Book chapter

Towards the development of delivery systems of bioactive compounds with eyes set on pharmacokinetics

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Abstract – Delivery systems carrying natural bioactive compounds for enhanced targeting and controlled release are capturing increasing attention. High loadings and sustained release are common design goals. However, in the case of compounds naturally present in human nutrition and physiology, further efforts are justified to optimize their bioactivity and promote clinical success. In this work, it is proposed a specific attention to the regulation of drug temporal presentation as important factor to obtain novel multifunctional delivery systems meeting higher therapeutic efficiencies. Case studies on the relation between drug release dynamics and biological responses are presented for some major delivery strategies and different bioactive molecules. Pharmacokinetic essential concepts and issues concerning the multi-target mode of action typical of the pharmacological properties of natural compounds are discussed in the perspective of improving the development of efficient drug formulations. Several classes of controlled release systems are considered through the chapter, and laboratory setups for testing films and particulate delivery systems are detailed, as well as the application of models for kinetic analysis. Descriptions are illustrated with experimental results obtained with caffeine and epicatechin in our laboratory. Future investigations will benefit from preclinical and clinical evaluation of the new formulations developed by emerging approaches and tools that are being suggested by diverse authors.

Keywords – Compartmental models; Controlled release; Dermal delivery; Drug absorption; Franz cells; Phytochemicals.

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1. INTRODUCTION

This chapter argues for a greater consideration of drug temporal presentation factors in the development of delivery systems for natural bioactive compounds. An overview of the therapeutic delivery of natural compounds and classification of release systems will be given in these first sections. Then, general concepts on the pharmacology of natural compounds and pharmacokinetic analysis will be systematized and discussed as we believe they may be critical for the development of effective formulations carrying those compounds. In section 5, some laboratory models useful to characterize the drug release and presentation properties of delivery systems will be presented.

Compounds produced by plants and microbes, such as the chemotherapeutics vinca alkaloids or doxorubicin, are a traditional resource for the discovery and development of drugs (Newman and Cragg, 2016). More recently, the incorporation of natural bioactive compounds in delivery systems and other biomaterials is gaining rising interest to achieve novel and safer therapies. For cancer treatment, vincristine, doxorubicin and paclitaxel (taxol) nanoformulations (e.g. Abraxane®) are already approved for clinical use, and numerous other systems are being investigated for delivering camptothecin (quinoline alkaloid) derivatives, the terpenoid celastrol, berberine (isoquinoline alkaloid) and different phenolic compounds with anticancer activity (Lagoa et al., 2020a).

Plant phenolics or polyphenols are a large and diverse group of compounds, including curcumin, resveratrol, vanilloids, and flavonoids such as tea catechins, quercetin and anthocyanins. These compounds show different chemopreventive, anti-inflammatory, cardiovascular and neuro-protective effects, among other biological activities, offering many possibilities for the development of novel drug formulations and delivery systems. Some representative examples of delivery systems carrying plant phenolics and other leading natural compounds are discussed below to give a panorama of the diversity of therapeutic applications at different development stages.

Liposomal systems are one of the most successful reaching the clinics, including anticancer products with doxorubicin (Doxil®), vincristine and the irinotecan camptothecin (Bobo et al., 2016; Lagoa et al., 2020a; Tiwari et al., 2012). Liposomal and phytosomal formulations of curcumin have also been trialed in cancer patients, while those of resveratrol and several other potential drugs are being studied in animal models (Kullberg et al., 2019; Lagoa et al., 2020a). Liposomes encapsulating amphotericin B with reduced side effects and morphine with prolonged release were approved for fungal infections and analgesia, respectively (Bobo et al., 2016; Tiwari et al., 2012). And, liposomes incorporated into ointment and gel bases can be used in dermal delivery (Tiwari et al., 2012).

Also approved for clinical use, a capsaicin dermal patch (Qutenza®) able to deliver a high concentration of the vanilloid directly to the skin is effective to relief peripheral neuropathic pain in patients (Simpson et al., 2017). Polysaccharide-based mucoadhesive patches or films are interesting carriers for buccal delivery of resveratrol (Ansari et al., 2018) and catechins (Miksusanti et al., 2020). Aiming other applications, alginate-chitosan scaffolds loaded with catechin were tested for the culture of chondrocytes exhibiting protective effects (Türk et al., 2013), while nanoparticles (NPs) of green tea polyphenols were prepared by conjugation to keratins and showed biological activity *in vitro* comparable to pure catechins (Yi et al., 2018).

NPs of poly(lactic-co-glycolic acid) (PLGA) with encapsulated quercetin were tested in a rat model of gastric inflammation and the results indicated a consistent protective action at the gastric mucosa (Chakraborty et al., 2012). PGLA NPs loaded with quercetin and other natural products increased the bioavailability and showed beneficial effects in streptozotocin-treated rats and in other

models for diabetes (Hussain et al., 2020). NPs modified with hyaluronic acid favored naringenin absorption in the mucosa of small intestine *ex vivo*, and colon targeting of alginate-chitosan microparticles loaded with icariin was reported in an animal model of ulcerative colitis (Lagoa et al., 2020a; Wang et al., 2016).

Icariin and the aglycone icaritin are also regarded for their osteogenic activity. Huang *et al.* (2018) tested a bone-targeted liposomal form of icaritin in an animal model of estrogen depletion-induced osteoporosis, whereas Reiter *et al.* (2019) encapsulated icariin in gelatin-coated bioactive glass scaffolds and obtained prolonged drug release profiles (L. Huang et al., 2018; Reiter et al., 2019).

Nanoemulsions are other interesting vehicle being tested to improve phytochemical delivery. Son et al., (2019) prepared a quercetin nanoemulsion with caprylic/capric triglyceride, Tween 80, alginate and soy lecithin in water, and reported hypocholesterolemic effects in rats fed a high-cholesterol diet (Son et al., 2019). Micelles and other nanocarriers of quercetin are also being considered for neurological diseases (Amanzadeh et al., 2019). Nanoemulsions loaded with quercetin and kaempferol increased the permeation of the flavonoids through intestine-mimicking membrane and porcine nasal mucosa, respectively, and both preparations improved absorption of the drugs *in vivo* (Colombo et al., 2018; Lagoa et al., 2020a). Exploring a different route, a black raspberry gel was developed and tested in humans by multiple applications in the tongue for 6 weeks, being able to modify the expression of several genes implicated in inflammation and cell death (Mallery et al., 2008).

Although various natural compounds reaching clinical evaluation show a good tolerability and partial beneficial responses, most of the formulations still did not ensure an acceptable efficacy. Looking at the clinically approved formulations, the successful bioactives tend to be the more target-specific and high-potency compounds, namely the above-mentioned alkaloids, doxorubicin or capsaicin. However, natural compounds very abundant in human diet, such as catechins and other flavonoids, don't have a precise or predominant therapeutic target. Instead, the biological activity of these compounds is the result of multi-target actions that can be highly dependent on pharmacokinetic (PK) factors. A critical attention to the PK provided by delivery systems opens new means to investigate the bioactivity of natural compounds and deploy their pharmacological potential in the development of effective drug formulations.

2. DRUG DELIVERY SYSTEMS

2.1. Release mechanisms and classes of delivery systems

Controlled drug delivery systems are conceived to release drug in a controlled manner over time. Usually the aim is to release the drug over long periods at a constant (zero-order) rate, confined to the drug's therapeutic window, although in some therapies a constant release is not desirable.

The mechanism of drug release can be used as a basis for classifying drug delivery systems. Robert Langer proposed a widely accepted classification for polymeric drug delivery systems, first in 1980 (Langer, 1980) and then a revised version in 1983 in collaboration with Nikolaos Peppas (Langer and Peppas, 1983). These publications established four general classes of delivery systems: diffusion-controlled, chemically-controlled, solvent-activated and magnetically-controlled delivery systems (Figure 1).

