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Design and Application of Microfluidic Systems for *In Vitro* Pharmacokinetic Evaluation of Drug Candidates

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Abstract

One of the fundamental challenges facing the development of new chemical entities within the pharmaceutical industry is the extrapolation of key *in vivo* parameters from *in vitro* cell culture assays and animal studies. Development of microscale devices and screening assays incorporating primary human cells can potentially provide better, faster and more efficient prediction of *in vivo* toxicity and clinical drug performance. With this goal in mind, large strides have been made in the area of microfluidics to provide *in vitro* surrogates that are designed to mimic the physiological architecture and dynamics. More recent advancements have been made in the development of *in vitro* analogues to physiologically-based pharmacokinetic (PBPK) models – a mathematical model that represents the body as interconnected compartments specific for a particular organ. In this review we highlight recent advancements in human hepatocyte microscale culture, and describe the next generation of integrated devices, whose potential allows for the high throughput assessment of drug metabolism, distribution and pharmacokinetics.

Keywords

Microfluidics; human hepatocyte; bioreactor; coculture

1. INTRODUCTION

Recent advancements in chemistry, such as parallel and combinatorial synthesis, have resulted in a multi-fold increase in the number of compounds that are available for evaluation in new drug discovery. Furthermore, other improvements using a variety of structural chemistry tools, and molecular biology tools, provide the pharmaceutical industry with an unprecedented level of structure-based designs to further guide the synthesis of new chemical entities (NCEs) as potential drug leads. Along with the advancement of chemistry and biology, new automated screening tools have became commercially available which can

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carry out complex, programmable and adaptable robotic operations to test hundred of thousands of compounds in a speedy and precise manner. As a result, these new factors have worked together to increase our ability to create NCEs that exhibit targeted pharmacological activity. Hence, the task of screening compounds for their pharmacokinetic properties, such as permeability, distribution, and metabolic stability, has become a major challenge in drug discovery. This change, in turn, has compelled the invention and implementation of high-throughput screening methods that predict *in vivo* pharmacokinetic behavior of NCEs in humans.

One of the most challenging tasks in drug discovery is predicting a NCE's pharmacokinetic behavior in humans using data derived from *in vitro* model systems [1, 2]. For an orally administered drug, the primary goal during discovery lead optimization, for a compound with a systemic target, is to improve oral bioavailability and systemic half-life. Therefore, during lead optimization it is essential to identify NCEs with predicted sufficient oral absorption using a variety of *in vitro* and *in vivo* assays. It is well recognized that in order for a NCE to achieve reasonable oral absorption, it will need to have adequate aqueous solubility, as well as intestinal permeability [3–5]. Drug absorption through the GI tract following oral administration is a rather complex and dynamic process. Passive diffusion can occur through the cell membranes of enterocytes (transcellular) or the tight junctions between the enterocytes (paracellular) [6–9]. Influx and efflux through various drug transporters also play roles in dictating drug absorption. Since many processes are occurring simultaneously, it is often impossible for a single *in vitro* model to simulate the entire *in vivo* process. However, two *in vitro* screening models, have been developed over the last decade and are currently used by most major pharmaceutical companies in their drug discovery efforts. The first is the Caco-2 human intestinal cell line that is widely used to measure permeability of drug candidates, while the second is a non-cell based assay termed Parallel Artificial Membrane Permeability Assay (PAMPA) used to predict passive, membrane permeability of drug candidates.

