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Animal Models for Evaluation of Oral Delivery of Biopharmaceuticals

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Abstract

Biopharmaceuticals are increasingly important for patients and the pharmaceutical industry due to their ability to treat and, in some cases, even cure chronic and potentially life-threatening diseases. Most biopharmaceuticals are administered by injection, but intensive focus on development of systems for oral delivery of biopharmaceuticals may result in new treatment modalities to increase patient compliance and reduce product cost.

In the preclinical development phase, use of experimental animal models is essential for evaluation of new formulation designs. In general, limited oral bioavailability of biopharmaceuticals, of just a few percent, is expected, and therefore, the animal models and the experimental settings must be chosen with outmost care. More knowledge and focus on this topic is highly needed, despite experience from the numerous studies evaluating animal models for oral drug delivery of small molecule drugs. This review highlights and discusses pros and cons of the most currently used animal models and settings, and in addition also the influence of anesthetics and sampling methods for evaluation of drug delivery systems for oral delivery of biopharmaceuticals primarily with examples on insulin.

Keywords

Peptides, proteins, insulin, *in situ* perfusion, *in vivo,* macromolecules

Abbreviations: API, active pharmaceutical ingredient; BE, bioequivalence; CLSM, confocal laser scanning microscopy; DDS, drug delivery system; ELISA, enzyme-linked immunosorbent assay; EMA, European Medicines Agency; FDA, U.S. Food & Drug Administration; FITC, fluorescein isothiocyanate; GI, gastrointestinal; GLP1, glucagon-like peptide 1; HPLC, high-performance liquid chromatography; IV, intravenous; IVIVC, *in vitro in vivo* correlations; IVIVR, *in vivo in vitro* relationship; LC-MS, liquid chromatography–mass spectrometry; P_{app}, apparent permeability; P_{eff}, effective permeability; PET, positron-emissions-tomography; QSAR, quantitative structural activity relationship; SC, subcutaneous; SEM, standard error of the mean; SPECT/CT, single-photon emission computed tomography; TEM, transmission electron microscopy

1. Introduction

During the last decades, biopharmaceuticals (e.g. peptides and proteins) have become a growing part of the pharmaceutical industry, and the drugs of choice for treatment of numerous chronic and potentially life-threatening diseases e.g. cancer, inflammatory diseases and diabetes [1,2]. At the time being, subcutaneous or intravenous administration of biopharmaceuticals is still the most widely used route of administration. Currently, approximately 100 biopharmaceutical drug compounds are on the market worldwide, and seven of these are in top 10 of the most selling drugs [3–6]. It is estimated that approximately 270 peptides are currently tested in clinical trials and more than 500 are in preclinical development [5]; numbers providing good indications towards a rapidly growing market. Oral delivery of drugs is the preferred route of dosing due to ease of administration, high patient convenience and thus, compliance and relatively low costs [6,7]. Desmopressin, a synthetic analogue of vasopressin, serves as a positive example of a marketed oral peptide drug formulation, along with promising results for oral delivery of semaglutide, a GLP-1 analogue. But despite these successes, there are many obstacles to deliver biopharmaceuticals in general via the oral route. Among those obstacles are the large molecular size of the drug together with their low stability in biological fluids, mainly caused by enzymatic degradation and low pH in the gastrointestinal (GI) environment. Moreover, biopharmaceuticals are known to have a low permeation across the intestinal mucosa [1,3,5,8–10], resulting in a very low bioavailability after oral dosing [11]. Due to the limited bioavailability, selection of the correct animal model and experimental settings are key elements when evaluating oral delivery of biopharmaceuticals and the appurtenant drug delivery systems (DDS). Furthermore, all experimental variables need to be assessed, including how they can potentially affect the readout of the experiment. A recent review by Sjögren *et al.* [12] addresses the importance of anatomy and physiology variability between various species when conducting animal studies. The aim of the present review is to give some guidelines when conducting animal studies, both *in vivo*, *in situ* and *ex vivo,* to assess the potential of oral DDS containing biopharmaceuticals. The models will be described and discussed including their respective advantages and disadvantages.

In the following, *ex vivo* is defined as studies, where the organs are placed in an external environment, whereas in *in situ* studies, the organ is studied as a whole in the living animal. Furthermore, *in vivo* studies are described, when investigating the biopharmaceutical in the whole living animal. In addition, *in vitro* models, refers to experiments with cells or excised tissue outside their normal biological environment, and these will only briefly be described. For a more detailed review on *in vitro* models, the reader is referred to recent reviews [12,13]. *In silico* modelling will also only be briefly touched upon, as this is excellently addressed in a recent review [14].

2. Drug delivery system designs for oral delivery of biopharmaceuticals

After almost 100 years of research within the area of oral delivery of biopharmaceuticals [10], more knowledge is still needed to succeed within this topic. As of today, the most promising attempts to succeed with oral delivery of biopharmaceuticals include a combination of enteric coating for delivery to the site of absorption. Moreover, addition of protease inhibitors and permeation enhancers to the DDS may enhance the absorption of the biopharmaceuticals through the intestinal membrane [10]. Novel approaches of utilizing e.g. microneedles in the GI tract may further facilitate the membrane transport [15]. These approaches optimally ensure delivery of an intact drug molecule at or into the surface of the intestinal membrane (the site of absorption), and the transport through the membrane. Delivery of intact and solubilized drug to the site of absorption is challenging due to varying pH in the GI tract, ranging from pH 1–

2 in fasted stomach to pH of 5.5–6.5 in the duodenum, and pH 5.5–7.0 in the large intestine [6]. In both the stomach and intestine, numerous digestive enzymes are present together with an intestinal flora, the microbiota, providing a very unstable environment for the biopharmaceuticals [16]. By utilizing an enteric-coated DDS for protection, it is possible to avoid degradation of the drug and have the biopharmaceutical to pass the stomach and reach the small intestine for absorption. Moreover, it is important to carefully consider the impact of the physicochemical properties, e.g. molecular weight, biophysical stability in the harsh GI environment, lipophilicity and ionization constant of the specific drug for the delivery potential. This needs to be assessed in relation to the biological barriers considering proteolysis in the stomach, variable pH values and poor permeation through the biological membranes, restricting the absorption from the GI tract. It is of course essential to ensure that the biological activity of the biopharmaceutical is maintained when developing an oral DDS [6,17]. The majority of ongoing research includes calcitonin and insulin as model drugs due to their frequent dosing and clinical importance thus, high economic impact [5]. In literature, a variety of *in vivo*, *in situ* and *ex vivo* models have been used involving various animal species, but also many different experimental settings have been utilized [12]. Table 1 and 2 provide an overview of the animal studies in literature with oral DDS for insulin (Table 1) and other biopharmaceuticals (Table 2).

3. Barriers to overcome for successful oral delivery

Apart from preventing degradation, a main obstacle for successful oral delivery of biopharmaceuticals is the limited permeation across the intestinal membrane (Figure 1). Thus, researchers aim to increase the permeation across the biological membrane by various means [3,8,10]. Often, the complexity and variability of the gut physiology and the influence that this may have on absorption is underestimated, when designing DDS to be absorbed from the small intestine. It is essential to include animal studies in the early development phase in order to integrate the dynamic processes happening simultaneously in the body, whereby the iterative design process towards an optimized DDS will have a greater chance of success [18]. Two major determinants for successful absorption from the GI tract are dissolution and permeation, and as biopharmaceuticals are generally freely soluble in aqueous medium with a logP value <0 dissolution will usually not be the rate-limiting step [19,20]. It can therefore be useful to assess the membrane permeability to the given biopharmaceutical *in vitro* before moving to animal models. Examples of *in vitro* permeability experiments include use of excised tissue, cultured cells, artificial membranes and isolated mucosal cells [18,19]. Following positive *in vitro* permeability results, it is essential to perform animal studies. When selecting an animal model, it is important to keep in mind the impact of the anatomical and the physiological differences and similarities between and within species. Even though, the morphology of the intestinal membrane may be seen as comparable in broad terms across species, drug transporter proteins, intestinal metabolizing enzymes, microorganisms, fluid volume and flow, and concentrations of intestinal secretions can differ from species to species, which is crucial to keep in mind [18]. Furthermore, pH values in the stomach and intestine may also differ from the animal in comparison to humans, and the total absorptive area of the intestine is different [12]. In addition, the physiology of the intestine will change with age, and will thus, be different in children and in the elderly population compared to middle-aged adults. This review does not go into depth with the differences in GI physiology, and how it will influence the permeability of intestinal mucosa to oral biopharmaceuticals.

