (y) versus doxazosin concentration (x). The calibration curve (mean (SD), n = 3) could be described by the equation:

$y = 0.134\ (\pm\ 0.017)\ x + 0.006\ (\pm\ 0.068)$

In all three replications, intercept was not significant.

5.7.2.3. Precision, accuracy, and recovery

In the last few years various authors have made effort to improve the existing methods for determination of doxazosin in human plasma. Most of these methods were applied to pharmacokinetic studies of 4–10 mg doses of doxazosin tablets (Jackman et *al.*, 1991, Kim et *al.*, 2006, Kwon et *al.*, 2007, Wei et *al.*, 2007, Ma et *al.*, 2007, Ji et *al.*, 2008).

To our best knowledge Sripalakit *et al.*, 2006, were the only who administered single 2 mg dose of doxazosin tablets to humans in the fasted state. In that study, 500 µl of sample was used for extraction *vs.* 150 µl used in the present study for achieving comparable accuracy, precision, and recovery.

The precision of the proposed LC-MS/MS method was examined in spiked canine plasma samples. After preparing and measuring QC samples of three different concentrations of doxazosin, each in triplicate, values of intra- and inter-day relative standard deviation (RSD) were calculated. Results showed that intra-day relative standard deviation was less than 7 %, while the corresponding inter-day value was less than 8 %. Even at concentration level close to the limit of quantification, RSD values were in accordance with the relevant guidelines (Green, 1996, Shah et *al*., 2000) where RSD for LOQ did not exceed 20 %.

Accuracy of the developed method was examined on QC standards at three concentration levels by comparing the measured value with the nominal values. These

standards were quantified using calibration curves prepared in plasma matrix. The results are summarized in Table 16 and are in agreement with the relevant guidelines (Green et *al*., 1996, Shah et *al*., 2000).

Table 16. Relative standard deviation of measurements, % recovery and % accuracy of three doxazosin concentrations spiked in canine plasma by using the method developed in the present study*

Nominal Concentration (ng/ml)	**RSD (%)**	**Mean relative recovery ± SD (%)**	**Accuracy (%)**
Intra-day			
2	6.8	97.2 ± 6.6	−2.8
5	6.1	101.0 ± 6.1	1.0
10	5.2	99.7 ± 5.2	−0.3
Inter-day			
2	6.8	94.1 ± 6.4	−5.9
5	4.9	103.6 ± 5.1	3.6
10	7.9	101.3 ± 8.0	1.3
*Each standard was prepared and measured three times on three different days			

Recovery was calculated by comparing ratios of integrated peak area of doxazosin to internal standard from the quality control samples to those from the standard solutions having the same concentrations of doxazosin and internal standard (direct injection of the corresponding unextracted standard solutions). The mean recovery of doxazosin from canine plasma at the concentrations of 2 ng/ml, 5 ng/ml and 10 ng/ml was over 94.1 % (Table 16).

5.7.2.4. Stability

Doxazosin was found to be stable at room temperature for at least 24 h. Recoveries were 91.0 % and 92.2 % for 5 ng/ml and 10 ng/ml samples, respectively. Similarly, doxazosin was stable at –20 °C for at least 60 days. Recoveries at 5 ng/ml and 10 ng/ml were 93.2 % and 91.3 %, respectively.

5.7.2.5. Limits of detection and quantification and carryover effects

LOD and *LOQ* values for doxazosin were found equal to 0.4 and 1.2 ng/ml of plasma sample, respectively.

Most of the reported methods have *LOD* and *LOQ* values close to the values obtained in our study. Only in one recently developed method that utilizes UPLC-MS/MS (Al-Dirbashi 2006) *LOD* and *LOQ* values are lower (0.02 ng/ml and 0.07 ng/ml, respectively) than those of the method developed in the present study.

Carry over was tested at the concentration of 30 ng/ml and it was determined to be equal or less than 0.3 %.

A simple, rapid, and selective LC-MS/MS method for determination of doxazosin plasma concentrations after oral administration was developed and validated. Specific advantages over previously published methods include the low sample volume (150 µl), the short retention times (of both doxazosin and IS) and high sensitivity.

Developed and validated LC-MS/MS method was employed for determination of doxazosin assay in samples collected in pharmacokinetic studies on dogs, in fasted and fed state.

5.8. Assessment of early exposure after the administration of DB and DM tablets by using canine data in the fasted state

Canine plasma levels in fasted state were measured after administration in 4 mongrel dogs.