Since the liver is one of the most important organs responsible for systemic drug metabolism, hepatic clearance has been another primary focus for lead optimization. Many approaches have been developed to predict hepatic clearance using *in vitro* methodologies. For example, human liver microsomes (CYP450 containing membrane vesicles) have been used extensively for obtaining metabolic clearance and half-life data for the prediction of human clearance [10, 11]. It was suggested that the presence of plasma in the microsomal mixture might lead to better prediction of *in vivo* clearance [12, 13], in that it accounts for the effect of protein binding which is present *in vivo*. Additionally, clearance rates can be corrected by taking into account the free fraction of the test article in plasma [14]. While these corrections may provide a more accurate clearance prediction, they still do not taken into account other factors such as hepatocyte transporter-mediated uptake, which may also play a rate-limiting role [15]. Isolated primary human hepatocytes offer the next step in drug metabolism studies [16–27]. In order to push the envelop of high throughput *in vitro* screening systems, however, a focus is now being direct to integrate these various systems with microfluidics systems into microscale devices. In this review, we provide an overview of current technological progressions that are aimed at the development of microscale devices.

2. DESIGN OF MICROFLUIDIC SYSTEMS FOR AN INTEGRATED *IN VITRO* MODEL

Development of a microscale devices and screening assays may provide more efficient prediction of *in vivo* clearance and half-life in humans. Such microscale devices have the potential to accurately produce physiologically realistic parameters and would more closely

model the desired *in vivo* system being tested. Currently, the gold standard for the pharmaceutical industry is the use of isolated human hepatocytes, either in suspension, or in a static culture configuration, where the cells are attached to a collagen-coated plate. Isolated human hepatocytes, which carry most of the livers detoxification work load, are generally recognized as one of the most relevant first line screens in pre-clinical candidate drug assays. However, serious limitations in predicting human liver responses to drugs still exist [28–37]. Limitations include the rapid loss of liver-specific function during in-vitro culture as well as the scarcity of healthy cells. Significant differences between human and animal metabolism necessitate the use of human cells. However, healthy human livers are used for orthotopic liver transplants and only marginal livers, which are rejected for transplantation, are used as a source for hepatocyte isolation.

The limitations of assays utilizing isolated hepatocytes, as well as the aforementioned goal of integrating multiple assays into a single high throughput device, have driven the development of microscale in-vitro assays with the goal of better modeling in-vivo physiological liver metabolism. In their final embodiment, these microscale devices must integrate a stable, and highly functional hepatocyte cell culture, with a fluidics device that recapitulates the *in vivo* environment, with respect to cell to volumes ratios, shear stress, and a host of other process and physiologically relevant parameters. As a case in point we focus on the microfluidic device offered by the Hurel Corporation, as it has been described in publication in terms of drug metabolism centric studies. However other devices also exist, and have been oriented toward use in toxicity studies, such as the devices by Park *et al.,* Sudo *et al.,* Toh *et al.* and Carraro *et al.* [38–52]. We will also focus on the scientific/ technological viability and importance of introducing flow and operating under pharmacokinetically modeled conditions, to engineer cell based assays with higher predictive capabilities, as well as offer up new solutions to other areas studied under the guise of drug metabolism and pharmacokintetics (DMPK).

2.1. Physical Structure – Microscale Perfused Cultures

To better model *in vivo* conditions several groups are now designing physical replicas of physiologically based in silico pharmacokinetics (PBPK) models [53–60]. These physical models, and resulting cell based assays, are designed to match relevant *in vivo* parameters. In the realm of DMPK, pharmacokinetic parameters of interest include interactions between cell liquid residence time, liquid to cell ratios, relative size of organs (or tissue compartments), metabolism by cells, shear stress, and the like. By providing a pharmacokinetic-based culture system that mimics the natural state of cells, the predictive value and in-vivo relevance of screening and toxicity assays is enhanced. As a case example, the Hurel microscale culture device [41] comprises a fluidic network of channels segregated into discrete but interconnected compartments. The specific compartment geometry is designed to provide cellular interactions, liquid flow, and liquid residence parameters that correlate with those found for the corresponding cells, tissues, or organs *in vivo*. In its initial configuration [61–63] the device implemented microfluidics chips with two cell compartments, or compartments, seeded with HepG2 human hepatoma cells and L2 lung cells or 3T3 fibroblasts.