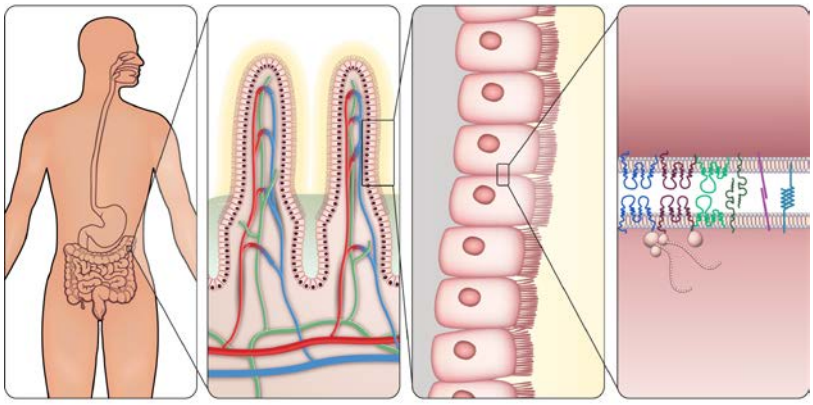

Figure 1: Graphic showing the *in vivo* barriers in the intestine following oral administration.

Table 1: Overview of studies evaluating oral delivery of insulin in animals

Administration route	Specie	Blood sampling	Quantification method	References
Colonic injection	Rats, diabetic	Portal vein	Blood glucose	[21]
Duodenal administration	Rats	Jugular vein	Blood glucose, ELISA and radioimmunoassay	[7,22–25]
Duodenal cannulation	Rats, diabetic	Carotid artery	Blood glucose	[26]
Duodenal cannulation	Rabbits	Carotid artery	Blood glucose	[27]
Ex vivo ileum	Rabbits	N.S.	P_{app} via HPLC	[28,29]
Ex vivo ileum	Sheep	N.S.	P_{app} via HPLC	[29]
Ex vivo jejunum	Sheep	N.S.	Histology test	[29]
Ex vivo jejunum, duodenum and ileum	Rats	N.S.	HPLC and CLSM	[30–32]
Ex vivo jejunum and colon	Rats	N.S.	Lactate dehydrogenase assay	[33]
Ex vivo permeation of colon	Rats, diabetic	N.S.	HPLC	[21]
In situ duodenal and ileal loop	Rabbits, diabetic	Jugular vein	Blood glucose	[34]
In situ ileal loop perfusion	Rats	Caudal vein	Blood glucose	[35]
In situ isolated intestinal loop	Rats, diabetic	N.S.	Histology of follicular mucosa (Peyer's patches) up to 4 h using fluorescence microscopy	[36]
In situ jejunum, ileum and colon	Rabbits	Mesenteric vein	Radioimmunoassay	[34]
In situ single pass perfusion	Rats	N.S.	HPLC	[37]
	Rats	Jugular vein	Blood glucose, ELISA, PET imaging	[28,38–41]
			PET imaging and ELISA	
Intestinal loop (injection)	Rats	N.S.	Fluorescence microscopy	[31,42,43]
Intraduodenal injection	Rats, diabetic	Tail vein	Blood glucose, enzyme immunoassay kit and blood glucose	[44–46]
Intraduodenal injection	Rats, diabetic	N.S.	Blood glucose	[47]
Intragastric injection	Rats	Tail vein	Blood glucose	[48–50]
Intragastric gavage	Rats, diabetic	Eye	Glucose oxidase and plasma glucose	[51]

Table 1. Continued.

Administration route	Specie	Blood sampling	Quantification method	References
Intragastric gavage	Rats, diabetic	Tail vein	Blood glucose and ELISA	[52–54]
Intragastric gavage	Rats, diabetic	Tail vein	Blood glucose and HPLC	[55]
Intragastric gavage	Rats, diabetic	Leg vein	Blood glucose and ELISA	[56]
Intragastric gavage	Rats, diabetic	Eye	Blood glucose and ELISA	[57]
Intragastric injection	Rats, diabetic	Tail vein	Blood glucose	[50]
Intragastric injection	Mice, diabetic	Tail vein	Blood glucose and ELISA	[58]
Intragastric placement	Pigs	Femoral vein	Blood glucose, ELISA and radiographs	[15]
Intraileal injection	Rats	Tail vein	Blood glucose	[59]
Intrajejunal administration	Rats	Tail or jugular vein	Blood glucose, ELISA and histology	[60]
Intrajejunal injection	Mice	Tail vein	ELISA	[61]
Intrajejunum injection	Pigs	Descending aorta	Blood glucose and ELISA	[62]
Oral administration (tablet, deep in the throat)	Mice, diabetic	Eye	Blood glucose	[63,64]
Oral administration (tablet, deep in the throat)	Rats	Tail vein	Blood glucose and ELISA	[65–69]
Oral administration (tablet)	Rats, diabetic	Tail vein	Blood glucose and ELISA	[70]
Oral gavage (capsules)	Rats, diabetic	Tail vein	Blood glucose and ELISA	[71–77]
Oral gavage (capsules)	Rats, diabetic	N.S.	Blood glucose	[78]
Oral gavage (capsules)	Rats	Tail vein	Blood glucose and ELISA	[30,59,79,80]
Oral gavage (capsules)	Rats, diabetic	Eye	Blood glucose, histology and mucoadhesion	[81]
Oral gavage (capsules)	Rabbits	N.S.	ELISA	[82]
Oral gavage of hydrogel	Rats, diabetic	N.S.	Blood glucose	[83]
Oral gavage of suspension	Mice, diabetic	Tail vein	Blood glucose	[84–86]
Oral gavage of suspension	Mice, diabetic	Eye	Blood glucose and ELISA	[87,88]

Table 1. Continued.

Administration route	Specie	Blood sampling	Quantification method	References
Oral gavage of suspension	Mice	Tail vein	Blood glucose and ELISA	[61,85,89]
Oral gavage of suspension	Rats, diabetic	Eye	Blood glucose, peroxidase, radioimmunoassay, ELISA and CLSM	[33,51,90–104]
Oral gavage of suspension	Rats, diabetic	Tail vein	Blood glucose, ELISA and SPECT/CT	[24,31,36,42,43,72,77,105–125]
Oral gavage of suspension	Rats, diabetic	Femoral artery	Blood glucose and ELISA	[126]
Oral gavage of suspension	Rats	Eye	Blood glucose and ELISA	[127]
Oral gavage of suspension	Rats	Tail vein	Blood glucose, ELISA, imaging and HPLC	[27,106,124,128–130]
Oral gavage of suspension	Rats, diabetic	N.S.	Fluorescence microscopy, CLSM, blood glucose and ELISA	[54,57,131,132]
Oral gavage of suspension	Rats	N.S.	CLSM	[111]
Oral gavage of suspension	Rats	Subclavian vein	Blood glucose and radioimmunoassay	[88,133]
Oral gavage of suspension	Dogs, diabetic	Jugular vein	Blood glucose	[134]
Oral gavage of suspension	Rabbits		Radioimmunoassay	[29]
Oral gavage of suspension	Rabbits, diabetic	Ear vein	Blood glucose	[135]
Oral gavage of suspension	Mice		Imaging via eXplore Optix system	[136]
Oral gavage of suspension	Mice, diabetic	Eye	Blood glucose	[136]

Table 2. Overview of studies evaluating oral delivery of biopharmaceuticals (except for insulin) in animals.