Individual plasma profiles are shown in Figure 25. In every profile, a second peak was observed at about 6 hours post dosing suggesting that doxazosin is enterohepatically circulated in dogs as it has been observed in humans.

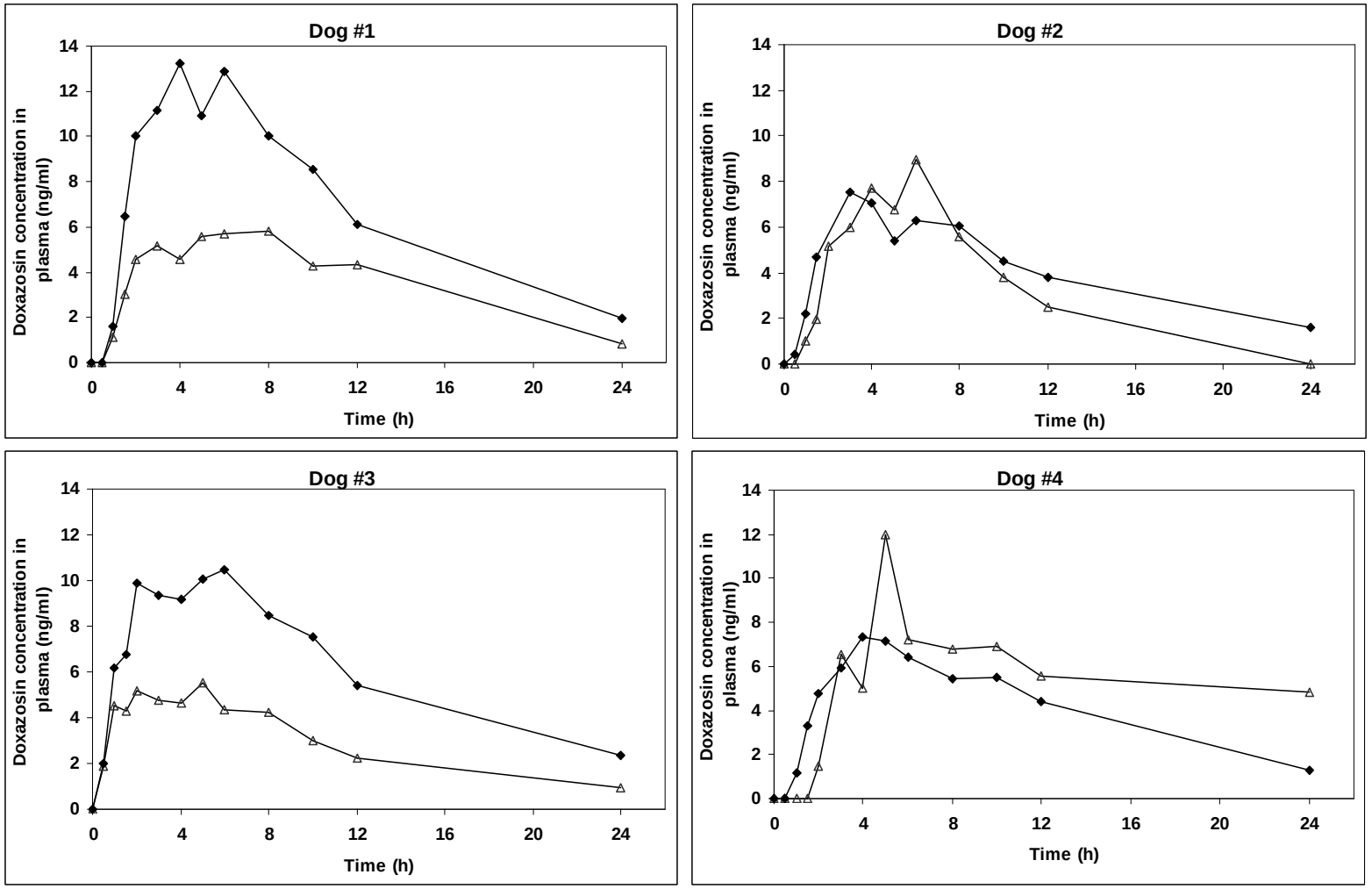


Figure 25. Individual doxazosin plasma profiles after single administration of one DB tablet (△) and one DM tablet (♦) to four dogs in the fasting state

Total exposure after DB administration was not statistically different from that measured after DM administration (p = 0.171). The first peak of doxazosin concentration in plasma after DB administration ranged 5.1–7.7 ng/ml and was observed 2–4 h post dosing (Figure 25). There are no human data in literature after administration of DB. The first peak of doxazosin concentration in plasma after DM administration ranged 7.3–13.2 ng/ml and was observed 2–4 h post-dosing (Figure 25), i.e. not much different from human data collected in this (Figure 21a) and in previous studies (~ 9 ng/ml, 2–3 h post-dosing, Elliott et *al*., 1987). Oral bioavailability of DM has been reported to be similar in dogs and humans (Kaye et *al*., 1986).