A key roadblock in the development of microscale devices, involving hepatocytes, is the rapid loss of hepatocyte liver-specific function during in-vitro culture and although some conclusions can be drawn with the application of appropriate pharmacokinetic principles, there are still substantial limitations. One concern is that current screening assays utilize cells under conditions that do not replicate their function in their natural setting. Therefore, to create a more “liver like” environment one would need to design an optimized microfluidic system with a potentially long-term, stable culture of primary human

hepatocytes. Along these lines there have been previous reports of microreactor systems that couple microfluidics with hepatocyte cultures, by various groups [39, 41, 42, 45, 51, 64].

2.2. Analysis of Fluid dynamics in the Microscale Perfused Culture

When developing an optimized microfluidic system as an *in vitro* microfluidic surrogate for DMPK assessment of NCEs, special attention needs to given to the fluid dynamics that exist within the *in vitro* surrogate. The dominant parameters one must address when designing a device are: 1) chemical gradients within the device; 2) flow rates used to assess a variety of DMPK aspects of the new chemical entity; 3) shear stress within a device; 4) efficient mixing given the laminar flow characteristics that are present in a majority of fluidic devices.

Analogous to the liver *in vivo*, in a microfluidic hepatic based *in vitro* device, concentration gradients can exist for nutrients, oxygen, metabolic wastes, as well as the NCEs being studied. *In vivo*, a gradient of oxygen, nutrients and hormones is thought to give rise to zones within the liver, leading to changes in phenotype across the lobule. These phenotypic changes lead to a heterogeneous distribution of drug metabolizing enzymes and transporters in the liver. *In vitro*, oxygen is rapidly depleted due to its relatively low solubility in cell culture medium and the high metabolic activity of the cells, especially at very high cell to volume ratios. While some work has been done to attempt and recapitulate the *in vivo* zonation phenomena *in vitro*, a larger focus has been on ensuring that all cells within a fluidics device receive enough oxygen [65]. When considering these aspects up front, one can ensure that cells remain viable, and reactions that are important for DMPK analysis are not mass transport limited. In this regard it is important to design flow rates, length scales, and cell to volume ratios based on known or predicted cellular requirements. Experimental values of cellular utilization, of oxygen for example, are available in literature and can be used in conjunction with computational fluid dynamics to design proper geometries [66–71]. When designing in this manner, one must be vigilant of the potential boundary conditions that exits, those being either hepatocytes in suspension (with a high surface to volume ratio) thereby having a high consumption rate, or a static plated configuration of hepatocytes (limited by mass transport and a static boundary layer) [72–75]. In order to overcome diffusion limitations in that later case, microfluidics can be applied to add a convective driving force used for the transport of oxygen and nutrients, as well as parent compounds that are to be screened with the *in vitro* surrogate. It should be noted though, that while there are many benefits due to the addition of flow there lurks the potential hurdle of added shear stress. It has been shown that while a small amount of shear stress is beneficial to cultures of hepatocytes (below 5 dynes/sq cm), while larger amounts are shown to be detrimental (above 5 dynes/sq cm) [65, 76, 77]. To ensure a system that promotes viability and cellular function, a balance must exist between the flow rate applied to the system, as well as the geometry of the system, without disturbing the beneficial transport effects gained through the application of flow. As a final point of consideration, mixing within the device should also be optimized. By their nature, microfluidics devices often exist at very low Reynolds numbers, laminar flow, and thus mixing and transport often occur through diffusion. A wide range of studies have been conducted to induce mixing within microfluidics devices, and include, but are not limited too, the inclusion of baffles, using pulsitile flow, applying electrokinetic driven flow, hydrodynamic focusing, and a whole slew of other techniques [78–85]. This again presents an opportunity, up front, for the use of computational fluid dynamics to ensure the proper design of the device, prior to introducing hepatocytes into the system. By designing a device that provides the proper mixing, and mass transport, while minimizing the amount of shear stress applied to the hepatocytes, one can then potentially provide a device with better *in vivo* predictive capabilities [71].