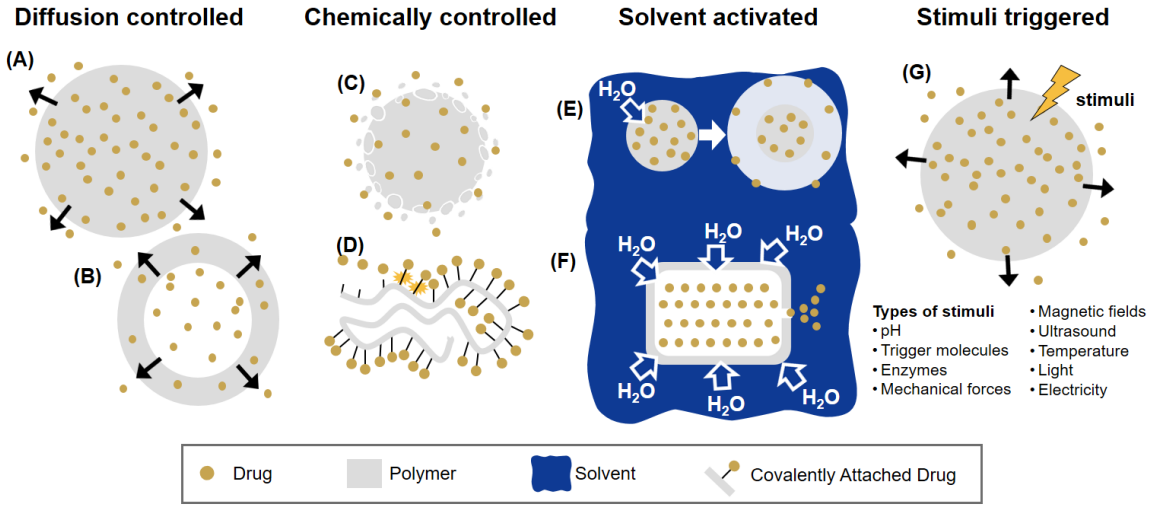

Figure 1. Categorization of drug delivery systems according to the release mechanism. Diffusion controlled systems can be (A) matrix or (B) reservoir based; in chemically controlled systems the drug can be released by erosion, more specifically (C) surface erosion, or from (D) pendant-chain systems; solvent activated systems release the drug by (E) swelling or by (F) osmotic pressure; and (G) stimuli triggered systems release the drug upon some external or environmental stimuli.

In diffusion-controlled delivery systems, the diffusion of drugs through the polymer matrix and/or porous channels limits the release rate. This type of delivery system may be a matrix system (Figure 1-A) where the drug is distributed in a polymer phase, or reservoir system (Figure 1-B) in which a polymer surrounds a drug core. Mathematical modeling of diffusion-controlled systems depends on the type of delivery system (reservoir or matrix), the pore size of the polymer phase (non-porous, microporous or macroporous), the drug solubility in the delivery system (dissolved or dispersed) and the geometric characteristics of the delivery system. The reader is referred to the aforementioned papers (Langer, 1980; Langer and Peppas, 1983), which provide additional explanation of these variables and guidance on appropriate mathematical modeling for each case.

In chemically-controlled delivery systems, hydrolysis or enzymatic cleavage or other *in vivo* chemical mechanisms control the drug release. Bioerodible systems consist of a polymeric carrier that is eroded over time allowing the drug to escape. When drugs of low diffusivity are used, the effects of erosion can outweigh diffusion, but in many cases both diffusion and erosion occur. Erosion can be heterogeneous (surface erosion), characteristic of hydrophobic polymers (Figure 1-C); or occur homogeneously in the volume of the polymer (bulk erosion), characteristic of hydrophilic materials that allow water intrusion, although both types of erosion can coexist. Surface erosion may be desirable for drug delivery, as the release rate is easily controlled over time and can be constant when there is a uniform distribution of the drug, a negligible diffusion and a surface area that remains practically constant as the polymer erodes, which is the case for thin slabs and hollow cylinders. Furthermore, bulk erosion can compromise the mechanical integrity of the delivery system, while surface erosion does not. Since bioerodible systems are absorbed *in vivo*, they do not

need to be surgically removed. However, the degradation products may be toxic, carcinogenic, or immunogenic, which is a potential disadvantage of this type of system. Siepmann and Göpferich (2001) offers explanation of mathematical models applied to erosion-based delivery systems (Siepmann and Göpferich, 2001).

The pendant chain type are also chemically-controlled delivery systems (Figure 1-D), in which the drug is connected to the polymer backbone and is released by hydrolytic or enzymatic cleavage of the drug-polymer linkage. The release rate of this system depends on the rate of cleavage (reaction) but also on the diffusion rates of the cleaving agent and of the drug through the polymer matrix. The release rate may depend entirely on the rate of cleavage if it is much slower than the molecular diffusion rates through the matrix.

In solvent-activated delivery systems, drug release is controlled by the permeation of a solvent into the polymeric delivery system, either by swelling or by osmosis. In swelling-controlled systems (Figure 1-E), the drug carrier consists of a hydrophilic glassy polymer that is solvent-free and/or compressed. Upon application, the environmental fluid enters the polymeric matrix causing it to swell and allowing the release of drug. An ideal swelling-controlled system only allows drug release in swelled regions of the polymer, so the release characteristics of the system are largely dependent on the velocity of the swelling interface (that separates swollen and unswollen regions of polymer), which in turn depends on the rate of diffusion of the solvent into the polymer.

Osmotically-controlled systems rely on osmotic pressure and various osmotic pump systems have been developed. The simplest of an osmotic pump (Figure 1-F) consists of a single reservoir with dispersed or dissolved drug encapsulated by a semi-permeable, rigid membrane, where the drug itself drives osmosis. Water enters through the semi-permeable membrane displacing the drug outward through an orifice. In this type of system using dispersed drug, zero-order release can be maintained while the drug remains saturated. This system can be further elaborated in order to achieve longer zero-order release using an additional compartment with an osmotic agent (other than the drug) that expands and pushes the adjacent drug compartment leading the drug outward. Osmosis can also drive release in matrix systems, where the solvent entering the polymeric matrix forms a network of porous channels, usually resulting from dissolution of drug particles and enabling the release of drug. The reader is referred to an extensive review (Herrlich et al., 2012) of various osmotic-controlled systems and mathematical modelling of these systems.

Magnetically-controlled delivery systems were first established as a separate class (Langer, 1980). However, it can be included in a larger class of stimuli triggered, or “intelligent” delivery systems (Figure 1-G). This type of delivery systems does not aim to achieve a constant release, but rather a pulsatile on-demand release that is required in some therapeutics, for instance the case of delivery of insulin and other hormones (Langer, 1990). This type of delivery systems releases drug or enhances the release rate, by accelerating diffusion or erosion, in response to external stimuli, such as magnetic fields, ultrasound, temperature, light or electricity, or to environmental stimuli such as pH, trigger molecules, enzymes, or mechanical forces such as shear force from blood (Langer, 1990; Zhang et al., 2016). Developing such delivery systems might require incorporation of magnetic particles in the case of magnetic triggered release, immobilization of an enzyme in the case of release triggered by a molecule, or other approaches that ensure an appropriate response to a given stimuli. Another advantage of these systems is that they allow localized release of drug.

2.2. From the release dynamics to the therapeutic performance

The essential rationale for designing a delivery system is to increase efficacy and/or reduce adverse effects of the drug. Operationally, the most common objectives are to develop a biocompatible system, able to increase the stability of the drug, release it at target tissues (targeted delivery) and at a controlled rate (Lagoa et al., 2020a; Tiwari et al., 2012). Slow, extended or/and sustained release of the drug is usually a desired goal (Reiter et al., 2019; Tiwari et al., 2012), although methods to define the optimal release rate for enhanced therapeutic or biological response are scarce (Table 1).

Drug release dynamics depends on the amount of drug loaded into the carrier matrix, on the drug solubilities in the matrix and in the release medium, and on properties of the matrix affecting drug diffusion kinetics (Langer and Peppas, 1983; Tiwari et al., 2012). The loading amount remains mostly an empirical factor in the development of drug-carrying systems, although experimental methods become available to anticipate at least the minimal effective loads (Silva et al., 2019), so the common preference goes to large loadings able to maintain high concentrations in blood or tissues.

However, there is evidence that temporal gradients of the bioactive molecule may be more effective than a constant concentration for inducing biological responses. Silva and Mooney (2010) found that temporal gradients of vascular endothelial growth factor (VEGF) delivered by polymeric systems, in particular a high early concentration followed by lower concentrations, induces *in vitro* angiogenic responses more potently than constant or gradually increasing VEGF levels. This finding was reinforced by *in vivo* investigation of the importance of total VEGF dose and delivery kinetics for therapeutic effects (Silva and Mooney, 2010). Furthermore on the importance of regulating the temporal presentation of bioactive molecules, Bai et al. (2018) developed a delivery system with distinct release kinetics for three growth factors and found their sequential delivery resulted in significant angiogenic differentiation of endothelial cells (Bai et al., 2018). Additional systems indicated in Table 1 showed the influence of temporal exposure of anticancer drugs (Hu et al., 2015), and how H_2O_2 toxicity to HeLa cells depends on concentration and concentration curves over time (Huang et al., 2016).

The key roles of concentration and time are recognized in pharmacology (and toxicology), but the systematic study of the interrelation between dose, pharmaco/toxicokinetics and pharmaco/toxicodynamics is a long standing challenge (Gabrielsson et al., 2019; Miller et al., 2000).

In this context, drug delivery systems are a precious tool to generate temporal (and spatial) gradients of drug presentation at the target tissues, and could be more explored to investigate how these factors determine therapeutic actions. Some advances have been obtained with conventional drugs that might be useful to extend in the investigation of natural compounds bioactivity. For example, efforts have been devoted to reproduce pulmonary drug delivery conditions *in vitro* and investigate the biological action of inhalation drug formulations (Rohrschneider et al., 2015; Zellnitz et al., 2019).