However, this study shows that early exposure after DM administration to dogs is higher than after DB. Specifically, $AUC_{p,base}$, ranged from 10.2 to 16.4 ng/ml/h after DB and from 15.4 to 29.2 ng/ml/h after DM administration and difference is significant (p = 0.048). The apparent discrepancy compared with the minimal (if any) difference in early exposure after DB and DM administration in humans (Figure 21b) could be attributed to species related differences. Dissolution data in gastric pH of the dogs (Figure 14) used in this study and solubility data in their gastric aspirates suggest that dissolution in the canine stomach occurs similarly to that in the human stomach. Therefore, the reasons for the inappropriateness of canine data may relate to the faster gastric emptying rates of dogs (Smeets-Peeters et *al.*, 1998, Reppas et *al*., 1991) and different dissolution characteristics of DB and DM in the upper small intestine of dogs; individual bile acids identity and concentrations in the contents of the canine upper small intestine are different between dogs and humans (Kalantzi et *al*., 2006)

5.9. Assessment of early exposure after the administration of DB and DM tablets by using canine data in the fed state

Individual doxazosin plasma concentration profiles after administration of DB and DM 2 mg tablets to four dogs in fed state are shown in Figure 26. Similar to fasting study, in most cases, more than one peak has been observed, which is in agreement with literature data indicating that doxazosin is enterohepatically circulated (Cardura®, Summary of product characteristics).