2.3. Optimized Human Hepatocyte Culture

Another facet of the microscale device is the generation of a stable long-term culture of human hepatocytes. Optimization of culture conditions to maximize in-vitro adult primary hepatocyte function has been well characterized. Studies have shown that duration of in-vitro function depends upon culture conditions such as the type of substrate used, spatial orientation of the cultured cells, addition of growth factors, and the combinatorial effects of these parameters [69, 86–89]. Many studies have examined the functional response of hepatocytes to the physical and chemical properties of culture substrates. The sandwich model, which incorporates human hepatocytes cultured between two thick layers of collagen, provides an in-vivo like environment that increases certain hepatocyte functions and extends their retention time [90–94]. In one example, primary hepatocytes were cultured in a number of collagen and Matrigel configurations including monolayer, collagen sandwich, Matrigel sandwich or composite (collagen/Matrigel) sandwich. Collagen sandwich and Matrigel cultures yielded superior and comparable albumin secretion for at least 2 weeks. The data also showed that hepatocyte polarity could be manipulated by the overall ECM composition independent of the actual morphologies of the cells in different substrates. A recent study has shown that hepatocytes cultured in a collagen sandwich and the presence of CYP450 inducers produced cells where expression ratios of phase I and Phase II genes closely resembled the *in vivo* liver [95].

Other methods to maintain function of isolated hepatocytes include the co-culture of primary hepatocytes with nonparenchymal cells. These co-cultures mimic the cell-cell interactions that are important in all facets of embryonic and adult physiology. It has been shown that when primary rat hepatocytes are cultured with fibroblasts, there is a marked increase in hepatocyte function as compared to hepatocytes cultured alone [39, 46, 47, 69, 86, 87, 96]. Recent studies indicate that a model which incorporates both human hepatocytes as well as Caco-2 cells to project oral bioavailability is more favorable than the use of hepatocytes in suspension culture. In general, systems yielding the most promising results are based upon the aggregation of hepatocytes into spheroids, which markedly increases hepatocyte function. In one system, hepatocyte aggregation was induced by plating hepatocytes on low-density fibronectin [26]. Hepatocytes on this ECM initially attach and remain rounded, and over a few days reassemble into spheroids. Such self-aggregated spheroids show an increase in cytochrome P450 1A1 (CYP1a1) activity as compared to hepatocytes that remain in a monolayer configuration. In another study, hepatocyte aggregates and hepatocyte monolayers were cultured in collagen gels [97–99]. Here again, hepatocyte aggregation occurred with increased function as aggregates expressed an average two-fold increase in urea and albumin production compared to monolayer-plated cells. The increase in function seen in this system was attributed to an increase in cell-cell contact also seen in co-cultures, as well as maintenance of spheroid morphology. Thus, to date there are three prevailing hepatocyte culture systems: co-culture, three dimensional networks and aggregate culture. Through these approaches, with a focus towards maximizing homotypic hepatocyte interactions [69, 87, 88], one may potentially derive a long term stable culture, with potential incorporation into a microscale device.

2.4. Producing an Integrated Scalable Device

With an analysis of the key components needed to create a microscale device, we next turn our attention to the integration of these components into a system which incorporates continuous flow of culture media over isolated hepatocytes cultures. In the past, development of a small-scale bioreactor incorporating rat hepatocytes for drug metabolism studies was first reported by Bader et al [100]. The studies reported that primary rat hepatocytes cultured in a small–scale flat membrane bioreactor in a sandwich configuration maintained drug biotransformation capacity of Uripidil for at least 14 days. A similar study

utilizing porcine hepatocytes showed that cells cultured in a flat membrane bioreactor maintained their phase I and phase II activities and responded to inducing drugs over a 3 week period. Studies done with hepatocellular carcinoma cell lines have shown similar results indicating that this model system is useful in studying drug metabolism [62]. Although these initial studies more closely resembled in-vivo resemble physiological conditions, they are still deficient in that they do not mimic physiological conditions accurately enough for predictive studies. Therefore, the resulting assay data is not based on the pattern of drug or toxin exposure that would be found in an animal.