Pulmonary drug delivery presents advantages in the treatment of respiratory diseases and aerosolizable controlled release systems can improve patient's convenience and compliance (Tiwari et al., 2012). Inhalable nanocarriers loaded with phytochemicals as berberine and genistein are being developed for lung cancer (Kamel et al., 2020; Lagoa et al., 2020a). By inhalation, the local pulmonary deposition of the drug facilitates a targeted treatment of respiratory pathologies avoiding high dose exposures by other routes of administration, but drug formulation with adequate

aerosolization using non-toxic excipients is a challenge (Kamel et al., 2020; Tiwari et al., 2012). In an interesting study, Rohrschneider *et al.* (2015) improved a dissolution test of orally inhaled drug products in a modified Transwell system containing a surfactant, and was able to obtain dissolution rates in agreement with the absorption rates of inhaled drugs observed in human pharmacokinetics (Table 1).

Methods of different complexity have been used to study *in vitro* dissolution–permeation relationship in oral drug absorption, and Li *et al*. (2011) introduced a device simulating drug dissolution and absorption that can be applied to complete oral solid formulations (Li et al., 2011). The device includes a drug-dissolving vessel with a basket, stomach/intestine pH adjustment, and a side-by-side diffusion chamber with mounted rat jejunum to monitor permeation (Table 1). Using this tool, dissolution and permeation properties of active ingredients in Tangzhiqing tablet were studied and the *in vitro-in vivo* correlations investigated with human PK data (Li et al., 2018). More recently, microfluidic systems have been used to study intestinal and mucosal permeation. Permeation of caffeine and other compounds over mucus barriers was described and tested with porcine intestinal mucus, deserving further pharmacological validation (Elberskirch et al., 2019). Other barrier-on-chip system was fabricated for small intestine modelling with an epithelial cell layer responsive to capsaicinoids (Winkler et al., 2020). A farther elaborated system combining different organ chips fluidically linked (Table 1) was proposed to mimic absorption-metabolism-excretion-toxicity and predict PK/pharmacodynamics of drugs (Herland et al., 2020).

Exploring mucosal absorption, the nasal delivery has some particular appeals for brain-targeting drugs and immune-modulating products, due to the olfactory receptor neurons pathway nose-CNS, the relatively high permeability of the nasal epithelium, the low enzymatic activity and the presence of immunocompetent cells (Lagoa et al., 2020a; Tiwari et al., 2012). A recent work introduced a semi-mechanistic compartmental model to describe systemic and brain drug pharmacokinetics following intranasal delivery, and formulation-specific parameters revealed the differences in transport processes between aqueous solution and nanoemulsion dosage forms (Kadakia et al., 2019).

For topical/dermal delivery, modelling drug permeation through skin indicated that control of the disposition/concentration and bioactivity at target tissues can be used to optimize drug release for enhanced therapeutic benefits (Silva et al., 2017). Conditions of dermal and pulmonary applications, as well as presence of active drug metabolites/mixture, are specific cases where dose-response-time analysis were recommended (Gabrielsson et al., 2019). These analyses take in account the dose, route and rate data in the pharmacological model of response; explanation in Gabrielsson et al. (2019).

It is clear that more studies investigating how temporal gradients of natural compounds or small-molecule drugs influence therapeutic performance are warranted (Table 1). Most of the models and *in vitro* systems collected herein can be useful tools but were still not fully explored with drug delivery systems to characterize the release dynamics-therapeutic response link. Considering the pharmacological particularities of natural bioactive compounds discussed in the next section, comparable to growth factors referred above, it is possible that a careful regulation of the temporal presentation of these compounds is necessary to achieve robust or potent therapeutic effects.

Table 1. Models and studies addressing the relation between drug presentation or release dynamics and the induced biological responses.

Reference	Description
Miller *et al.* (2000)	Generalized Haber's rule as power law curves relating concentration and duration of exposure to a fixed level of response (endpoint). Modelling the three-dimensional surface relating concentration, time and response.
Silva and Mooney (2010)	Influence of the temporal changes in vascular endothelial growth factor concentration, the dose and the spatial distribution provided by delivery system on angiogenesis.
Hu *et al.* (2015)	Localized co-delivery system consisting of paclitaxel nanoparticles and lapatinib microparticles in a thermosensitive hydrogel, with a short-term release of paclitaxel and long-term release of lapatinib. Influence on *in vitro* and *in vivo* antitumor activity.
Rohrschneider *et al.* (2015)	Improvement of a Transwell-based method to reproduce descriptors of *in vivo* absorption (pharmacokinetics) of inhaled corticosteroids.
Huang *et al.* (2016)	Intracellular H_2O_2 generation and measuring system able to create kinetic curves of H_2O_2 with a variety of amplitudes and durations. Influence on cell death.
Silva *et al.* (2017)	Prediction of catechins' bioactivity gradients at skin strata for different superficial concentrations.
Li *et al.* (2018)	Application of a drug dissolution/absorption simulating system to complete oral formulations, and *in vitro-in vivo* correlation analysis with the actual absorption profiles in humans.
Gabrielsson *et al.* (2019)	Presents basic concepts of dose-response-time analysis and case studies.
Herland *et al.* (2020)	System integrating gut, liver and kidney chips, with an arterio-venous reservoir, predicts (oral) nicotine pharmacokinetic parameters observed in human studies. Bone marrow, liver, kidney chips and arterio-venous reservoir system recapitulates the pharmacokinetics and organ toxicity responses after intravenous injection of cisplatin in humans.

3. ISSUES ON THE PHARMACOLOGY OF NATURAL COMPOUNDS

3.1. Challenges in the therapeutic application

Natural bioactive compounds may present several properties that complicate their therapeutic use:

- low aqueous solubility;
- poor chemical stability;
- low gastrointestinal absorption;
- significant metabolization;
- rapid excretion;
- uneven tissue distribution;
- present in complex mixtures;
- multi-target mechanisms of action;
- low-potency actions.

The first of these points are well-known limitations of some natural compounds, and delivery systems are capable to stabilize active formulations, increase absorption and, therefore, favor their oral bioavailability (Kullberg et al., 2019; Lagoa et al., 2020a; Nasery et al., 2020; Peng et al., 2018).

Metabolization in the gut, liver and other tissues can also be problematic as it transforms the parent compound to derivatives with variable bioactivity capacities. It should be noted that some metabolites retain or show augmented pharmacological activities compared to the parent compounds (Basu-Modak et al., 2003; Boesch-Saadatmandi et al., 2011; Edwardson et al., 2015). Degradation and excretion decrease the residence time of the drug in the blood, so an important objective of delivery systems (e.g. PEGylated liposomes) has been to protect the bioactive compounds from metabolization and clearance to prolong their circulation time (Huang et al., 2018; Lagoa et al., 2020a).

Alike other drugs, natural compounds don't distribute equally through the body and might not accumulate enough in the diseased tissues/organs. Diverse targeted carriers are being explored, as well as local delivery strategies (e.g. peritumoral implants), to avoid metabolic conversion and to get drug release in the target region/cells while simultaneously lowering its systemic concentration.

In reality, the first points in the above list of pharmacological difficulties are not exclusive of natural compounds, but are common to many drugs. Nevertheless, natural compounds and especially those that are found in the habitual human diet may present these properties amplified together with additional particularities.

Natural bioactive compounds can be used in purified forms, identically to typical synthetic drugs, but also as extracts and complex preparations, being certain botanical "defined mixtures" recognized by regulatory agencies, for example solasodine glycosides for chemotherapy (Curaderm®) and pollen allergens for allergy immunotherapy by sublingual administration (Newman and Cragg, 2016). Other natural products or derived mixtures going through clinical evaluation are tea catechin extracts, silymarin, berries, grapes and curcuminoids (Lagoa et al., 2020b; Tatti et al., 2010), all these can be highly complex (multi component) mixtures. An improved tricomponent defined mixture containing specified amounts of isovanillin, harmine and curcumin is affording promising anticancer results (Booth et al., 2020).

The co-presence of multiple active compounds, derived from the original preparation or generated by the metabolism, adds extra factors to the prediction of the pharmacological effect and the rational design of delivery systems. Gabrielsson *et al.*, 2019 signaled the cases of unusual concentration-time profiles due to mixture kinetics, or co-presence of active drug metabolites, where a pharmacological analysis using biophase-driven response-time course may be more useful (Gabrielsson et al., 2019).

Lastly but possibly a critical issue to manage in the successful design of the delivery strategies is the multi-target, typically low-potency, modes of action of many natural compounds, discussed in the next section.

3.2. Multifactorial actions of natural compounds

The mode of action is not known for all the drugs available, but synthetic drugs are identified and improved for potent action on a biological target (molecular component or process) clearly linked to the pathological condition to treat. On the other hand, bioactive compounds like most phenolics, alkaloids and others naturally occurring in human environment, nutrition and metabolism don´t have a chief therapeutic target. Indeed, the pleiotropic actions are seen as a potential advantage of natural compounds, although it constitutes a hurdle to predict their therapeutic outcome.