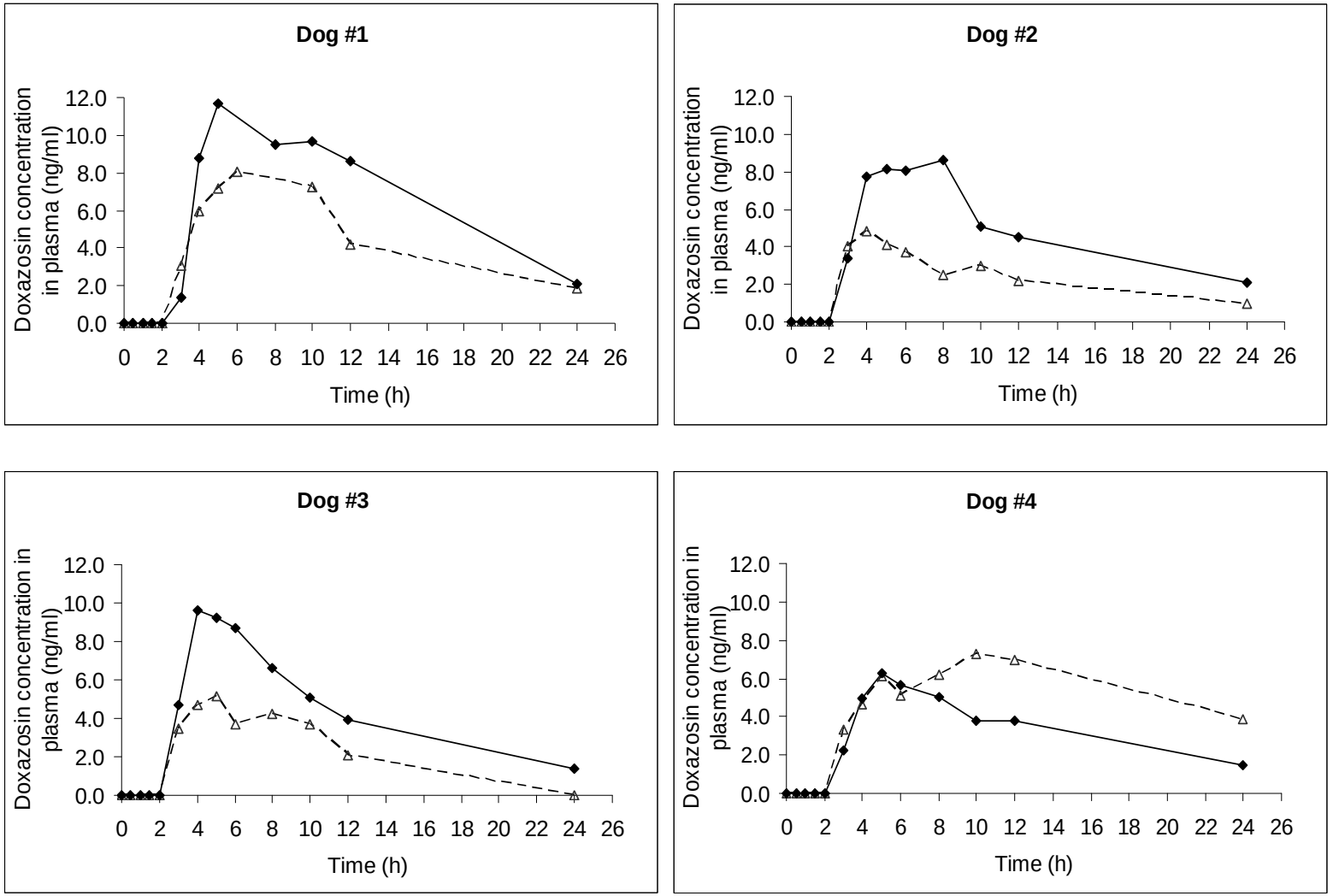


Figure 26. Doxazosin plasma profiles after single administrations of one DB tablet (△) and one DM tablet (♦) to four dogs in the fed state

As said previously, in men the peak plasma level of approximately 9 ng/ml is achieved within 2 to 3 hours after a single oral dose in the fasted state (Elliot et *al.*, 1987). Taking doxazosin with food delays its absorption by about 1 hour, but this does not alter pharmacokinetic parameters significantly, making it possible to take the drug in conjunction with food (Conway et *al.*, 1993).

In the canine fed study the maximum doxazosin plasma concentration of DB (c_{max}) ranged from 4.9 to 8.1 ng/ml and was achieved 4 to 10 hours post-dose (t_{max}). DM achieved maximum concentrations of 6.3 to 11.6 ng/ml 4 to 8 hours after administration.

Based on $AUC_{p,base}$ values, the use of mesylate salt seems to lead to slightly faster increase of plasma concentrations in dogs in the fed state (Table 17).

Table 17. Individual $AUC_{p,base}$ values (ng/ml/h) after the single dose (2 mg) administration of DB and DM of doxazosin to four dogs in the fed state

	DB	**DM**
Dog #1	20.2	27.3
Dog #2	6.6	7.3
Dog #3	10.7	18.9
Dog #4	11.0	10.4

Again, the superiority of the mesylate salt in fed state cannot be evaluated on a statistical basis due to the small number of dogs available.

When fed state results are compared to the results of fasting study, it is evident that the presence of food delays t_{max}, and slightly decreases c_{max} (especially for DM), while the $AUC_{p,base}$ does not change significantly (Table 18).

Table 18. Comparative c_{max}, t_{max} and $AUC_{p,base}$ values in fasting and fed state in four dogs (range)

	Fasted state		**Fed state**	
	DB	DM	DB	DM
c_{max} (ng/ml)	5.1–7.7	7.3–13.2	4.9–8.1	6.3–11.6
t_{max} (h)	2–4	2–4	4–10	4–8
$AUC_{p,\ base}$	10.2–16.4	15.4–29.2	6.6–20.2	7.3–27.3

Since gastric emptying of dogs in the fed state is similar with that of humans (De Zwart et *al*., 2009) and also terminal elimination half life in the tested dogs ranged from 4.0 to 15.1 h, which was similar to that in humans, from 9 to 22 h (Babamoto et *al*., 1992), it can be claimed that the slightly lower plasma profiles in dogs and delayed t_{max} are both due to the fed state conditions.

It should be noted that the results of food effect in canine study for DM correlate well with the literature data regarding the food effect in humans.