Biopharmaceutical	Administration route	Specie	Blood sampling	Quantification method	References
Antihypertensive peptide (Val-Leu-Pro-Val-Pro-Arg)	Oral gavage of suspension	Rats, hypertensive	N/A	Blood pressure by the tail cuff method	[137]
Antide	Oral administration (tablet, deep in the throat)	Rats	Tail vein	LC-MS of plasma	[138]
Buserelin	Intraduodenal injection	Rats	Carotid artery	Radioimmunoassay	[139]
Exendin-4	*In situ* perfusion	Rats	Heart puncture	Immunoassay kit	[35,140]
Exendin-4	*In situ* perfusion	Rats	N.S.	Fluorescence microscopy	[141]
Exendin-4	Intraintestinal injection	Mice, diabetic	Tail vein	Blood glucose	[141]
GLP1	Jejunal placement	Rats	Tail vein	Blood glucose	[49]
GLP1	Oral gavage of suspension	Mice	N.S.	Blood glucose	[142]
GLP1	Oral gavage of suspension	Mice, diabetic	Tail vein	Radioimmunoassay, intraperitoneal glucose tolerance test, blood glucose, near-infrared imaging and X-ray	[143,144]
GLP1	Oral gavage of suspension	Rats	Jugular vein, carotid artery and eye	ELISA	[143,145]
GLP1	Oral gavage of suspension	Rats, diabetic	Tail vein	Blood glucose, ELISA and pancreatic insulin after euthanisation	[146,147]
Granulocyte colony-stimulating factor	Oral gavage of suspension	Rats	Tail vein	ELISA	[148]
Heparin (conjugate)	Oral gavage of suspension	Mice	Heart puncture	Anti-factor assay kit	[149]
Leuprolide	*Ex vivo*, intestine	Rabbits	N.S.	Radioimmunoassay	[150]
Leuprolide	Intrajejunum, intraileum or intracolonic injection	Rats	Portal vein and aortic artery	Radioimmunoassay	[150]

Table 2. Continued

Biopharmaceutical	Administration route	Specie	Blood sampling	Quantification method	References
Leuprolide	Oral administration (tablet, deep in the throat)	Rats	Tail vein	LC-MS of plasma	[151]
Leuprolide	Oral gavage of suspension	Rats	Tail vein	LC-MS of plasma	[11]
Myrcludex B	Oral gavage of suspension	Rats	Sacrificed	Radioactive liver count	[152]
Protein Alpha crystallin	Oral gavage of suspension	Mice	Eye	ELISA	[153]
Salmon calcitonin	*Ex vivo*, intestine	Rats	Retroorbital	Fluorescence spectroscopy, ELISA and histology	[154]
Salmon calcitonin	*In situ* single pass perfusion	Dogs	Portal vein	Radioimmunoassay	[155]
Salmon calcitonin	Intraduodenal injection	Rats	Tail vein	ELISA	[156]
Salmon calcitonin	Intraduodenal injection	Rats	Eye	Colorimetric calcium by UV spectrophotometer	[26]
Salmon calcitonin	Intrajejunal injection	Rats	Tail vein	ELISA	[157]
Salmon calcitonin	Intrajejunal injection	Rats	Heart puncture	Colorimetric method	[158]
Salmon calcitonin	Intrajejunal injection	Rats	Jugular vein	ELISA	[159]
Salmon calcitonin	Oral administration (tablet, deep in the throat)	Rats	Tail vein	Chromogenic assay	[160]
Salmon calcitonin	Oral gavage (capsules)	Rats	Jugular vein	Photometry, radioimmunoassay and	[155,161]
Salmon calcitonin	Oral gavage (capsules)	Rats	Tail vein milking	ELISA	[157]
Salmon calcitonin	Oral gavage of suspension	Rats	Intestinal tissue	CLSM and fluorescence	[162,163]
Salmon calcitonin	Oral gavage of suspension	Rats	Jugular vein	Calcium assay	[163,164]
Salmon calcitonin	Oral gavage of suspension	Rats	Saphenous vein	Calcium assay	[165–167]
Salmon calcitonin	Oral gavage of suspension	Rats	Tail vein	Calcium assay, colorimetric method and ELISA	[168–172]

Abbreviations used in the tables: CLSM: Confocal laser scanning microscopy, ELISA: enzyme-linked immunosorbent assay, HPLC: High-performance liquid chromatography, LC-MS: Liquid chromatography–mass spectrometry, N/A: Not applicable, N.S.: Not stated, UV: ultra-violet.

4. *Ex vivo* and *in situ* models

Ex vivo models refer to experiments in live animals with the organs placed in external environments ensuring lowest possible change in native conditions. Similar to studies with *ex vivo* models, *in situ* models may also be used and has the advantage that the whole organ is studied intact in a living animal (Table 3). *Ex vivo* and *in situ* studies count for 14 and 11 % of the total number of conducted animal studies, for studies with insulin (Figure 2A) and other biopharmaceuticals, respectively (Figure 2B) (information from Table 1 and 2). In Figure 2, the *in situ* studies and intestinal administration have been divided into two columns, these can be similar investigations, but the intestinal administration refers to either injection or placement of the DDS in the intestine, whereas the *in situ* studies describes investigations utilizing a flow of medium through the intestinal segment(s). *In situ* perfusion of intestinal segments in the GI tract of rodent, typically rats or alternatively rabbits, are frequently used to study the permeation and absorption kinetics of drugs. Under those experimental settings, intestinal segments can be cannulated and the drug formulation in solution or suspension with or without DDS can be flushed through the isolated intestinal section. This procedure is referred to as the single-pass perfusion model, but as an alternative is the Doluisio approach, a closed-loop model, where the intestinal segment is filled with the solution or suspension throughout the entire experiment [173,174]. Both models have shown to provide intestinal membrane permeability values correlating closely to human data for small molecules [173]. The biggest advantage of the *in situ* methods compared to *in vitro* techniques is the presence of an intact blood and nerve supply in the live animals [18]. Rat and human jejunum effective permeability estimates of passively absorbed drugs in solution correlate highly for small molecules, and both can be used with precision to predict *in vivo* oral absorption of such drugs in man [175].

An advantage with *in situ* perfusion studies is that the whole intestine can be perfused or merely selected small segments, depending on which investigations are initiated. The predictability of the rat *in situ* perfusion model appears to be useful for the prediction of active uptake in humans, as rats have similar patterns of expression of the small intestinal membrane transporters as humans [176]. A recent study used *in situ* closed intestinal loops in rats to identify the region-dependent effect of potential absorption enhancers, penetratin and penetramax, indented for oral delivery of insulin [40]. The intestinal segments studied were duodenum, jejunum, ileum and colon, and test solutions were administered directly to the loop segments 30 min after surgery. The experiment concluded that ileum and colon appeared to be the most effective target sites for the tested permeation enhancers, as explained by the higher level of protease activity in the upper small intestine [40]. In the same study, it was shown that the maximal absorption detected depended on the enhancer used. Carrier peptides are used in some studies as intestinal absorption enhancers in combination with for example insulin, and for e.g. L-penetratin, the most pronounced effect was observed in the ileum, followed by jejunum, duodenum and colon. In contrast, D-penetratin resulted in the highest blood concentrations of insulin after dosing in the colon, and less after dosing in the duodenum, jejunum and then ileum, respectively [40]. Thus, due to such DDS dependent regional differences, it seems that no general recommendation is clear regarding which region to administer the formulation to. In general, knowledge of GI regional differences related to intestinal drug absorption and effect on the specific evaluated DDS is crucial when setting up an animal experiment. A recent review focused on the intestinal absorption pathways of insulin nanoparticles in animal models [177]. That review concluded that intestinal absorption of insulin-loaded nanoparticles is closely related to accumulation of the particles in Peyer's patches, primarily located in the distal ileum [178], whereas the pathway of delivery for DDS targeting enterocytes and/or tight junctions remains unclear [177].