Returning back to our case example of the Hurel Device, we can see that the most promising results lie in an integrated system with optimized microfluic considerations, and a robust coculture system [39, 41]. It was demonstrated with this system that for a wide range of compounds, that the integrated system provides much higher predictive capability then hepatocytes under static conditions, or under flow, without the optimized coculture configuration [39, 41]. In addition, production rates of metabolites, a key capability for metabolism identification groups, is much higher in this system.

3. CURRENT AND FUTURE APPLICATIONS OF MICROSCALE SYSTEMS

3.1. Evaluation of Hepatic Clearance

The primary and current use, as aforementioned, of the Hurel microscale system is in the area of drug metabolism, more specifically in the application of predicting *in vivo* clearance values from *in vitro* clearance data. Fig. (1) illustrates the relationship between the clearances of drugs by the liver compartment of the microfluidic device and the drug concentrations in the reservoir. The liver compartment in the system functions as the sole eliminating compartment and is connected to a non-eliminating compartment, the reservoir. The liver compartment and the reservoir are connected via the medium flow with a flow rate of Q_h in a recirculation loop. Unlike traditional static culture systems, where the predictive hepatic clearance may be obtained by using the experimental intrinsic clearance with the well-stirred model to incorporate the hepatic flow parameter, the microfluidic device itself has a flow component. Therefore, the device itself represents a well-stirred model. The clearance data obtained using the flow device can be scaled up directly to predict the hepatic clearance. Extraction ratio concept is used to scale-up the clearance data obtained using the flow device to the estimated human hepatic clearance as shown below:

$$CL_h = \frac{(C_0 - C_t) \cdot V}{AUC_{0-t}}$$

where CL_h (μL/min/chip) is the clearance of the microfluidic system, C_0 and C_t are concentrations (μM) of compound in the reservoir at time 0 and t respectively, $AUC_{0\text{-}t}$ is the area under the concentration-time curve from 0 to t, and V is the volume of incubation. The extraction ratio of microfluidic system, E_h, is

$$E_h = \frac{CL_h}{Q_h}$$

The predicted human hepatic clearance from the flow device can be obtained by up-scaling the extraction ratio:

$$CL_H = Q_H \times E_h$$

The predicted data are comparable to the conventional intrinsic clearance calculation [101] with the model compounds tested, indicating that human hepatocytes cultured under flow condition are at least as metabolically active as the ones cultured under a traditional static surrogate system. In addition, the device provides the apparent benefit of avoiding mathematical modeling to predict *in vivo* parameter value(s), yield higher predictive capability, as aforementioned.

3.2. Evaluation of Cell Permeability and Bioavailability

Oral administration of drug remains the most popular route of administration and with the aforementioned increase in NCEs it becomes necessary to develop high throughput approaches for evaluating permeability. These methods have to be effective, low in cost and robust considering the immense number of potential compounds being identified [102]. Biological evaluation of NCE, looking at parameters such as lipophilicity and absorption potential [103] as well as the utilization of systems like immobilized artificial membrane chromatography and parallel artificial membrane permeability assay, has been used in predicating the likely degree of absorption in small intestine. Furthermore, these approaches have led to robotic multi well plate systems which provide high throughput analysis. However, these methods fail to account for many of the physiological aspects of the *in vivo* absorption and therefore fail to predict absorption accurately [102]. Several animal tissue models have been implemented, but are very difficult to scale down for high throughput evaluation and are very costly requiring large amounts of compound. The most widely used *in vitro* model for NCE cell permeability determination is cell monolayer cultures with endothelial cells over a diffusion surface. Various different cell types have been employed in these platforms with the most popular cell type being the caco-2 cell. These platforms have been effective, but their scalability has been hindered due to surface area limitation created with smaller well plates. The Hurel micro-device, as illustrated in (Fig. 2), can overcome this limitation allowing for the miniaturization of these cellular models via the precise control over fluid flows possible with micro-fabricated systems. This will facilitate the development of a miniaturized compartment containing the *in vitro* cellular permeability assay. Computational modeling previously described will be implemented to design a compartment with appropriate volume disruption and flow. Flow through an absorption compartment will result in a more physiologically relevant model considering that there is no static situation in the small intestine and equilibrium values can never be reached. Samples from above and below the endothelial cell diffusion layer will be sampled. The above sample will be used to determine cell permeability and the bottom layer will indicate the bioavailability of the drug. Furthermore, these cellular systems can be established in series with hepatocyte systems to have a robust device that can simultaneously measure bioavailability and drug metabolism (Fig. 3).