Based on all the results presented in this chapter, the following conclusions may be brought:

- Regarding the importance of adequately simulating luminal composition, it seems that, regardless of the degree of simulation of luminal composition, potential differences in dissolution between DB and DM are not substantial to affect doxazosin absorption rates; i.e. the differences in dissolution data between DB and DM are small to have an impact on early exposure;
- According to human data in fasted state, there is no significant difference in early exposure between DB and DM tablets;
- In fasted state canine data overestimated the differences in early exposure between DB and DM;
- Regarding the evaluation of differences in early exposures between DB and DM, in fed state canine studies DM also appeared in plasma earlier that DB, which should be taken with caution, as showed in fasted state study;
- The presence of food resulted in delayed t_{max} in canine study which is in agreement with the literature data for humans (for DM), which indicates suitability of the canine model for evaluation of food effects in case of doxazosin mesylate;
- Finally, regarding the usefulness of *in vitro* data for prediction of food effects in humans, in case of DM similar dissolution characteristics in fasted and fed stomach and not clearly different dissolution profiles in fasted and in the fed small intestine suggest that any difference in absorption in the fed state should be attributed only to the delayed gastric empting in the fed state (i.e. slower apsorption should be expected). This is in correlation with the *in vivo* human data. In case of DB, the differences in dissolution in fasted and fed stomach should not be important due to the

small dose and longer gastric residence time in fed state stomach. However, it seems that difference in dissolution between fasted and fed state in small intestine environment is bigger for DB. Therefore, there is a possibility for more significant food effect after administration of DB.

6. CONCLUSIONS

Results of the present study revealed several interesting findings.

1. Dosage form performance in the gastric media

a) Solubility of DB in FaSSGF-V2 (pH 1.6) is about ten times higher than solubility of DH in the same medium. The similar dissolution profiles of DB and DH in FaSSGF-V2 suggest conversion of DB to DH during dissolution of the dose.

b) Solubilities of DB and DM in HGF were forecasted by data in fasted state simulating gastric fluid containing physiological components (FaSSGF-V2) but not by data in CGF or in $HCl_{pH\ 1.6}$.

c) Based on the data in FaSSGF-V2, and HGF, solubility of all three substances is inversely proportional to the ionic strength of medium. Solubility data in CGF are in line with this observation. Solubility of DH is much lower than solubility of DM in all acidic media, which can be attributed to its higher thermal stability and common ion effect.

d) In contrast to the solubility data, incomplete dissolution of the dose in acidic media was noticed, presumably due to ion exchange interactions with either placebo components from the tablet (e.g. croscarmellose sodium) or components of biorelevant media (e.g. sodium taurocholate) and formation of insoluble salt(s) in the dissolution medium.

e) In the fed state simulated gastric conditions, lipophilic components of milk affected dissolution profile of DB but not DM or DH. Due to the longer gastric residence times in the fed stomach, however, differences between dissolution of DB and DM are not expected to be important to the doxazosin absorption rates.

2. Dosage form performance in media simulating the composition in the upper small intestine

a) DM dissolves more efficiently than DB, but incompletely, for potentially similar reasons as in media simulating the gastric conditions.

b) In biorelevant media, differences between dissolution profiles of DB and DM are decreased.

c) Regarding the effect of anion of the buffer system, it was generally minimal.

d) Dissolution of DB was significantly higher in FeSSIF than in FaSSIF. For DM the advantage in the fed state simulating media is not so clear and it depends on the buffer used in experiments.

3. Early exposure after DB and DM administration

a) Simulated cumulative % in plasma vs. time profiles during the first 2 h after administration constructed by using data in simple aqueous buffers or in biorelevant media, gastric emptying rate in humans, the average bioavailability and enterohepatic circulation led to adequate prediction of early exposure after DM administration to humans. In addition, in the fasted state, early exposure in humans should not be affected substantially by the form of doxazosin that has been administered (base or mesylate). This conclusion is reached regardless of the type of dissolution data used (in biorelevant media or simple aqueous buffers), at least for the period between 0.5 and 2 h after administration.

b) Data in dogs indicate early exposure is higher after DM administration in the fasted state and therefore, canine model is not useful for this comparison.

c) In the fed state, t_{max} after DM administration is delayed in dogs as it has been found previously in humans. In the fed state, t_{max} after DB administration is also slower in dogs. There are no data in humans but it expected that t_{max} will also be delayed.

d) Would food effects be quantitatively different between DB and DM? Taking into account the longer gastric residence times in the fed state and the *in vitro* dissolution profiles of DM and DB tablets in media simulating the fed gastric conditions, DB should arrive in the fed small intestine at similar dissolved percentages as DM. The *in vitro* dissolution profiles of DM and DB tablets in media simulating the fed state conditions in the small intestine show that dissolution of DM is more complete than dissolution of DB tablets. Therefore, in the fed state, absorption after DB administration is expected to be slower than after DM

administration. This is in line with canine data collected in this study, suggesting that, although the dog is not a good model for predicting differences between DB or DM in the fasted state it may be a good model for predicting differences between DB and DM in the fed state.

e) It would be interesting to explore if conclusions on early exposure are maintained at higher strengths (doses) of tablets.