These compounds can affect multiple targets, usually by way of low-potency actions, modulating different interconnected biochemical processes (Figure 2). These fragile but multifunctional interactions are possibly a result from an adaptation of human physiology to the frequent exposure to those compounds.

Natural bioactive compounds have been described to target innumerous cell components of prime relevance, including:

- membrane and nuclear receptors;
- membrane transporters;
- metabolic, carbohydrate and lipid modifying enzymes;
- RNA and DNA processing enzymes;
- protein processing enzymes;
- electron transport chain’s components, antioxidant and redox proteins;
- transcription factors;
- among others.

By modifying the function of different molecular targets, as illustrated in Figure 2, natural compounds can exert a consistent modulation of a range of biological processes at the levels of metabolic and cell fate regulation, gene expression, epigenetic modifications, redox homeostasis, kinase cascades and other cell signaling mechanisms. It is important to note that by targeting key regulatory elements or central nodes/hubs strongly interacting with multiple actors in regulatory networks, as microRNAs or antioxidant interactomes (Bendokas et al., 2019; Boesch-Saadatmandi et al., 2011; Kim et al., 2019; Lagoa et al., 2017), it is possible to put forward potent mechanisms of action. Likewise, it is conceivable that certain actions antagonize others, annulling or inhibiting any significant effect of the drug. Not being exhaustive describing the pharmacological actions of natural compounds in this text, some examples of references for supplementary reading are indicated:

Gutierrez-Merino *et al.* (2011), Bendokas *et al.* (2019), Kim *et al.* (2019), Rengasamy *et al.* (2019), Booth *et al.* (2020), Hussain *et al.* (2020), Lagoa *et al.* (2020b).

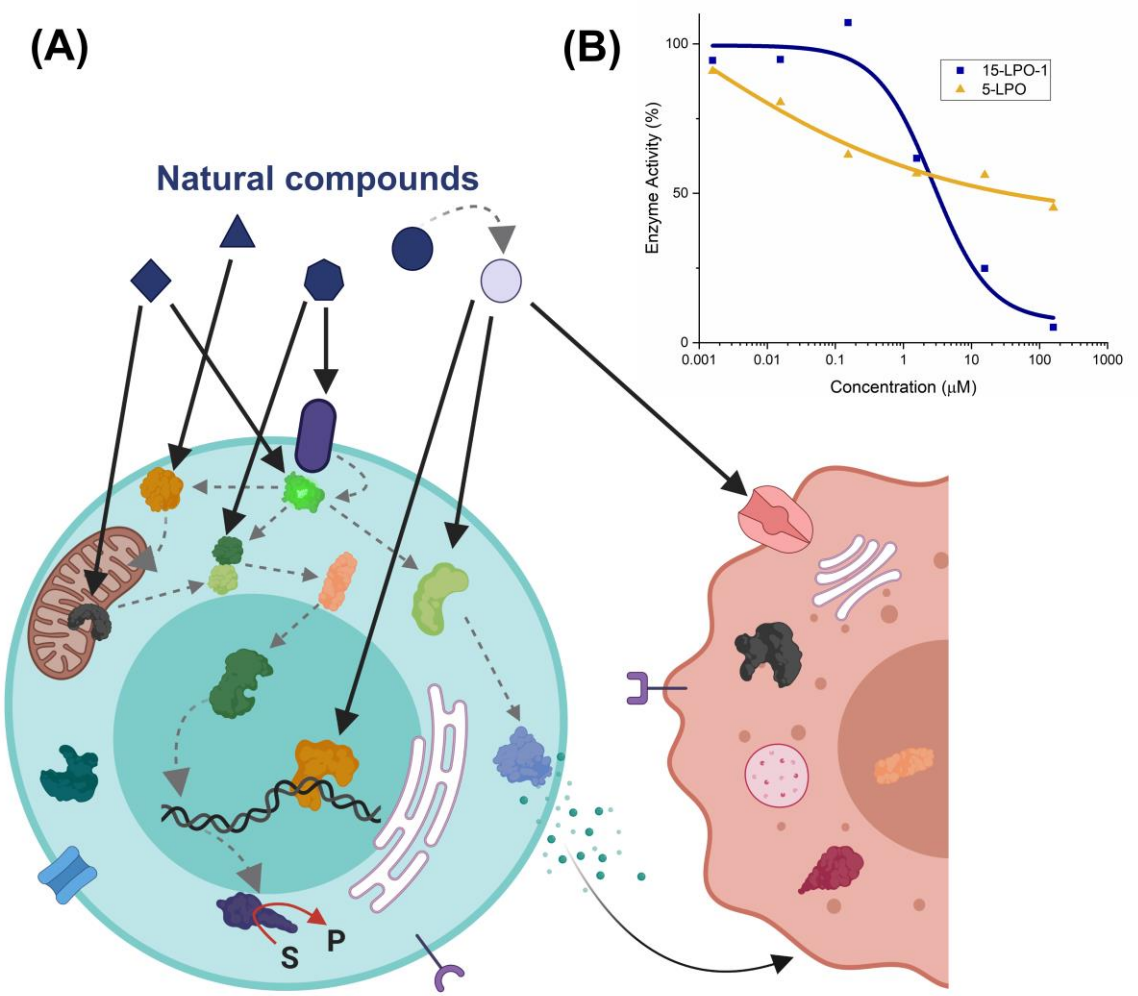

Figure 2. Multifunctional actions of natural compounds and mixtures. (A) Natural compounds, mixtures of natural compounds and/or metabolites can target multiple cell components in metabolic and signaling pathways. (B) Concentration dependences of the inhibition of 15-lipoxygenase-1 (15-LPO-1) and 5-lipoxygenase (5-LPO) by liguroside A, according to the work by Kawakami *et al.* (2019). The experimental data is modelled with a Hill curve to illustrate the different dose-effect relationships.

In addition to multi-targeting, the data available also indicates that the potency or dose-response relationships can vary significantly between targets of the same compound and between compounds affecting the same target. In a previous work, flavonoids were found to inhibit mitochondrial complex I with dissimilar potencies (R Lagoa et al., 2011). Figure 2-B illustrates how the same compound can affect similar enzymes with different potencies and dose-effect relationships (Kawakami et al., 2019). In many cases, dose-response relationships can follow the

general Hill equation, but different curves are found in the literature of natural bioactive compounds. For example for tea catechins, concatenated data for fibroblast protection and for melanoma inhibition revealed different types of response curves, hyperbolic and sigmoidal shapes, respectively (Silva et al., 2017). And comparing the concentration dependency, curcumin appears to have one order of magnitude lower potency against melanoma cells (Lagoa et al., 2020a). There are also hormetic (biphasic) dose responses and, in these cases, high doses pose the risk to mask/overwhelm the capacity to induce therapeutic effects (Calabrese et al., 2019).

Furthermore, since the effects of compounds on the different biological targets will also follow dissimilar binding-transduction kinetics (Danhof, 2016), it can be anticipated that the final outcome of treatments with natural bioactive compounds will depend on the dose, time schedule and cell/tissue's sensitivity at the time frame(s) of exposure to the drug. In this line, Zhou *et al.* (2020) recently described circadian pharmacological effects of berberine on chronic colitis, justified by diurnal rhythm of both the drug target (a clock protein) and disease severity (Zhou et al., 2020).

Facing time-dependent pharmacological effects of natural compounds, a thoughtful attention must be given to the time frame of administration and the temporal gradients/PK of the compounds, and therefore to the delivery strategy used in the interventions.

Often in the design of drug delivery systems, a zero-order release kinetics to provide a PK profile similar to zero-order is attractive (Tiwari et al., 2012). However, sustained blood levels of natural compounds are not observed in the nutritional settings that show beneficial effects. With compounds present in food/diet, the temporal variation of concentration in blood typically follows a bi- or tri-phasic curve with absorption, elimination and eventually distribution phenomena (model PK curves in Figure 3).

Recalling the studies with growth factors mentioned in the previous section (Bai et al., 2018; Silva and Mooney, 2010), constant concentrations of the bioactive molecule is not necessarily the optimal drug presentation pattern for therapeutic efficiency. Silva and Mooney (2010) showed that a VEGF profile consisting of initially high levels, followed by decreasing concentrations, noticeably increased cellular responses.

It is plausible that issues on the pharmacology of natural compounds discussed throughout this section critically affect the therapeutic efficiency of interventions. While the understanding of the biological actions of natural compounds in humans is advancing, further consideration of their pharmacological particularities can contribute to the development of more successful delivery systems and treatments.

The PK of compounds after oral intake in common conditions, like in nutrition, resumes the effects of endogenous factors (e.g. compound absorption and metabolism) that determine the temporal presentation of the drug. We hypothesize that delivery systems affording drug release rates compatible with the PK of natural compounds observed in specific conditions will show higher therapeutic efficiency. Available and relevant conditions at the moment are the habitual nutrition or clinical trials where the natural products proved positive results. The application of this new approach in the development of delivery systems requires the analysis of existing PK data for the natural compound of interest.