3.3. Estimation of Volume of Distribution

Tissue binding greatly affects drug bioavailability overtime, in many cases determining that drugs half-life, which has a major effect on dosing regiments. At present, the methods used to predict volume of distribution (Vd) include: the extrapolation of animal data [104, 105], physiologically based pharmacokinetic (PBPK) modeling and in silico approaches that employ quantitative structure-pharmacokinetic relationships [106]. Animal models are time consuming and costly, as well as the data extrapolated may not always translate into accurate human Vd. In silico approaches while providing high throughput screening power, are theoretical models that do not account for all the variables that are associated with Vd *in vivo*. Until this point *in vitro* models have not been established for assessing Vd and with the amount of throughput demanded by today's pharmaceutical markets current methods will be costly. A reason for this being, tissue engineering has only recently provided means of culturing adult human cells effectively. Furthermore, the advent Micro-fabricated devices

provide the control necessary to develop *in vitro* platforms for assessing drug Vd. The Hurel device will have a multi- tissue compartment designed to represent plasma distribution into different tissues. The amount of cells will be determined based on their percentage exposure to plasma *in vivo*. This compartment can be implemented in the chip to measure the volume of distribution a given drug has. The precise control over volume, flow and cell ratios can be established in a compartment to mimic the volume distributions in the body. In this manner a compartment will be created which will represent the tissues *in vivo* contributing to Vd.

The proposed device (Fig. 4) will be a three compartment device with two flow systems. The first flow system representing the absorption pathway *in vivo* will have a diffusion layer into the second flow system. The amount of drug in the second flow system will represent the bioavailability of the drug, which will be circulating through two compartments representing the liver and all other tissues in the body. With this design several different DMPK parameters can be assessed simultaneously and to a greater degree of accuracy. The size of the micro-device allows for the design of high throughput systems able to evaluate DMPK characteristics for several NCEs. This micro-device will be the first multi-compartmental micro-device for evaluation of DMPK properties. Overall, the proposed next generation chip is a robust highly integrated micro device designed to facilitate high throughput screening of several different DMPK drug characteristics.

4. SUMMARY

Traditional methods of predicting human response to drugs utilize surrogates-typically either static, homogeneous in-vitro cell culture assays or *in vivo* animal studies. Static *in vitro* cell culture assays are of limited value because they do not accurately mimic the complex *in vivo* environment and thus cannot accurately predict human risk. Similarly, while *in vivo* animal testing can account for these complex intercellular and inter-tissue effects not observable from in-vitro cell-based assays, in-vivo animal studies are extremely expensive, labor-intensive, time consuming, and often the results are of doubtful relevance when correlating human risk. The development of microscale screening assays and devices that can provide better, faster and more efficient prediction of in-vivo toxicity and clinical drug performance is of great interest in a number of fields. A significant body of knowledge currently exists on development of microscale models to better mimic *in vivo* metabolism. Such a microscale device offers a unique opportunity to study drug metabolism with *in vivo* relevant parameters which would more closely model the desired *in vivo*, physiologically realistic, system being tested. In designing a microscale system, special attention must be paid not only to the device itself, but also the cell culture that is introduced within the system. Upon successful integration, such devices have the potential to yield higher predictive capabilities then other non-microscale device alternatives. In addition these microscale devices provide a platform to which other assays can be coupled to potentially provide an “all-in-one” device for assessment of DMPK parameters of a new chemical entity [39, 41, 71].