LIST OF ABBREVIATIONS

AUC – Area under curve of the drug concentration time profile

AUC_p – Partial area under curve of the drug concentration time profile

$AUC_{p,base}$ – The partial area under the plasma profiles, i.e. from $t = 0$ up to time, t, at which the first peak on plasma profile, after the administration of DB to the specific dog

BA – Bioavailability

BCS – Biopharmaceutics Classification System

BE – Bioequivalence

BPH – Benign prostatic hyperplasia

CCS – Croscarmellose sodium

CGF – Canine gastric fluid

c_{max} – Maximum concentration in plasma

D/S – Dose-solubility ratio

DB – Doxazosin base

DH – Doxazosin hydrochloride

DM – Doxazosin mesylate

DSC – Differential scanning calorimetry

DVS – Dynamic vapour sorption

FaSSGF – Fasted state simulated gastric fluid

FaSSGF-V2– Fasted state simulated gastric fluid, updated composition

FaSSIF – Fasted state simulated intestinal fluid

$FaSSIF_m$ – Fasted state simulated intestinal fluid containing maleic anhydride

FaSSIF-V2 – Fasted state simulated intestinal fluid, updated composition

FeSSGF – Fed state simulated gastric fluid

FeSSIF – Fed state simulated intestinal fluid

$FeSSIF_c$ – Fed state simulated intestinal fluid containing citrate buffer

FeSSIF-V2 – Fed state simulated intestinal fluid, updated composition

FTIR – Fourier transforms infrared spectroscopy

GI – Gastrointestinal

GMO – Glycerol-monooleate

^{1}H NMR – Nuclear magnetic resonance

HGF – Human gastric fluid

HPLC – High performance liquid chromatography

IMMC – Interdigestive migrating myoelectric complex

IS – Internal standard

IVIVC – *In vivo in vitro* correlations

K_{sp} – Solubility product constant

LC-MS or LC-MS/MS – Liquid chromatography coupled with mass spectrometry

LE or LLE – Liquid extraction or liquid-liquid extraction

LOD – Limit of detection

LOQ – Limit of quantification

MW – Molecular weight

pH – The negative logarithm of the hydrogen ion (H^+) concentration

PK – Pharmacokinetic

p*K*a – The negative logarithm of the acid dissociation constant, *K*a

PP – Protein precipitation

QC – Quality control

Rpm – Rotations per minute

SEM – Scanning electronic microscopy

SPE – Solid phase extraction

t_{max} – Time to reach maximum concentration

XRPD – X-ray powder diffraction

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CURRICULUM VITAE

Marijana Erceg (maiden name Peko)

Personal information: I was born on 8th March 1976 in Split. I am a citizen of Republic of Croatia. I have finished grammar and high school in Split. I live and work in Zagreb.

Education: I have started the study of pharmacy at the Faculty of Pharmacy and Biochemistry at the University of Zagreb in 1994. I have defended my graduation thesis under the name "Dissolution testing of diclofenac sodium from Voltaren tablets-development and validation of method" in 1999 and received a B.Pharm. degree. I have enrolled in the program of Postgraduation study of Pharmaceutical sciences in 2000 on the same Faculty.

Working experience: I have been working in Pliva Croatia Ltd. since November 1999, first as an analyst and later on as a project group leader in Analytics of pharmaceutical products, Research and Development of generic products.

Scientific interests: my scientific interests are directed towards development and improvement of methods for determination of solubility and dissolution testing, in order to increase their biorelevance and predictive power so that they can be used more successfully in forecasting absorption processes *in vivo*.

Scientific papers:

1. Erceg, M., Vertzoni, M., Cerić, H., Dumić, M., Cetina-Čižmek, B. and Reppas C. *In vitro* vs. canine data for assessing early exposure of doxazosin base and its mesylate salt". *Eur J Pharm Biopharm*, in press
2. Erceg, M., Cindrić, M., Pozaić-Frketić, L., Vertzoni, M., Cetina-Čizmek, B. and Reppas, C. (2010). A LC-MS/MS method for determination of low doxazosin concentrations in plasma after oral administration to dogs. *J Chromatogr Sci*, 48, 114–119.
3. Lusina, M., Cindrić, T., Tomaić, J., Peko, M., Pozaić, L. and Musulin, N. (2005). Stability study of losartan/hydrochlorothiazide tablets. *Int J Pharm*, 291(1–2), 127–137.