4. PHARMACOKINETIC ANALYSIS

4.1. Essential concepts

Pharmacokinetics (PK) is defined by IUPAC as “the process of the uptake of drugs by the body, the biotransformation they undergo, the distribution of the drugs and their metabolites in the tissues, and the elimination of the drugs and their metabolites from the body over a period of time” (IUPAC, 1997).

The evolution of substances in the body is often described in four phases (ADME):

- Absorption is the movement of the drug into the blood; the hydrophobicity of drug is a key determinant of drug absorption, if a drug is too hydrophilic it cannot cross the lipid cell membrane of gut wall cells, on the other hand if it is too lipophilic it will not be able to cross the extracellular aqueous medium. After gut wall absorption, the drug is delivered to the liver before entering the systemic circulation (Katzung, 2012).
- Distribution is the drug’s movement from one location of the body to another; usually distribution refers to the transfer from systemic circulation to lowly perfused tissues or to the drug’s effector site.
- Metabolism is the set of chemical transformations applied to the drug; it can occur in the gut wall and liver before absorption (the first pass effect) or after absorption mainly in the liver, although all cells can potentially metabolize drugs.
- Excretion is the removal of drug and its metabolites from the body. The drug and its metabolites are eventually removed from the body, mainly through renal excretion, although it can occur through the lungs, milk, sweat, tears, skin, hair, or saliva.

Related with excretion and metabolism (elimination) is the concept of clearance, which is the volume of plasma that is cleared of drug per unit time. Half-time of elimination ($t_{1/2}$) is the time needed to clear half of the absorbed drug.

Another important concept in PK is that of bioavailability (F), which is the fraction of unchanged drug that is absorbed into the systemic circulation. It is determined by the ratio of the area under the curve (AUC) of a PK profile (blood concentration *vs*. time) of a given administration route and the AUC of intravenous administration of the same dose, so by definition, intravenous administration has a bioavailability of 100%. The bioavailability of orally administrated drugs varies greatly, from 5% to 100% depending on the drug; the greatest limiting factor of this route for conventional drugs is the first pass metabolism (Katzung, 2012). Transdermal delivery for instance may yield greater bioavailability (80% to 100%), but the absorption of the drug through the skin is slow and in some cases impossible (Katzung, 2012). AUC, maximum blood concentration (C_{max}) and maximum blood concentration time (t_{max}) are standard measurements in PK studies, depicted in Figure 3-A.

The volume of distribution (V_d) is the volume that a drug would have to occupy theoretically if it were to be homogeneously distributed in the body at blood concentration. It is estimated by the ratio of the amount of bioavailable drug in the body and the concentration of the drug in blood.

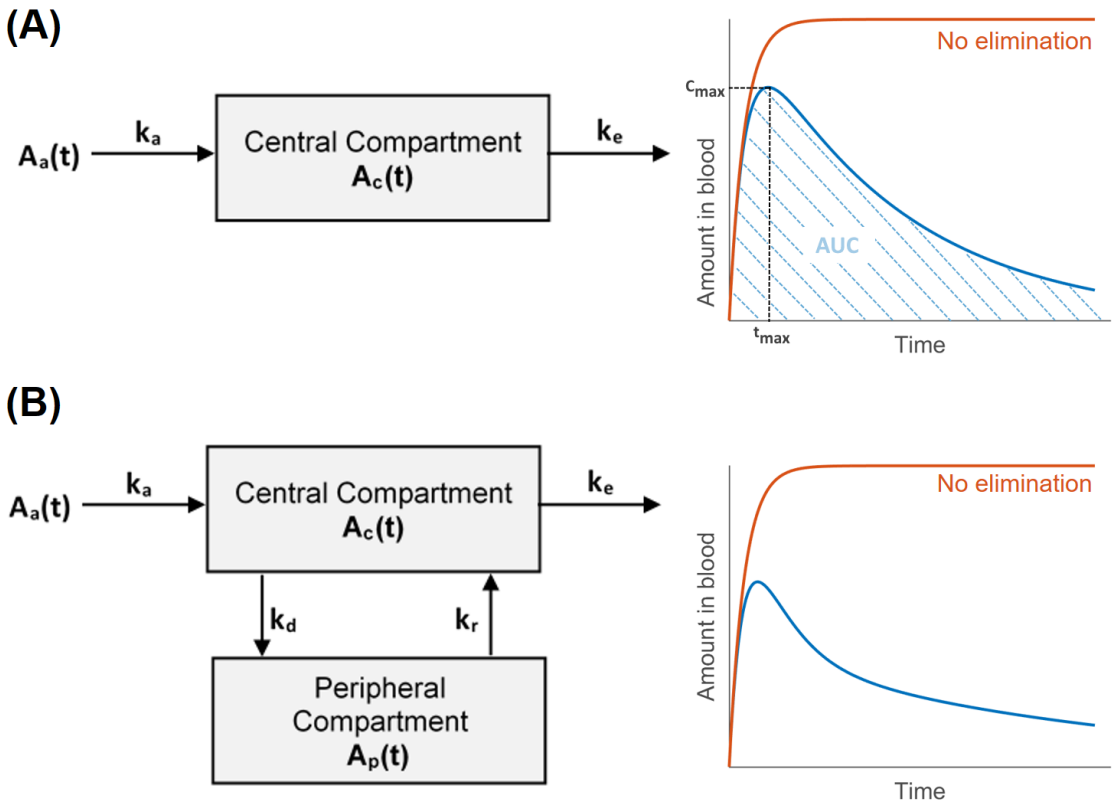

Figure 3. Schematic representation of 1-compartment (A) and 2-compartment (B) pharmacokinetic models. In the example plots, the orange lines represent the drug absorbed assuming no elimination or distribution occurs, while the blue lines represent the time evolution of drug assuming absorption and elimination (and distribution in B). Area under the curve (AUC), maximum blood concentration (C_{max}) and maximum blood concentration time (t_{max}) are illustrated in A. The transfer rate constants used for the exemplified 1-compartment model were k_a = 1 h^{-1} and k_e = 0.1 h^{-1}, and for the 2-compartment model were k_a = 1 h^{-1}, k_d = 0.2 h^{-1}, k_r = 0.001 h^{-1} and k_e = 0.1 h^{-1}.

4.2. Pharmacokinetic compartmental models

Compartmental models of PK assume the drug in the body moves between compartments with a determined rate. Such systems can have either a descriptive or a predictive use. Physiologically based pharmacokinetic models simulate the human body with each compartment representing a single or a small group of similar organs and have predictive value (Danhof, 2016). On the other hand, when PK data of the natural compound of interest is available, descriptive models can be used to obtain PK parameters.

Simple compartmental models, such as 1- and 2-compartment models, are widely used for the description of PK data (Figure 3). While the 1-compartment model considers that the drug instantly distributes through a single V_d, represented by the central compartment, the 2-compartment model

distinguishes tissues where the drug diffuses more slowly in a peripheral compartment. Usually in this model, the central compartment represents blood and highly perfused organs including the heart, lungs, liver and kidneys. The peripheral compartment might represent fat, muscle tissue and/or cerebrospinal fluid.

The kinetics of transfer (absorption, elimination, distribution and redistribution) are defined as first-order kinetics for most drugs, although in some cases Michaelis-Menten kinetics are applied for elimination (Dubois et al., 2011). The first-order transfer is characterized by an increase/decrease of a rate of the current drug amount in a compartment and, mathematically, the compartmental models are represented as a system of differential equations of which the solution is a sum of exponentials. For the 1-compartment model, the system that explains the transfer of drug through the compartments is defined as (Tomás, 2014):

$$\begin{cases} A_a'(t) = -k_a \cdot A_a(t) \\ A_c'(t) = k_a \cdot A_a(t) - k_e \cdot A_c(t) \\ C_c(t) = \frac{A_c(t)}{Vc} \\ A_a(0) = F \cdot Dose \\ A_c(0) = 0 \end{cases} \quad \text{(Eq. 1)}$$

, where A_c is the amount of drug in the central compartment, A_a is the amount of administered bioavailable drug, k_e is the elimination rate constant, k_a is the absorption rate, and F is the fraction of bioavailable drug. The concentration in the central compartment, C_c is obtained dividing the amount A_c by the volume of distribution in the central compartment, V_c. Following this model, C_c can be expressed as:

$$C_c(t) = \frac{F \cdot Dose}{V_c} \cdot \frac{k_a}{k_a - k_e} \cdot (e^{-k_e \cdot t} - e^{-k_a \cdot t}) \quad \text{(Eq. 2)}$$