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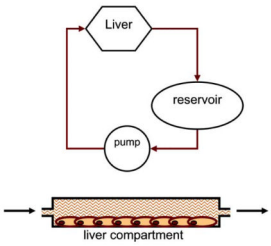

Fig. 1.

Top: Diagram of the metabolism microfluidic device comprises of a liver compartment and a reservoir. The liver chamber is the sole elimination compartment of the system. Bottom: the side view of the liver chamber, where the hepatocytes are cultured. The fluid (culture medium or buffer) from and to the reservoir can flow through the liver compartment.

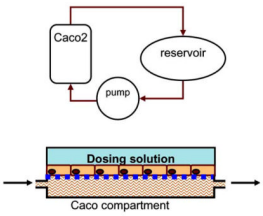

Fig. 2.

Top: Diagram of an absorption microfluidic device comprises of an absorption compartment and a reservoir. The absorption chamber has apical and basolateral sides separated by a permeable membrane. Bottom: the side view of the absorption chamber, where Caco-2 cells are cultured on a permeable membrane. The fluid (culture medium or buffer) from and to the reservoir can flow through the absorption compartment. The dosing solution is in contact with the Caco-2 cells from the top.

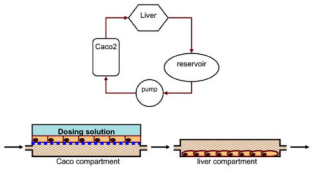

Fig. 3.

Top: Diagram of a bioavailability microfluidic device comprises of an absorption compartment, a metabolism compartment and a reservoir. Bottom: the side view of the absorption and the metabolism compartment. The fluid (culture medium or buffer) from the reservoir flows through the absorption compartment first, then the metabolism compartment. The drug solution first permeates through the Caco-2 monolayer, and then passes through the liver compartment to encounter the first-pass metabolism.

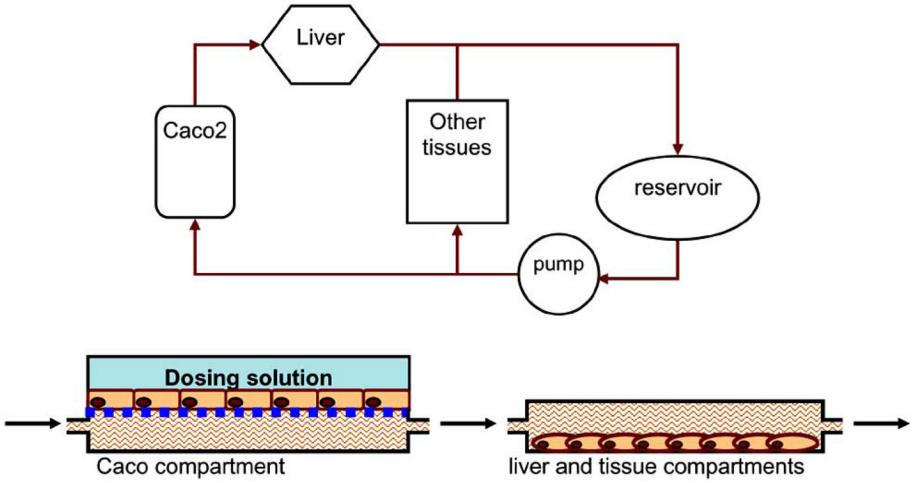

Fig. 4.

Top: Diagram of a pharmacokinetic microfluidic device comprises of an absorption compartment, a metabolism compartment, a biodistribution compartment and a reservoir. The fluid (culture medium or buffer) from the reservoir splits in certain proportion and flows through the absorption compartment or the biodistribution compartment. The fraction flows through the absorption compartment then enter the metabolism compartment simulating the first-pass metabolism. The fraction flows through the biodistribution compartment provides tissue binding effect. Both fractions merge before entering the reservoir. Bottom: the side view of the absorption and the metabolism compartments.