Participation on scientific meetings:

1. *In vitro vs. canine data for assessing early exposure of doxazosin base and its mesylate salt*, poster presentation, AAPS 2011 Annual Meeting and Exposition, October 2011, Washington DC.
2. *The influence of formaldehyde traces on impurity profile of mucolytic agent*, poster presentation, Fourth Croatian Congress of Pharmacy with International Participation, 2010, Opatija, Croatia.
3. *Dissolution behaviour of doxazosin base, mesylate salt and hydrochloride salt in biorelevant media,* poster presentation, Third Croatian Congress of Pharmacy with International Participation, 2005, Cavtat, Croatia.
4. *Razrada metode za ispitivanje brzine oslobađanja i validacija spektrofotometrijske metode za određivanje oslobođene količine aktivne supstancije iz dražeja,* poster presentation, Second Croatian Congress of Pharmacy with International Participation, 2001, Cavtat, Croatia.

Other presentations:

1. *Dissolution testing,* oral presentation, PLIVA Research and development, January 2004.
2. *Solubility - chemical vs. pharmaceutical point of view,* 4th PLIVA-R&D Days, December 2005.
3. *Development of generic drug - in vitro and clinical investigations,* oral presentation, Croatian Pharmaceutical Society, April 2006.

APPENDICES

Publication 1

Publication 2

BASIC DOCUMENTATION CARD

University of Zagreb
Faculty of Pharmacy and Biochemistry **Doctoral Thesis**

EFFECTS OF SALT FORMS ON THE ORAL ABSORPTION OF HIGHLY PERMEABLE WEAK BASE DOXAZOSIN

Marijana Erceg

Pliva Croatia Ltd., Generics Research and Development, Zagreb

Summary

In this study, the usefulness of biorelevant *in vitro* data and of canine data in forecasting early exposure after the administration of two phases of a BCS Class II compound, i.e., doxazosin base (DB) and its mesylate salt (DM) was evaluated. DB, DM, and doxazosin hydrochloride (DH) were prepared and extensively characterized.

The solubility of prepared substances was tested *in vitro* in various media, including human aspirates, using the shake flask method. Dissolution experiments were performed in simple buffer media and biorelevant media simulating gastric and intestinal fluids in the fasted and fed state. Pharmacokinetic (PK) studies were performed in dogs with DB and DM tablets in the fasting and fed state, while the results form human PK study on DM tablets in the fasted state were available from previous Pliva´s study. Analytical method for determination of doxazosin in canine plasma was developed and validated using canine samples collected in the fed state in order to assure the suitability of the method for measurement of low concentrations of doxazosin in plasma and high specificity of the method despite the number of interfering compounds in the fed state plasma samples.

Solubilities of DB and DM in human gastric fluid were forecasted by data in fasted state simulating gastric fluid containing physiological components (FaSSGF-V2) but not by data in $HCl_{pH\ 1.8}$. Unlike data in FaSSGF-V2, dissolution of DB and DM tablets in $HCl_{pH\ 1.6}$ is rapid. Dissolution of DB tablet in FaSSGF-V2 is incomplete and conversion to DH seems to occur. Differences between DB and DM in dissolution in the small intestine are overestimated in the absence of physiological solubilizers. Using the *in vitro* data and previously described modelling procedures, the cumulative doxazosin profile in plasma was simulated and the 0–2 h profile was used for evaluating early exposure. Individual cumulative doxazosin profiles in plasma, after single DM tablet administrations to 24 adults in fasting state, were constructed from corresponding actual plasma profiles. Compared with *in vitro* DM data in aqueous buffers, DM data in biorelevant media led to better prediction of early exposure. Based on intersubject variability in early exposure after DM administration and simulated profiles, the administered phase, DB or DM, does not have a significant impact on early exposure in fasting state. Early exposure in dogs (evaluated based on partial *AUC*s) was significantly higher after administration of DM to dogs. Therefore, dog is not a good model for predicting differences between DB or DM in the fasted state, but it may be a good model for predicting food effects and differences between DB and DM in the fed state.

Thesis is deposited in the library of the Faculty of Pharmacy and Biochemistry and University Library
(138 pages, 26 figures, 18 tables, 130 references, original in English language).