For the 2-compartment model, the initial conditions are:

$$\begin{cases} A_a'(t) = -k_a \cdot A_a(t) \\ A_c'(t) = k_a \cdot A_a(t) - k_e \cdot A_c(t) - k_d \cdot A_c(t) + k_r \cdot A_p(t) \\ A_p'(t) = k_d \cdot A_c(t) - k_r \cdot A_p(t) \\ C_c(t) = \frac{A_c}{V_c} \\ A_a(0) = F \cdot Dose \\ A_c(0) = 0 \\ A_p(0) = 0 \end{cases} \quad \text{(Eq. 3)}$$

, where A_p is the amount of drug in the peripheral compartment, k_d and k_r are the distribution and redistribution rate constants respectively. The concentration in the central compartment is described by:

$$C_c(t) = \alpha_1 \cdot e^{-\beta_1 \cdot t} + \alpha_2 \cdot e^{-\beta_2 t} - (\alpha_1 + \alpha_2) \cdot e^{-k_a \cdot t} \quad \text{(Eq. 4)}$$

, where α_1, α_2, β_1 and β_2 are auxiliary terms defined as:

$$\begin{cases} \alpha_1 = \frac{Dose \cdot F}{V_c} \cdot \frac{k_a \cdot (k_r - \beta_1)}{(k_a - \beta_1)(\beta_1 - \beta_2)} \\ \alpha_2 = \frac{Dose \cdot F}{V_c} \cdot \frac{k_a \cdot (k_r - \beta_2)}{(k_a - \beta_2)(\beta_1 - \beta_2)} \\ \beta_1 = \frac{1}{2} \cdot (k_e + k_d + k_r + \sqrt{(k_e + k_d + k_r)^2 - 4k_r k_e}) \\ \beta_2 = \frac{1}{2} \cdot (k_e + k_d + k_r - \sqrt{(k_e + k_d + k_r)^2 - 4k_r k_e}) \end{cases} \quad \text{(Eq. 5)}$$

In 2-compartment models, the transfer from the central to the peripheral compartment can be quite rapid, leading to a lower C_{max} than that of a 1-compartment model (Figure 3). Usually the elimination in a 2-compartment model ends up being slower, especially if redistribution rate constant (k_r) is low because the drug needs to leave the peripheral compartment before being eliminated.

To the above equations for 1- and 2-compartment models, t can be replaced by (t - t_{lag}), with t_{lag} being an additional variable that accounts for an eventual delay in the start of drug absorption.

4.3. Application of pharmacokinetic models to a prototypical polyphenol

Epicatechin is a prototypical green tea polyphenol, catechin-type flavonoid and bioactive natural compound widely present in human diet. Epidemiological data and varied laboratory studies reinforce important health beneficial effects of epicatechin, which have been associated to multi-target mechanisms of action (Bondonno et al., 2019; Gutierrez-Merino et al., 2011; R Lagoa et al., 2017; Rengasamy et al., 2019). Unfortunately, most human trials of tea polyphenols formulations are returning only partially positive outcomes (Kessels et al., 2017; Lagoa et al., 2020b), so there is a great interest in developing more effective delivery systems for these compounds (Lagoa et al., 2020; Miksusanti et al., 2020; Silva et al., 2020; Türk et al., 2013; Yi et al., 2018).

The PK of epicatechin and other catechins has been studied previously in humans by Lee *et al.* (2002). In this study, 20 mg/kg of green tea solids dissolved in 200 mL of water was orally administered to 8 healthy individuals after overnight fasting, and the plasma concentration of catechins was followed for 24 h (Lee et al., 2002). The authors repeated the trial in 3 separate occasions with the same 8 individuals and each trial was identified as GT1, GT2 and GT3. Then, the PK data was analyzed by a 1-compartment model and the study reported the values for C_{max}, T_{max}, AUC and $t_{1/2}$ shown in Table 2. These are the descriptive parameters routinely reported in PK and bioavailability studies, but for guidance in the development of drug delivery systems, more mechanistically relevant parameters as the absorption rate (k_a) are needed.

To obtain additional PK parameters from the data by Lee *et al.* (2002), we collected the average PK profiles of plasma epicatechin concentration for each trial described in the original publication and carried out our own analysis using 1- and 2-compartment models. The two models were compared using the adjusted coefficient of determination (R^2_{adj}) and the 1-compartment model performed better in all situations (GT1, GT2 and GT3), so the 2-compartment model was discarded.

The values of the various PK parameters obtained using our 1-compartment model are similar to those obtained by Lee et al. (2002) (Table 2). The 1-compartment model was fitted to average PK curves, instead of the individual PK curves used by the authors in their analysis (curves not available), which justifies the small differences in parameter values between the two analysis. Despite this difference, all values we obtained are well within the standard deviation intervals presented by Lee *et al.* (2002). Using our compartmental analysis, the kinetic values of absorption (k_a) and elimination (k_e) were additionally obtained (Table 2). The average values for these

parameters from the 3 trials are 1.61 h^{-1} and 0.38 h^{-1}, for k_a and k_e rates, respectively, and can be useful to develop delivery systems with release rates in accordance.

Table 2. Pharmacokinetic parameters of epicatechin in humans obtained by fitting of 1-compartment model to experimental data in Lee *et al.* (2002). The parameters from the original publication are averages of fits for data from each individual with standard deviations in parenthesis.

	C_{max} (ng/mL)		**T_{max} (h)**		**AUC (ng*h/mL)**		**$t_{1/2}$ (h)**		**k_a (h^{-1})**	**k_e (h^{-1})**
	Lee *et al.* (2002)	Our analysis	Lee *et al.* (2002)	Our analysis	Lee *et al.* (2002)	Our analysis	Lee *et al.* (2002)	Our analysis	Our analysis	Our analysis
GT1	120.8 (67.6)	110.5	1.26 (0.41)	1.19	436.5 (157.5)	400.3	1.53 (0.73)	1.43	1.386	0.485
GT2	133.0 (67.6)	127.7	1.35 (0.34)	1.25	593.8 (286.9)	563.8	2.05 (1.01)	2.01	1.616	0.344
GT3	118.3 (73.7)	110.7	1.18 (0.56)	1.19	558.2 (288.8)	509.5	2.52 (1.95)	2.22	1.839	0.312

5. STUDY MODELS AND APPLICATION IN DERMAL DELIVERY

To develop drug delivery systems with suitable release kinetics, relevant study models are necessary for testing and optimization. Franz cells and Transwell® systems are setups that can be used to mimic different drug absorption conditions and enable *in vitro* testing of pharmacological activities under variable concentration-time profiles.

5.1. Studies in Franz cell

5.1.1. Drug permeation studies

A Franz cell is an apparatus that consists of two chambers (donor and receptor), between which a synthetic membrane or an *ex vivo* biological membrane (e.g. a dermatomed skin sample) can be fixed (Figure 4-A). Placing a liquid or emulsified test compound in the donor chamber allows the study of the permeation through the membrane by sampling and measuring the compound concentration of the receptor fluid over time. A small stir bar keeps the receptor media under constant magnetic agitation.

Franz cells are routinely employed to characterize skin permeability of compounds, but also to study drug-loaded films and other vehicles intended for mucosal (buccal, intranasal, intestinal) delivery (Ansari et al., 2018; Colombo et al., 2018; Lago, et al., 2020a; Miksusanti et al., 2020). In conditions of transdermal and transmucosal delivery, the kinetics of drug absorption will depend on the drug release properties of the delivery system and the permeability properties of the biological barrier (Silva et al., 2017; Zellnitz et al., 2019).

Some synthetic membranes have been used for mimicking skin permeability, namely Strat-M® and silicone membranes. Uchida *et al.* (2016) proposed a silicone membrane showing validity as model of human skin permeation for different compounds (Uchida et al., 2016). We have also been using this membrane and Figure 4-B shows permeation profiles for caffeine. From the experimental data, the permeability coefficient (*P*), the diffusion parameter (DL^{-2}) and partition parameter (*KL*) can be calculated according to Equation 6.

$$\begin{cases} Flux = P \cdot C_v \\ DL^{-2} = \frac{1}{6 \cdot T_{lag}} \\ KL = 6 \cdot T_{lag} \cdot P \end{cases} \quad \text{(Eq. 6)}$$

, where the *P* is calculated from the *Flux*, which is the slope of the linear phase of the permeation profile divided by the effective permeation area, and from the concentration of the donor solution (C_v). The T_{lag} can be obtained from the intersection of the linear trend line of the linear phase with the x-axis, and is used to calculate the diffusion (DL^{-2}) and partition parameter (*KL*).