Ex vivo models are also utilized to investigate membrane permeation of the biopharmaceutical and/or interaction of the DDS with the intestinal membrane. A subcategory of *ex vivo* models is *ex situ* models, where organisms are moved from their natural environment. Often used models in relation to studies on oral delivery of biopharmaceuticals are *ex situ* barrier models assessing transport of compounds across excised intestinal tissue. The use of Ussing chambers to predict oral absorption has previously been reviewed, and the reader is referred to those excellent reviews for more details on the experimental setup [18,179,180]. In the reviews by Sjögren *et al.* [12] and Lennernäs [179], it is highlighted that more knowledge is needed from such *ex vivo* studies especially regarding the regional intestinal effective permeation to form the basis of improved *in silico* models [179]. Since the publication of those reviews, a study has evaluated the permeation of fluorescein isothiocyanate (FITC)-labelled insulin *ex vivo* using fresh rat ileum mucosal tissue and compared the findings to *in vitro* data from Caco-2/HT-29-MTX-E12 cell co-cultures [181]. The study showed that the apparent membrane permeability (P_{app}) of insulin dosed in trimethyl chitosan nanoparticles was 1.34-fold higher compared to unmodified nanoparticles and 1.87-fold increased as compared to the use of micelles [181]. When comparing with *in vitro* data, the same trend was observed both with and without the presence of mucus (e.g. 1.10 vs. 1.16-fold increase with mucus and 1.14 vs. 1.23-fold increase without mucus). Last, the study evaluated the DDS in animal studies after oral administration to diabetic rats. The blood glucose depression as observed 3 h after administration was found to be decreased 1.28-fold when comparing trimethyl chitosan nanoparticles to unmodified nanoparticles, whereas the decrease was 1.62-fold when comparing trimethyl chitosan nanoparticles to micelles [181]. Thus, all three models showed the same ranking of the formulations despite more pronounced difference between the formulations in the *in vitro* experiment than in the *in vivo* study. How those data and thus models are related to efficacy studies in man is yet to be addressed.

L-valine-appended PLGA particles for oral delivery of insulin has been studied using an *ex vivo* everted intestine method and applied complimentary to oral gavage administration to diabetic rabbits [182,183]. The *ex vivo* data showed 48 % insulin transport across the intestine for PLGA particles compared to 91 % for L-valine-appended PLGA after 60 min. When tested in an animal model, the L-valine-appended PLGA showed a slightly sustained hypoglycemic response compared to the non-conjugated particles [183]. Those findings highlight the complexity of relating *ex vivo* data to *in vivo* findings. A more complex barrier must be overcome when administering a formulation orally compared to studying permeation across tissue *ex vivo*, resulting in a less pronounced difference between the DDS tested. Despite the advantage of using animal tissue with functional cells acting as a barrier for drug uptake, such experiments are time consuming to set up, but can be useful for screening and comparing DDS containing the same biopharmaceutical and beneficial to perform prior to *in vivo* studies [18].

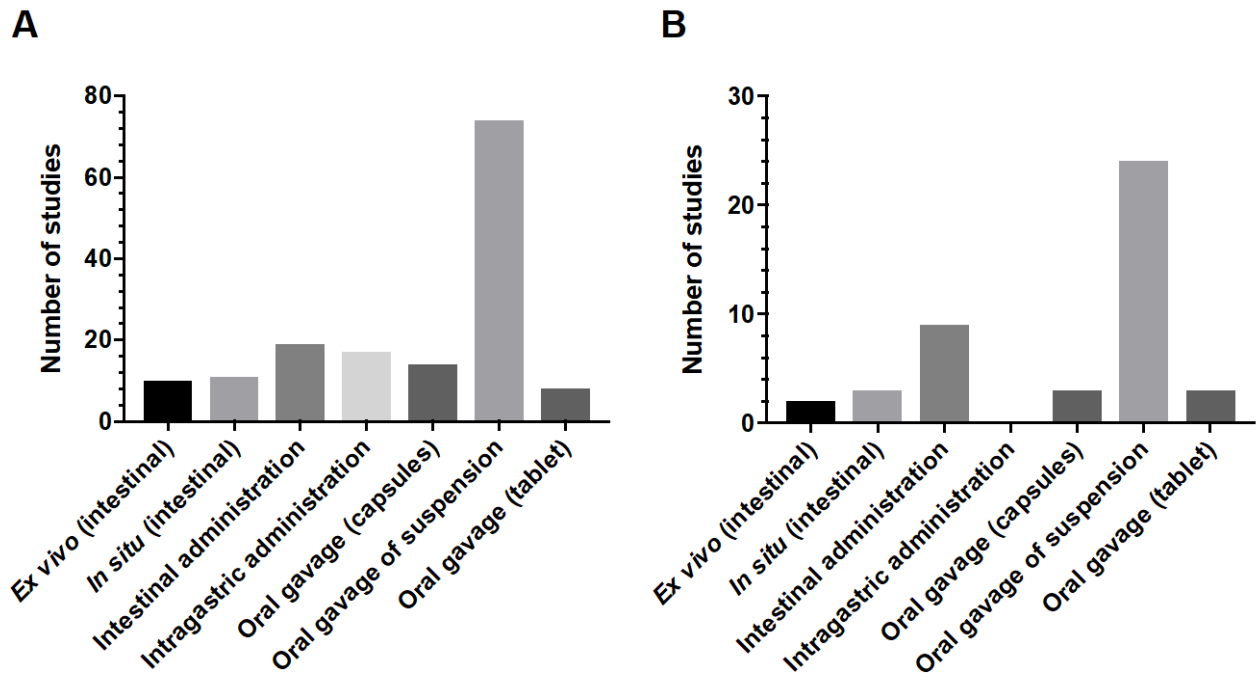

Figure 2: Overview of methods used to evaluate oral bioavailability of insulin (A) and other biopharmaceuticals (B) *in vivo*, *ex vivo* and *in situ* based on reviewed papers listed in Table 1 and 2.

Table 3: Overview of the pros and cons of the most used animal models for testing oral biopharmaceuticals

Model	Aim	Pros	Cons
Ex vivo	Permeation and absorption kinetics	Regional differences can be investigated	Organisms are taken out of the animal
In situ	Permeation and absorption kinetics	Regional differences can be investigated. Permeability data similar to human data	No data on passing through the stomach
In silico	Mechanistic or physiology-based pharmaceokinetic simulations	Does not include animals	Does not include in vivo solubility, stability and metabolism
In vivo, healthy animals	Oral PK, PD and bioavailability evaluation	Better animal welfare	Not conclusive to in regards to disease treatment
In vivo, diseased animals	Oral PK, PD, and bioavailability evaluation in diseased animals	Might be a more realistic scenario to the human situation	Large variations in the animal disease and translation of data to man

5. *In silico* models

In silico approaches refer to computer simulations, ranging from applying simple rules to advanced dynamic modelling [18]. Modelling of compound solubility and membrane permeability plays an increasingly

important role in drug discovery as they can be used as tools for early parameterization of mechanistic or physiology-based pharmacokinetic models or as starting points for refined models of a constrained series of chemical analogues [19,184]. Recently, *in silico* modelling has also been shown to be a useful tool to screen for new permeation enhancers and optimization of the physicochemical aspects of surfactant enhancer systems for oral delivery of proteins [185]. This study utilized a Random Forest Quantitative Structural Activity Relationship (QSAR) model, which was validated based on drug permeation data obtained from studies in Caco-2 cell culture models [185]. It was concluded that this approach serves as a robust strategy to systematically assess novel enhancers, but cannot, however, stand alone in the selection process. As for biopharmaceutical delivery, it is important to emphasize that the model as of today does not include aforementioned important parameters such as solubility, stability and metabolism [185]. A recent and very thorough review did, however, conclude that computational biopharmaceutical profiling is useful for early prediction of drug delivery strategies [14]. For more information on computational prediction, the reader is referred to this review [14].

Several commercial software for advanced *in silico* modelling are available, and three of the most commonly used, Simcyp 13.3, GastroPlus 8.0 and GI-Sim 4.1, were recently compared in relation to their capability to predict human intestinal drug absorption [186]. The study used *a priori* modelling with input data from 12 poorly water soluble drugs, all characterized by incomplete gastrointestinal absorption. It was concluded that the three types of software, all provide useful guidance in formulation development, with GI-Sim and GastroPlus favored over Simcyp due to better prediction of intestinal absorption of incompletely absorbed drugs [186]. Due to the black box nature of *in silico* software, it is generally recommended always to use several models to assess the same problem [12,187]. Moreover, it is very challenging to utilize for biopharmaceuticals due to the complicated degradation kinetics in lumen and during permeation. A highly important aspect to note is that accurate determinations of effective permeability (P_{eff}) is needed to serve as a basis for future *in silico* predictions of oral delivery of biopharmaceuticals [12]. Moreover, it should be emphasized that the current *in silico* models does not include the complex nature of the *in vivo* environments determining the dissolution behavior [188].