Key words: doxazosin base, doxazosin salts, absorption, early exposure, solubility, dissolution, dogs, humans

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Thesis accepted: 23 November, 2011

TEMELJNA DOKUMENTACIJSKA KARTICA

Sveučilište u Zagrebu
Farmaceutsko-biokemijski fakultet **Doktorski rad**

UTJECAJ RAZLIČITIH SOLI VISOKO PERMEABILNE SLABE BAZE DOKSAZOSINA NA ORALNU APSORPCIJU

Marijana Erceg

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S a ž e t a k

Glavni ciljevi ovog rada bili su istražiti da li postoje razlike u apsorpciji između lipofilne slabe baze doksazosina (DB) i njegove mesilatne soli (DM), procijeniti sposobnost predviđanja tih razlika kod ljudi temljem *in vitro* podataka, te *in vivo* podataka dobivenih farmakokinetskom studijom na psima. Obzirom da baze i ne-hidrokloridne soli mogu prelaziti u hidrokloridne soli u kiselom mediju želuca, pored DB i DM pripremljena je, karakterizirana i *in vitro* ispitana i hidrokloridna sol doksazosina (DH). Topljivost pripremljenih supstancija ispitana je *in vitro* u različitim medijima metodom zasićene otopine. Brzina oslobađanja ispitana je u jednostavnim vodenim puferima kao i biorelevantnim medijima koji simuliraju želučane i crijevne tekućine sa i bez hrane.

Farmakokinetičke studije na psima napravljene su sa DB i DM 2 mg tabletama, sa i bez hrane, dok su rezultati studije na zdravim dobrovoljcima nakon primjene 2 mg DM tableta bez hrane bile raspoložive iz prijašnje Plivine studije. Analitička metoda za određivanje doksazosina u psećoj plazmi razvijena je i validirana koristeći pseću plazmu prikupljenu u studiji s hranom, s obzirom da se u tom slučaju očekuju niže koncentracije lijeka te veći broj interferirajućih spojeva u plazmi koji bi mogli narušavati specifičnost bioanalitičke metode.

Rezultati *in vitro* ispitivanja pokazuju da je topljivost DB i DM u humanom želučanom soku moguće bolje predvidjeti koristeći biorelevantni medij (FaSSGF-V2) nego razrijeđenu kiselinu $HCl_{pH1.8}$. Također je pokazano da je brzina oslobađanja doksazosina iz DB i DM tableta puno brža u $HCl_{pH1.6}$ nego u FaSSGF-V2. Nepotpuno oslobađanje aktivne supstancije iz DB tableta u FaSSGF-V2 ukazuje na prelazak DB u DH. Rezultati također pokazuju da su u odsustvu fizioloških solubilizatora, razlike u brzini oslobađanja doksazosina iz DB i DM tableta u tankom crijevu precijenjene.

Moguće razlike u apsorpciji između DB i DM procijenjene su mjerenjem rane izloženosti, tj. brzine apsorpcije 0–2 h nakon primjene lijeka. Koristeći *in vitro* podatke, prosječnu bioraspoloživost nakon oralne primjene, enterohepatičku cirkulaciju doksazosina kod ljudi, te prethodno objavljene parametre *in silico* modeliranja, simuliran je kumulativni profil doksazosina u plazmi za DB i DM. Pojedinačni kumulativni profili doksazosina u plazmi nakon primjene jediničnih 2 mg doza DM tableta u 24 zdrava dobrovoljca, konstruirani su iz pripadajućih profila u plazmi. Rezultati pokazuju da u slučaju DM tableta, *in vitro* podaci u biorelevantnim medijima bolje predviđaju ranu izloženost nego podaci u jednostavnim puferima.

Uzevši u obzir varijabilnost u ranoj izloženosti među pojedincima nakon primjene DM tableta te simulirane profile za DB i DM, dolazi se do zaključka da vrsta administrirane supstancije (DB ili DM) nema značajnog utjecaja na ranu izloženost kod ljudi. U studiji s psima se pokazalo da je rana izloženost kod DM bila značajno viša nego kod DB što upućuje na zaključak da psi nisu prikladan model za procjenu razlika u brzini apsorpcije između DB i DM kod primjene lijeka bez hrane, no mogli bi biti koristan model za predviđanje utjecaja hrane kao i razlika između DB i DM nakon primjene lijeka s hranom.

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(138 stranica, 26 slika, 18 tablica, 130 literaturnih navoda, izvornik je na engleskom jeziku).

Ključne riječi: doksazosin baza, soli doksazosina, apsorpcija, topljivost, brzina oslobađanja, psi, ljudi

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