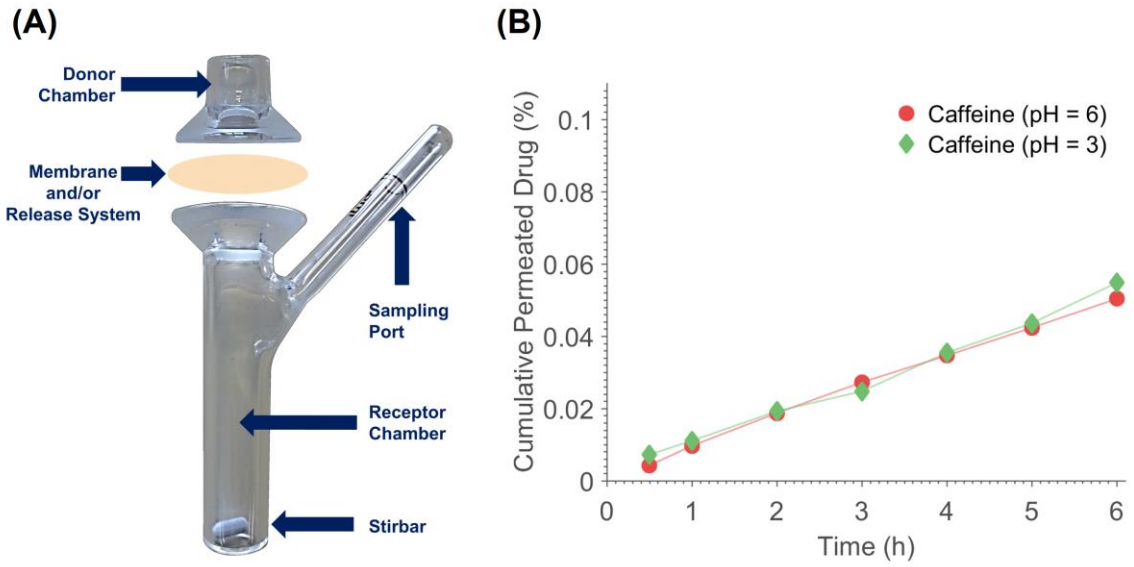

Figure 4. Franz cell setup for drug permeation and release studies. (A) Franz cell and its components; (B) Permeation of caffeine through a silicone membrane from Lintec Co., using donor saturated solutions in a Franz cell with effective permeation area of 0.636 cm^2 and a receptor volume of 5 mL saline (NaCl 0.9% w/v).

Caffeine could permeate the silicone membrane and the acidification of the saturated caffeine solution did not alter the permeation kinetics (Figure 4-B), suggesting that mild acid pH does not influence the permeability of the membrane. Caffeine is used as a model of positive drug in permeation devices (Elberskirch *et al.*, 2019). From the kinetic profile, the permeability parameters *P*, DL^{-2} and *KL* were calculated and are shown in Table 3. The obtained values were similar to the results by Uchida *et al.* (2016) and provide a good reproduction of caffeine permeation (*P*) through skin, encouraging the use of Franz cells with appropriate membranes as tool to investigate the transdermal administration and absorption kinetics of bioactive compounds. Ointments, gels and emulsion systems can be directly tested in this setup.

Table 3. Permeability parameters of caffeine through silicone membrane and skin. Values of permeability coefficient (P), diffusion parameter (DL^{-2}) and partition parameter (KL) are compared to the reported in the literature (Uchida et al. 2016). The literature values of *KL* and DL^{-2} were estimated as an interval from a graphical representation of the results. Swiss ADME is a free web tool for pharmacokinetic properties of compounds (Daina et al., 2017) using QSPR model to calculate skin permeation (Potts and Guy, 1992).

	P ($cm \cdot s^{-1}$)	**DL^{-2} (s^{-1})**	**KL (cm)**
Our work	1.8×10^{-7}	1.3×10^{-3}	$1.6 \cdot 10^{-4}$
Silicone membrane (Uchida et al., 2016)	2.2×10^{-7}	$[1 - 10] \times 10^{-3}$	1.0×10^{-4}
Human skin (Uchida et al., 2016)	1.8×10^{-7}	$[1 - 10] \times 10^{-5}$	1.1×10^{-2}
Swiss ADME	3.0×10^{-8}	-	-

5.1.2. Drug release studies

As indicated in the previous section, Franz cells can be valuable to study film-type or other drug release systems developed for dermal (skin patches) and mucosal delivery. For these applications, we choose alginate as support because this polysaccharide is well accepted for biomaterials and it allows the amenable encapsulation of bioactive compounds in different forms (Silva et al., 2020; Son et al., 2019; Türk et al., 2013). Moreover, alginate-based materials are already used in dermal applications and are proposed for colon-targeted delivery (Lagoa et al., 2020a; Wang et al., 2016). Using alginate-containing carriers, curcumin release was increased by acid pH in the case of chitosan/alginate NPs, and by alkaline pH in nanoemulsion-filled alginate beads (Nasery et al., 2020). A study with human volunteers showed that alginate-coated gelatin capsules can withstand the stomach environment and then migrate to the ileocecal region of the intestine where they disintegrate (Tiwari et al., 2012).

Following the hypothesis that a drug release system enabling kinetics of drug delivery similar to the rate of drug absorption (k_a) observed in PK studies will favor therapeutic efficiency, it is important to characterize the release kinetics of the systems under development. In the case of the polyphenol epicatechin, PK analysis after oral administration pointed to an average k_a value of 1.61 h^{-1} (section 4.3). It should be noticed that increased absorption promoted with catechin-rich oral

formulations, especially when taken under fasting conditions, may trigger adverse reactions (Oketch-Rabah et al., 2020).

Figure 5 displays kinetic curves of epicatechin release by alginate films measured in a Franz cell (as that represented in Figure 4-A). Results for alginate films with and without glycerol (plasticizer) are shown to illustrate how modifications in the delivery system can be used to tune release kinetics. Epicatechin was encapsulated into these films during gelation with calcium chloride, methods detailed in (Silva et al., 2020).

The experimental data of drug release in Franz cells can be analyzed using diverse modeling approaches, including kinetic models to compare with PK compartmental models. The kinetics of epicatechin release was fitted to a first-order model (Eq. 7) and the Weibull model (Eq. 8):

$$C = C_{\infty}(1 - e^{-kt}) \quad \text{(Eq. 7)}$$

$$C = C_{\infty}(1 - e^{-at^{b}}) \quad \text{(Eq. 8)}$$

, where *k* is the rate constant, and *a* and *b* are the Weibull constants. The first-order kinetics model, while not describing the physiochemical phenomena of release, allows a direct comparison with the transfer rates (absorption and elimination) of PK compartmental models (section 4).

As depicted in Figure 5, the first-order model fits quite well the epicatechin release data, and looking at the *a* and *b* values from the Weibull model (contains more independent variables) it converges approximately to a first-order kinetics. Remarkably, the release rate of epicatechin from alginate film (Figure 5-A) is similar to the rate of absorption of the polyphenol in humans (approximately 1.6 h^{-1}). Furthermore, the release rate of glycerol-supplemented film (Figure 5-B) is slower than of unsupplemented film, but afforded higher concentrations of epicatechin in the release medium probably because it accomplishes a higher encapsulation efficiency. In both cases,

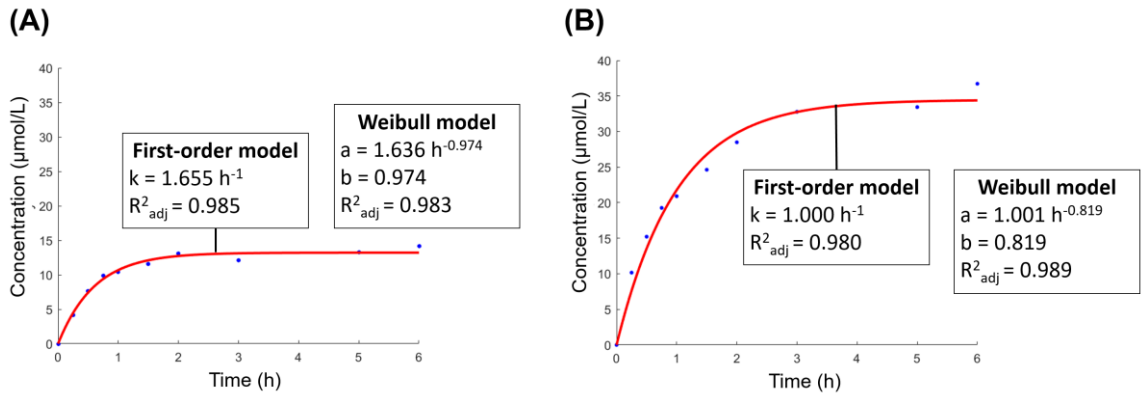

Figure 5. Release of epicatechin by calcium alginate (A) and glycerol (30% w/w)-supplemented calcium alginate (B) films in Franz cell (details in Figure 4 caption). Lines are the nonlinear regression fits of the experimental data to first-order models, and the parameters from Weibull model are also presented for comparison. In the release assays, alginate films were placed between the donor and receptor chambers, the receptor chamber was filled with 5 mL of saline ensuring contact with the film and 30 µL of the receptor liquid was sampled through the sampling port and replenished with saline at various time intervals. The polyphenol concentration in the samples was measured by HPLC.

these release systems afforded micromolar concentrations of the tea catechin (Figure 5), which were further confirmed in assays with *ex vivo* porcine skin (Figure 6). Envisaging a dermal application of such carriers, these micromolar levels of tea catechins at skin surface are compatible with simulations of high anti-melanoma bioactivity (Silva et al., 2017).