6. *In vivo* models

In vivo models comprise the use of living species and in these cases a biopharmaceutical or DDS (containing a biopharmaceutical) are dosed and the effect is tested after appropriate sampling and/or testing. The use of reproducible and reliable *in vivo* models is highly important and required for development and marketing of drugs for oral administration. Biopharmaceuticals are, due to previously described physicochemical properties, characterized by a poor absorption across intestinal epithelium resulting in a very low oral bioavailability, but results from *in vivo* studies highly can depend on the species used [11]. As previously mentioned, it is therefore crucial to utilize a highly sensitive and reproducible model, in order to be able to detect the relatively low changes in pharmacokinetic and pharmacodynamics parameters relating to increased oral bioavailability. Additionally, knowledge about how experimental conditions such as specie morphology, dosing method, anesthesia, sampling method, use of animal disease models resembling human diseases and finally choice of analytical method for sample evaluation is of great importance and will be discussed in the following sections.

6.1. Use of animal models with or without human disease symptoms and choice of specie

One of the first choices to take when conducting animal studies is which specie to choose, and as seen from Figure 3, rats are used in 80 % of the studies listed in Table 1 and 2. Mice represent another species often chosen, used in 11 % of the insulin studies and in 16 % of studies with other biopharmaceuticals. The physiological variations among species were recently reviewed [12], for which reason we will not go into detail with this topic, but rather focus on practical considerations when setting up an animal model. As rats are the most commonly used species in this context, it is important to know the basic differences compared to humans. The GI tract of a rat differs from that of man in several ways with the absence of gall bladder, higher nocturnal activity and different gut flora in the rat. In general, rats appear to provide good estimates for the prediction of absorption for compounds without dissolution problems such as biopharmaceuticals, and also highly reflect the human mucosal barrier in the intestine. Despite this, metabolic differences often lead to misleading predictions of oral bioavailability in humans [18,19].

Generally, when deciding on which animal model to apply, it is important to acknowledge that the bioavailability of biopharmaceuticals will be low even when avoiding the stomach and dosing directly to a specific part of the GI tract, due to enzymatic degradation and poor membrane permeability of large molecules. Bioavailability is, however, found to be slightly higher when drugs are administered directly to the jejunum as compared to other segments of the intestine [5]. One aspect is the low apparent bioavailability; another is the correlation to humans. A comprehensive study compared the absorption of a whole range of small molecule drugs after dosing to the intestine [176]. The study showed that almost no overall correlation exists between oral bioavailability in rat and human (r^2=0.29), whereas a correlation exists for intestinal permeability (r^2=0.8), both when considering carrier-mediated transport as well as passive diffusion mechanisms [176]. When evaluating the expression level of transporters in duodenum, a moderate correlation (r^2=0.56) exists between rat and human [176].

Another aspect to consider is whether to use animal models of human disease or healthy animals. Often the complexity and variability of gut physiology is underestimated, with only one or two variables being considered, this can either be in dosage form design or drug targeting approach [189]. Although strides have been made towards understanding the conditions and mechanisms responsible for absorption from a healthy gut, knowledge in this field is not yet complete. Even more significant is the lack of understanding the GI environment in the diseased state. Functionalized dosage forms cannot be evaluated in a reproducible manner without a comprehensive understanding of the conditions to which they are subjected during *in vivo* testing. Understanding and taking into account the intestinal environment will not only open up for improved evaluation of new dosage form designs, but also improve experimental settings for *in vitro* and pre-clinical tests in animal models leading to better *in vitro in vivo* correlations (IVIVC), and thus, opening new avenues for oral DDS for biopharmaceuticals [189].

When reviewing the existing literature (Figure 3), 63 % of the studies administering insulin (Table 1) have included use of animal models of human diseases, whereas this is only the case for 14 % of non-insulin biopharmaceuticals (Table 2). The overall purpose of insulin administration is to replace the partly or complete lack of insulin in diabetic patients to prevent hyperglycemia [190]. Therefore, animal models of human diseases, in this case diabetic animals, are commonly used in order to gain insight of the efficacy of the administered DDS, eventually combined with knowledge of the mechanistic behavior of DDS [191].

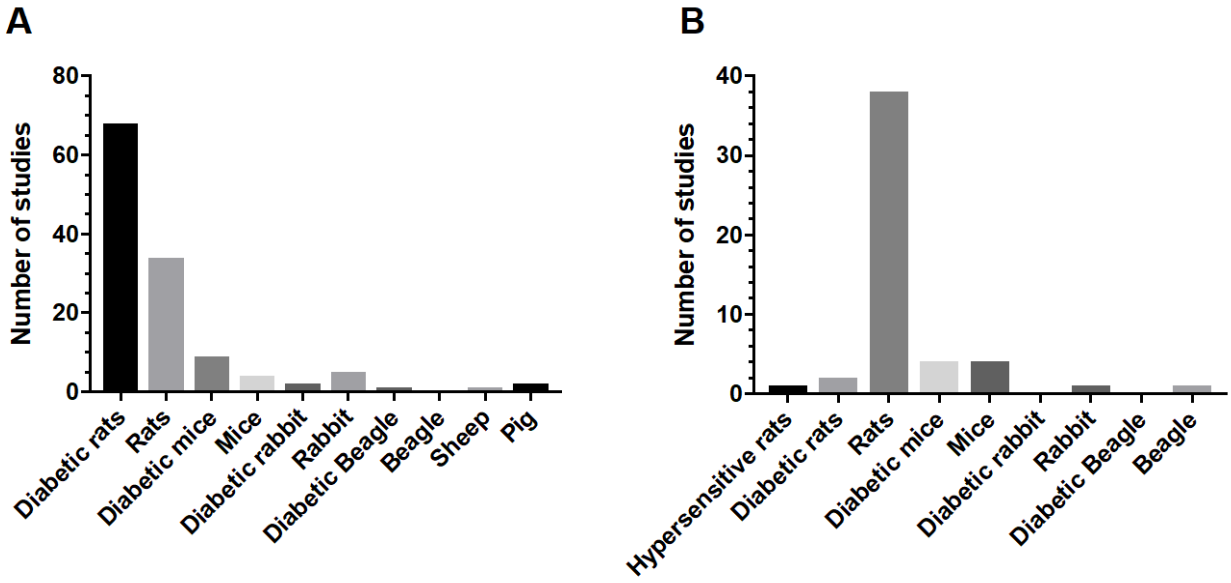

Figure 3: Overview of species used to evaluate oral bioavailability of insulin (A) and other biopharmaceuticals (B) *in vivo, in situ* or *ex vivo*. The data are based on reviewed papers, listed in Table 1 and 2.

Numerous diabetic animal models exist, ranging from type 1 diabetic with spontaneously developing autoimmune diabetes, chemical ablation of pancreatic β-cells to type 2 diabetic models, where both obese and non-obese animals are included. Moreover, transgenic and knockout mouse models are also used within diabetic research [190,192]. In the reviewed papers (Table 1), the most commonly used diabetic model is streptozotocin-induced diabetes in rats or mice, done by single intraperitoneal injection of 40-60 mg/kg streptozotocin to rats [77,124] or 65-150 mg/kg to mice [84,87] destroying the pancreatic β-cells [193]. The animals are considered diabetic once the plasma glucose level reaches ≥ 250 mg/dL for rats [77] and ≥ 300 mg/dL to 400 mg/dL for fasted (12 h) and fed mice [84,87]. Unfortunately, streptozotocin does not only harm the pancreatic β-cells [194], but also causes renal injury together with oxidative stress inflammation and endothelial dysfunction [195], which may influence the readout. Thus, as there are pros and cons associated with the various animal models and induction of human diseases in these, careful consideration should be taken to select animal model(s) representing the physiological diversity seen among human diabetic patients [191]. Animal disease models seldom copy all the aspects of the corresponding human disease, and are less characterized in the toxicology area compared to healthy animals. For securing this, several reviews suggests that more than one animal model of human disease should be included in the studies [190,192,196]. However, the exact same aspect of heterogeneity in diabetic expression and complications hereof considerably challenges data evaluation from animal studies, as it might be problematic to separate the drug-induced effect from disease-related complications [191]. Besides the always relevant discussion regarding the use of diseased animal models, it has been discussed that different species and strains behave differently both in relation to induction of diabetes and during treatment hereof [190]. In general, animal models cannot observe the differences seen between diabetic men and women when looking into for example cardiovascular complications [196]. Moreover, animals of different gender e.g. for diabetic rats, might also respond differently to experimentally induced stress and

other metabolic variations, thus leading to gender-biased results. This is not seen in the same way for humans, but can influence the results of the animal studies substantially [190,196].

No clear answer exists to the question of whether to use healthy or diseased animal models. Nonetheless, many caveats are associated with the use of animal models of human disease for assessment of oral DDS, when evaluating biopharmaceuticals with a known mode of action. Also, the animal welfare in terms of the complications associated with models of human diseases such as lack of histology control, diversity in disease expression leading to inclusion of more than one model of human disease, decreased life span and disease-related complications must be carefully considered [191,192].

In terms of species, healthy animals such as Sprague-Dawley rats, CD-1 mice, Beagle dogs, cynomolgus monkeys and mini pigs are the most commonly used models for evaluation of small molecule drugs due to good homogeneity [191]. For biopharmaceuticals, however, a more pronounced species specificity exists [191], as certain biopharmaceuticals are only active when administered to humans or chimpanzees and in other cases immunogenicity hampers full assessment in some species [197]. Such cases and alternative strategies to address such challenges have been thoroughly reviewed previously, for this reason the reader is referred here for further information [197].

6.2. Effect of anesthesia on the readout

Despite common knowledge in the scientific community of the fact that anesthesia is likely to affect the desired readout in animal models, not much literature exists addressing this aspect. When evaluating blood pressure, it is known that determination hereof is easier and with more accurate results when anaesthetizing the animals [198]. Contrary, anesthesia also introduces a significant variable, as it alters the blood pressure and cardiovascular reflexes among other physiological parameters [198].

It has been discussed from an animal welfare perspective and also from a scientific validity perspective within the area of musculoskeletal research that standard protocols for anesthesia and pain management should be developed and applied for animal models [199]. A study from 1983 shows that intraperitoneal injection of pentobarbital to healthy rats increases the blood glucose level by 33 % already 3 min after administration, and returns to normal level only after 40 min [200]. Figure 4 depicts the effect on blood glucose level following subcutaneous (SC) administration of insulin to healthy male Sprague Dawley rats anaesthetized using the most commonly used anesthetics for such studies. The experiments were conducted after 12 h.

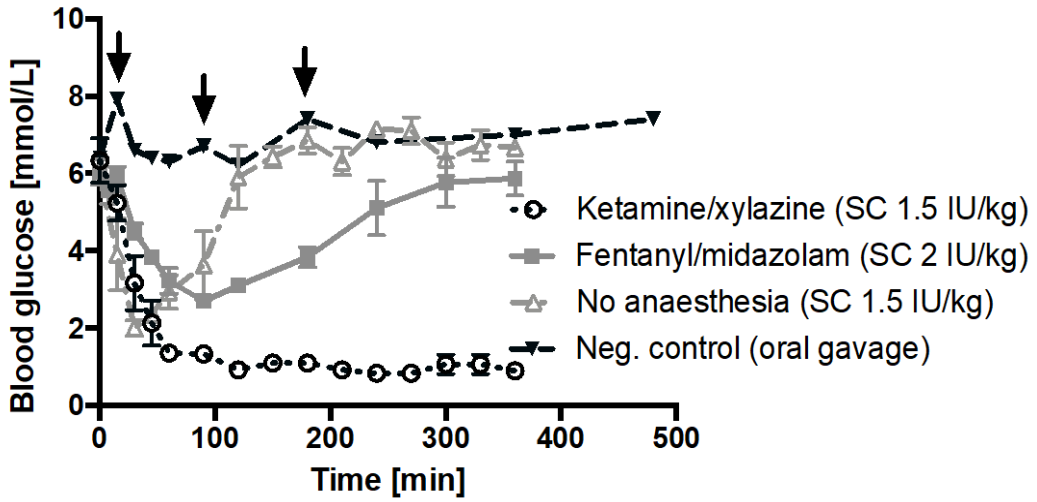

Figure 4: Effect of anesthesia on blood glucose level in healthy rats after subcutaneous (SC) dosing of insulin. The black arrows indicate momentary inhalation of isoflurane. The curves represent the average of three rats ± SEM, except for the negative control where n=1. Blood samples were collected via the sublingual tongue vein.

The data depicted in Figure 4 clearly shows that a combination of ketamine/xylazine significantly decreases the blood glucose level, which is highly problematic if evaluating the unbiased effect of orally administered insulin. Fentanyl/midazolam does not have the same pronounced effect, but still results in a different profile as compared to non-anesthetized animals. More precisely, the maximum effect on the blood glucose level following insulin administration is delayed 60 min in the anaesthetized rats when compared to non-anaesthetized rats, and the recovery period is likewise significantly prolonged. It could be speculated, however, that the reduced recovery period in the non-anesthetized animals when compared to the anaesthetized animals is not only related to the effect of anesthesia, but also the blood sampling procedure. Thus, blood collection via the sublingual tongue causes a stress-induced elevated blood glucose level. Having said that, the authors experienced no sign of stress during handling in terms of diarrhea, urine excretion, screaming, fear of handling upon repeated blood sampling etc., which was the case when repeating the experiment using a restrainer. Conclusively, the effect of anesthesia is the most plausible explanation for variation in blood glucose level.

In the negative control group, the rats were subjected to momentary inhalation of isoflurane (shown by black arrows in Figure 4), and this is shown to increase the blood glucose level, similar to the previously described effect of pentobarbital [200].

Summing up, unless the selected animal model requires rigid restraint or if it is unethical from an animal welfare perspective due to the burden applied to the animal in conscious state after e.g. surgery, it is favored to use conscious models to avoid the impact from anesthesia [198]. Having said that, the stress applied to animals during surgeries such as cannulation of the intestine affects the animal for up to four days after surgery, and therefore, a recovery period of one week is highly recommended before conducting the experiment.

6.3. Routes of administration and practical considerations

Choosing the optimal administration route to the animal models requires careful considerations in order to minimize the risk of potential adverse events [201]. Some of the aspects to be considered include the expertise or training required for successful administration, the volume or size of the dosage form needed for administration of a sufficient dose, the precise administration site, pH of the test sample and to which extent animal restraint is needed [201]. When evaluating the effect of orally administered DDS for delivery of biopharmaceuticals, the most frequently used dosing method is by far oral gavage (Table 1 and 2, Figure 3).

Oral gavage, mimicking the intended route of administration to humans, requires restraint of the animals and correspondingly moderate training of the research personnel [201]. It has been shown that such restrain induces increase in both blood pressure and heart rate for up to 1 h following the dosing with gavage together with an increased stress level for the animals [202]. This can, however, be significantly reduced if practicing the procedure with the animals in advance. For mice, the stress level is already normalized on the second day of training [202], whereas rats requires three training days to maintain normal heart rate and blood pressure during oral gavage [203]. Besides proper training, the stress level associated with oral gavage can be decreased by dipping the gavage device in sucrose before dosing [202]. This is, however, not recommended when evaluating compounds such as insulin and GLP-1, where blood glucose level can be the desired readout. Also, soft gavage tubes are favored over stainless steel, as it induces less stress to the animals. Although, a drawback of using soft tubes is the risk of the animals biting the tubes causing even more stress to the animals and potentially exclude the animal from the experiment [201]. Another important aspect to consider is the dosing volume, which is not recommended to exceed 5 mL/kg. Larger volumes are likely to induce passive reflux, aspiration pneumonia, irritation in or even rupture of the GI tract [201,204] together with gastric distension, as rodents are not able to vomit [201]. Last, the solution or suspension administered should have room temperature not to induce unnecessary stress to the animals.