Different tools are emerging to simulate drug release and absorption *in vitro* (Li *et al*., 2011; Rohrschneider *et al*., 2015; Li *et al*., 2018; Elberskirch *et al.*, 2019). In alternative to the standard agitation flask assays for drug release studies, the Franz cell reveals a useful laboratory model to test and optimize drug release systems in conditions that approximate physiological delivery, i.e. the temporal profile of drug presentation. For bioactive products with the particularities discussed in section 3, it can be anticipated that the testing conditions become even more critical to successfully develop drug delivery systems with improved therapeutic efficiency.

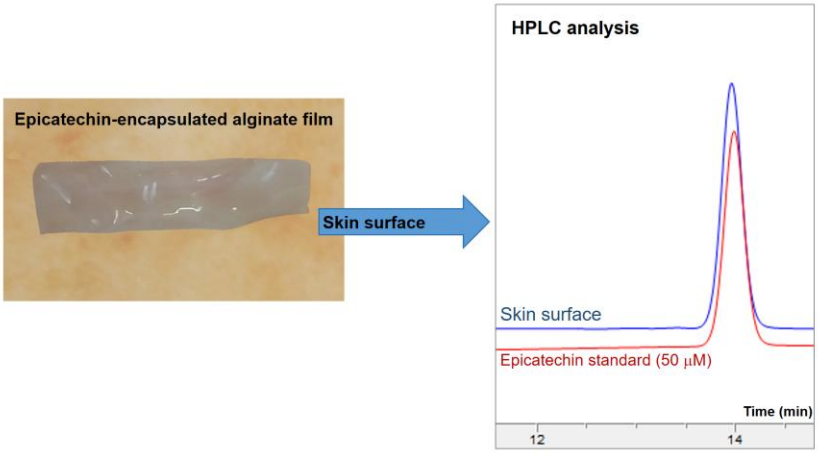

Figure 6. Assay of epicatechin delivery by calcium alginate film on *ex vivo* porcine skin. After 1 hour release, skin surface is sampled and analyzed by HPLC (absorbance signal at 278 nm). The chromatogram of an epicatechin standard with 50 μM concentration is shown for comparison.

5.2. Studies in Transwell system

Transwell® is an innovative but relatively easy-to-use setup that allows researchers to add complexity in a cell culture system when compared to normal cell culture methods. Some examples using Transwell culture systems include co-culture models (Wielgat et al., 2019), 3D cell printing (Kim et al., 2017) and *in vitro* invasion assays (Marshall, 2011). Interestingly, additional applications include the isolation of cancer stem cell (Wang et al., 2014) and permeability studies of blood-brain barrier models in the presence of different compounds (Veronika et al., 2020).

When used for drug delivery assays, the Transwell system can be compared to the Franz cell with a donor and a receptor chamber (or compartment) separated by a membrane (Figure 7). The definition of donor and receptor (or acceptor) chamber will always depend on which compartment the drug will be initially released as the drug diffusion can be studied in both directions, i.e. from upper to lower compartment or vice-versa depending on the objective of the study. Transwell systems are available with different membrane's pore diameter that, combined with other modifications, can be explored to achieve a desired *in vitro* drug behavior (Rohrschneider et al.,

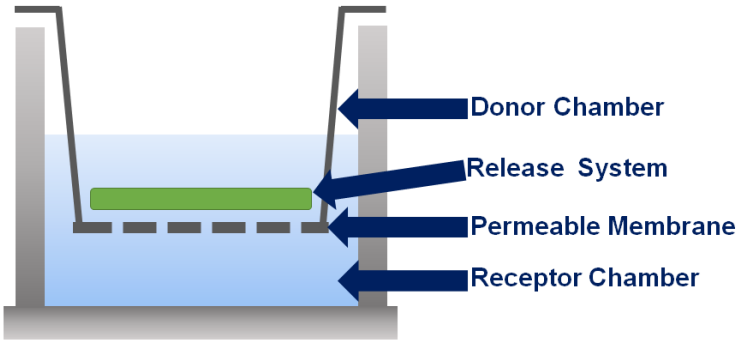

Figure 7. Schematic representation of the Transwell® setup for drug release studies. The delivery system is placed in the donor chamber (or compartment) and the drug concentration monitored in the receptor (or acceptor) chamber. Cells can be cultured in the system and exposed to a concentration-time profile that mimics the physiological temporal presentation of the drug.

2015). In this line, the possibility to mimic the PK properties of bioactive compounds in Transwell culture systems is very attractive for investigative delivery studies. This strategy would enable to deepen the pharmacological activities of natural compounds by exposing cells to different concentration-time profiles (Figure 7).

Polysaccharide films loaded with epicatechin (as discussed in section 5.1.2) tested in a Transwell system slowly released the polyphenol (representative curve in Figure 8-A), with a kinetic rate inferior to the k_a absorption rates observed in PK studies (>1 h^{-1}).

In addition to films, particulates and delivery systems with other geometries can be used in Transwells. We used dry alginate spheres prepared by the method described in (Ricardo Lagoa and Rodrigues, 2007), loaded with epicatechin and placed in the donor compartment. Monitoring the cumulative epicatechin concentration in the acceptor (Figure 8-B), the loaded spheres enabled a kinetic rate (0.9 h^{-1}) more similar to the absorption k_a rate observed in humans.

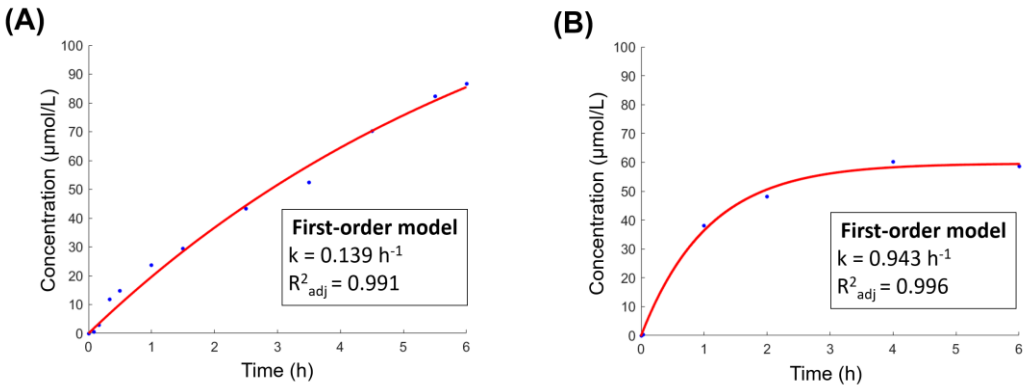

Figure 8. Time evolution of the epicatechin concentration in the acceptor compartment of a Transwell® using calcium alginate films (A) and spheres (B) loaded with the polyphenol in the donor compartment. A 24 mm Transwell system with a polyethylene terephthalate membrane insert was used. The release medium in the compartments was saline and epicatechin concentration was analyzed by HPLC.

The assays here described show the suitability of Transwell systems to screen different delivery systems, including those with different morphologies, and identify the ones presenting more suitable release properties. Moreover, since Transwells are already well-established cell culture supports for diverse functional studies, the possibility to generate different concentration-time profiles with varying drug delivery kinetics offers a useful tool to investigate novel features of the compounds' bioactivity in cells.

6. CONCLUSIONS

Natural bioactive compounds as polyphenol antioxidants ask for more effective delivery approaches that could increase clinical application of new therapies. Epidemiological and laboratory studies give support to the therapeutic potential of several of these compounds, but only in some cases human trials are returning the necessary efficacy for clinical approval, typically with the more specific and potent compounds as alkaloids or capsaicin.

Compounds naturally appearing in human diet and metabolism present important pharmacological features that can critically limit their therapeutic outcome. Low bioavailability, multi-target and low-potency action mechanisms make natural compounds-based treatments highly dependent on PK factors, administration dose and time schedule, absorption/excretion rates or cell time-changing exposition.

It is proposed PK parameters to be best accounted as possible in the design of delivery systems for natural bioactive compounds. Doses identified by epidemiological studies and existing clinical trials as affording beneficial effects should be analyzed to plan therapeutic approaches. PK data obtained in humans in these favorable conditions offer insights into the dynamics of absorption of the compounds and their course in the human body that may be the key to define successful drug delivery schemes.

In addition to usual goals of high loading and controlled release, a PK-guided regulation of temporal drug presentation in the development of delivery systems is hypothesized to lead to more effective treatments by natural compounds. Laboratory testing setups compatible with different delivery systems are presented herein, accompanied by illustrative examples of validation and application procedures.

A combination of physiology-based modelling, biomaterial design and *in vitro* screening tools are becoming available for the development of novel drug formulations. An overall goal of future research is to assess the therapeutic value of these promising approaches and the derived products in preclinical and clinical studies.

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