Oral administration of tablets or capsules is an alternative to oral gavage of liquids. As seen from Table 1 and 2, tablets are administered by placement in the deep throat thus, activating the swallowing reflex of the animal. The capsules are dosed by utilizing a commercially available steel device for the dosing of the capsules to the stomach. For both tablets and capsules, the size hereof must be scaled to the animal to which it is administered [201]. Although, certain sizes are recommended, it has been shown that enteric-coated capsules of a commercially available size scaled to rats (7.18 mm in length) do not reach the intestine after dosing to rats, but remains in the stomach, where they dissolve [205]. Interestingly, if shortening the capsules to a length of 3.5 mm, they may be emptied from the stomach to the intestine. The study also concluded on a faster gastric emptying and transit of the capsule to the intestine in fed state animals as compared to animals in the fasted state [205]. The potential drawback of using the shortened capsules is a very limited loading capacity and also difficulty in handling the small capsules. Moreover, one should aim for achieving a homogeneous coating of the capsules (or tablets), and avoid scratches in the coating during handling and dosing, as this is likely to significantly impact the *in vivo* faith of the dosage form thus, induce sample variation. Also, powders can be administered via oral gavage, using a positive displacement pipetting device [206].

Compared to oral gavage, intragastric and intraintestinal administrations are more invasive procedures requiring surgical skills of the research personnel and also utilization of anesthesia. Nevertheless, when considering the previously mentioned correlation (section 6.1) between bioavailability in rat and human being r^2=0.29 and r^2=0.8 (after intragastric administration) [176], these methods are highly relevant to

consider. Many variations of this procedure exist, including whether the DDS is administered by injection to the absorption site or dosed via an inserted cannula. In addition, the DDS may also be administered to different regions of the intestine and then it is important to consider if the DDS is administered under anesthesia (which is always the case for injections to the GI tract) or after a recovery period in conscious cannulated animals. Regarding the effect of anesthesia, the reader is referred to the discussion in section 6.2.

For injections or *in situ* studies, the material of the potential cannulas should be carefully considered [207]. A recent review provides, a very useful overview of pros and cons of the available materials [207]. In brief, the most important aspects to consider are the biocompatibility, the cannula inner wall diameter (in relation to the DDS administered) and risk of bacterial adherence. Moreover, flexibility of the material and chemical and temperature resistance are also important as a soft material of the cannula is less of a burden for the animal compared to a less flexible material [207]. The parameters are more or less essential depending on the length of the study and if the animals are to recover from surgery for a longer time before the experiment can start, or are anesthetized during the whole study. When working with conscious models, it is important to perform the surgical procedure under as clean conditions as possible, and therefore, autoclaving the cannula can be important [207].

Summing up, there are pros and cons for both oral gavage, intragastric or intraintestinal administration. Oral gavage is less invasive and requires moderate training of research personnel, whereas intragastric and intraintestinal administrations are invasive and requires intensive surgical training. Also, taking the one-week recovery period into account, the throughput is lower for intragastric and intraintestinal administrations compared to oral gavage. A significant disadvantage of oral gavage is, however, the very limited correlation to man, whereas a good correlation exists for intragastric administration. This is an important aspect to consider, due to the very limited oral bioavailability of biopharmaceuticals.

6.4. Blood sampling methods

When evaluating DDS in animal models, the most common readout is a pharmacological effect or pharmacokinetic profiling, either by quantification of blood glucose after dosing biopharmaceuticals such as insulin and GLP-1 or by compound-specific assays such as enzyme-linked immunosorbent assays (ELISA). Thus, collection of blood samples is essential, and as for all aspects of animal studies, this also involves careful consideration of the advantages and drawbacks of the methods available in order to induce least possible stress to the animals. In Figure 5, the used methods for blood sampling can be observed (compiled from studies reported in Table 1 and 2).

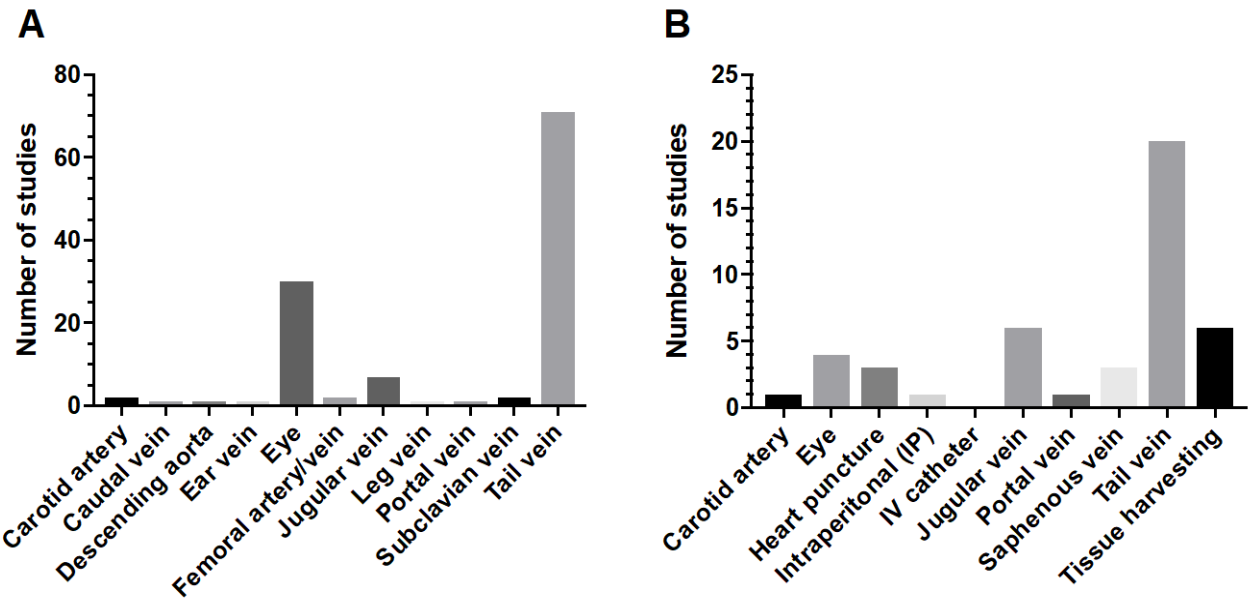

Figure 5: Overview of sampling methods used to evaluate oral bioavailability of insulin (A) and other biopharmaceuticals (B) following *in vivo, in situ* or *ex vivo* studies. The graphs are based on the reviewed papers listed in Table 1 and 2.

From Figure 5, it is clear that blood sampling from the tail vein is by far the most commonly used method in mice and rats. However, several methods exist to collect blood from the tail vein [201,208], and it can be performed on the animals either in conscious or anaesthetized state. One approach is to use a restrainer, where the animal enters with their head first and the tail is secured in place by a plug or stopper [207]. For minimizing applying stress to the animals, a red or dark tube is favorable [207], together with frequent washing to avoid cross infection and pheromonal deposition [208]. Once having fixated the rat, the blood can be collected either by vein puncturing using a lancet or needle, or by insertion of a temporary surgical cannula for repeated sample collection. Prior to the sampling, the tail can either be dipped into lukewarm water or placed under a heating lamp to ease access to the tail vein [208], and the blood is typically collected using a capillary tube. An alternative is milking of the tail, where a puncture on the vein is conducted, and the blood is milked out. Here, extreme care must be taken not to rub the tail too intensely, as this may result in leucocytosis and burns. Administration of local analgesic cream prior to sample collection can reduce the stress induced on the animals [208]. Alternative to a restrainer, a towel [207] or even the hands can be used to wrap the animals, keeping the tail free, but whereas the restrainer only requires one person, two persons are needed for these procedures.

Collection of blood from the eye is the second most used blood sampling method for assessment of orally administered biopharmaceuticals (Figure 5). The animals do need to be anaesthetized during blood sampling, and it is not recommended for repeated blood sampling as there is a potential damage of the eye, and in addition also much stress is induced to the animal [208].

For repeated blood sampling, insertion of a cannula should be considered in order to reduce the stress of the animal. According to Figure 5, the jugular vein or alternatively the carotid artery are commonly used in rats, although these methods require intensive surgical training of the research personnel. The surgery is conducted under full anesthesia, and blood samples can be collected in either the anaesthetized or

conscious state. During surgery, the jugular vein or carotid artery is localized, a small incision is made into the vein or artery and the cannula is carefully inserted and securely fastened. For studies with conscious animals, the cannula is tunneled under the skin to exit in the neck and a harness is employed [207,208]. The surgery must be done in a clean environment to avoid infections. The considerations regarding the choice of cannula are as described for intragastric and intraintestinal administrations in section 6.3. When collecting blood, the cannula is flushed with sterile saline added anticoagulant between sample collection, and it is highly important to minimize dilution of the blood by using the lowest possible volume of saline. Heparin and EDTA are the most commonly used anticoagulants, and it is of course important to consider a potential interference of the anticoagulant with the biopharmaceutical in the analytical assay.

Blood sampling from the oral cavity or the sublingual tongue vein is also a possibility. This is a fast and easy method, but there is a significant risk of contamination of these samples when the biopharmaceutical is dosed using oral gavage. Moreover, this method can only be conducted in conscious state, and requires restrain of the animals hence, risk of inducing unnecessary stress to the animals.

6.5. Analytical methods

An overview of the analytical methods used after drug administration is given in Figure 6. When evaluating insulin, blood glucose is the most common readout (Figure 6A). Besides, providing information of the pharmacodynamics regarding the effect of the administered biopharmaceutical, it is also a valuable tool to continuously monitor the animal burden while conducting the experiment, and thereby, preventing hypoglycemia in the animals. For testing other biopharmaceuticals than insulin, the preferred analytical method is compound-specific assays such as ELISA and radioimmunoassays providing pharmacokinetic data (Figure 6B), and these methods are often second choice when evaluating insulin-loaded DDS.

Supplementary to the aforementioned methods, microscopic and spectroscopic techniques can be used. Here, information regarding deposition and mechanistic behavior of the DDS can be gained. Those methods are usually conducted after euthanisation, and do therefore only provide information for specific time points. Consequently, if using these methods, more animals are used to assess the *in vivo* faith of a DDS over time. Alternative methods such as single photon emission computed tomography/computerized tomography (SPECT/CT) can be considered, and here the labeled DDS is administered via the chosen route of administration, and the *in vivo* faith of the administered sample is followed over time [209]. A significant drawback of this approach is, however, that it requires very expensive equipment and radiolabeling of the test compounds immediately prior to administration. However, the method allows for collection of images of whole animals, the distribution of the label can be quantified, and the method also allows for 3D imaging [209]. Fluorescence detection in animals is also possible, but can be difficult and also demands labeling of the DDS (or biopharmaceutical) with a fluorescence probe [210].

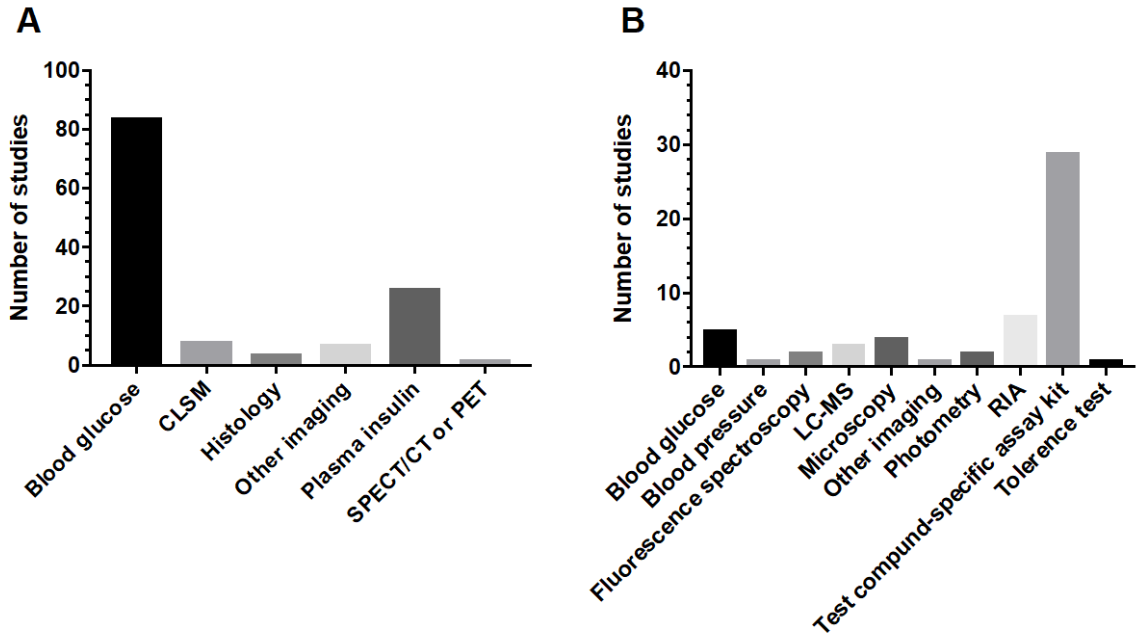

Figure 6: Overview of the analytical methods used to evaluate oral bioavailability of insulin (A) and other biopharmaceuticals (B) *in vivo, in situ* or *ex vivo*. This is based on the reviewed papers listed in Table 1 and 2.

7. Combining and correlating models

IVIVC (also referred to as *in vivo in vitro* relationship) is a major area of interest both for academia and industry, and is included in both the European Medicines Agency (EMA) and the Food & Drug Administration (FDA) guidelines [12]. A recent review by Sjögren *et al.* [12], thoroughly addresses IVIVC and its applications in relation to characterization of DDS, and it will be presented here in brief. IVIVC is mathematically derived as the predicted correlation between *in vitro* dissolution and/or cell models and *in vivo* exposure, yet the term is often used to link *in vitro* behavior to clinical prediction or results [12]. Knowledge about IVIVC is highly important, as it is used for understanding how, and to which extent, changes in the DDS or manufacturing process influence clinical safety and efficacy. Thus, it is a very important tool from an industrial and regulatory perspective, as it is also used as a quality control parameter after product launch [12].

8. Conclusions

Despite the increasing interest in oral delivery of biopharmaceuticals, crucial gaps still exist in relation to knowledge and development of animal models and suitable experimental settings for assessment of biopharmaceuticals dosed by the oral route. As of today, most knowledge of the assessment of oral drugs and the correlation between animal and human studies is based on small molecules. When evaluating orally administered biopharmaceuticals, it is even more crucial to keep in mind that the animal models will merely be models, and as the bioavailability is expected to be very low thorough considerations are essential for all the experimental details, in order to minimize experimental variability and risk of false readouts. This review provides an overview of some of the most important factors influencing the assessment of oral biopharmaceuticals. The review describes the available models and experimental setting used for testing biopharmaceuticals and serves to provide an overview of which animals and methods are

commonly used when testing oral delivery of biopharmaceuticals. Furthermore, it addresses considerations related to use of anesthesia and the effect this can have on the readout of the studies. Likewise, considerations related to blood sampling procedures and analytical methods are discussed in this review. It is impossible to generalize on which models and methods to utilize in specific studies, but this review presents the advantages and disadvantages of the various methods used so far, hence easing the test designs regarding animal models and methods for the evaluation of biopharmaceuticals to be administered by the oral route.

Conflicts of interest

None

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Captions

Figure 1: Graphic showing the *in vivo* barriers in the intestine following oral administration.

Figure 2: Overview of methods used to evaluate oral bioavailability of insulin (A) and other biopharmaceuticals (B) *in vivo*, *ex vivo* and *in situ* based on reviewed papers listed in Table 1 and 2.

Figure 3: Overview of species used to evaluate oral bioavailability of insulin (A) and other biopharmaceuticals (B) *in vivo, in situ* or *ex vivo*. The data are based on reviewed papers, listed in Table 1 and 2.

Figure 4: Effect of anesthesia on blood glucose level in healthy rats after subcutaneous (SC) dosing of insulin. The black arrows indicate momentary inhalation of isoflurane. The curves represent the average of three rats ± SEM, except for the negative control where n=1. Blood samples were collected via the sublingual tongue vein.

Figure 5: Overview of sampling methods used to evaluate oral bioavailability of insulin (A) and other biopharmaceuticals (B) following *in vivo, in situ* or *ex vivo* studies. The graphs are based on the reviewed papers listed in Table 1 and 2.

Figure 6: Overview of the analytical methods used to evaluate oral bioavailability of insulin (A) and other biopharmaceuticals (B) *in vivo, in situ* or *ex vivo*. This is based on the reviewed papers listed in Table 1 and 2.