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HUMAN RECONSTRUCTED MUCOSAL MODELS TO ASSESS DRUG PERMEATION: CAFFEINE CASE STUDY ON ORAL MUCOSA

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INTRODUCTION

FDA has been extending its consideration for *in vitro* permeation studies (IVPT) to support drug safety and toxicology. To gain relevance and predictivity *vs* humans, 3D reconstructed human mucosal models are the most promising alternative (to either human and animal explants) to assess as first instance the penetration kinetics and passage of active molecule and functional ingredients in food, pharma, medical device and cosmetic industries. Recently, the interest in the ergogenic effects of caffeine delivered in low doses (~200 mg or ~3 mg/kg body mass) and administered in forms other than capsules, sport drinks and coffee, such as chewing gum, gels and mouth rinses is increased. The evaluation of the absorption rate of these new forms through the buccal mucosa compared with capsule absorption in the gut become critical to define caffeine bioavailability as well as it could be important in military and sport situations where rapid caffeine effects are requested[(1)].

CAFFEINE AS REFERENCE HYDROPHILIC MOLECULE

Caffeine is a water-soluble compound (MW: 194.194 g/mole, LogKow-0.07) which is absorbed by passive diffusion mediated via Tight Junctions through the spaces between the cells (paracellular route). Caffeine is the reference molecule of low lipophilicity in OECD TG 428 (*in vitro* percutaneous absorption method) with well-defined penetration kinetic. Its penetration and absorption behavior has been extensively studied on many different *in vitro* models and thus represents a good reference compound for *in vitro* permeation studies[(2,3)]. Caffeine has also been used as probe to assess *in vitro* the attitude of a given product to form a protective film on the skin[(4)].

AIM OF THE STUDY

The aim of this research project is to quantify the caffeine 2% epithelial penetration and passage on a 3D Reconstructed Human Oral Epithelium (HOE) (Episkin, Lyon, France). This concentration have been selected as the higher average dose founded in literature and because at this concentration the caffeine is founded in many industrial products.

EXPERIMENTAL DESIGN: CAFFEINE PENETRATION AND ABSORPTION ON HOE

The requirements and acceptance criteria of the OECD TG 428 have been followed.
The transmucosal passage of a 2% caffeine in water solution (pH= 5.28) has been assessed on HOE model (HOE/S/5. 0.5 cm^2) for a total of 2h on three independent testing runs of 6 biological replicates from the same batch. In order to mimic the realistic exposure and passage kinetic, the receptor fluid (1 mL) in the basolateral compartment has been collected after 15min (the estimated realistic contact time of the molecule with the oral mucosa) and then after 30min, 1h and 2h. After 2h, caffeine solution residual was removed and collected and HOE tissues were homogenized. All samples, residuals, homogenates and fluid receptor aliquots were collected for caffeine content quantification by ULPC as illustrated in **figure 1**.

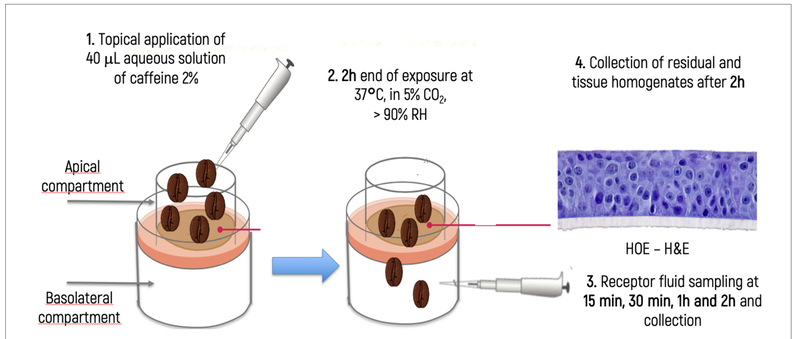

Figure 1. Experimental design for caffeine permeation study on HOE.

ANALYTICAL METHOD FOR CAFFEINE QUANTIFICATION

The liquid chromatographic system employed was an Agilent 1290 infinity UPLC system (Santa Clara, USA) equipped with a reverse phase ACQUITY UPLC BEH C18 column and the Empower 3 software (Waters, USA) for data acquisition and processing. The ULPC method has been developed for caffeine content in presence and absence of biological matrix before performing the study. The method sensitivity was of 0.1 mg/L in solution.

MULTIPLE ENDPOINT ANALYSIS (MEA) APPROACH APPLIED TO EPITHELIAL PASSAGE STUDIES

The assessment of integrity and permeability of the epithelial model after the penetration study has been performed in separate tissues (duplicate) treated in the same conditions but not used for the epithelial passage by evaluating different parameters:
- Transepithelial-electrical-resistance (TEER) measurements
- Lucifer Yellow (LY) assay
- Histo-morphological analysis

BARRIER ASSESSMENT

TEER was measured with the Millicell-ERS instruments (range 0-20 kΩ) to assess the integrity of the barrier membrane before (t=0h) and after treatment (t=2h). The results are presented in **figure 2** (left): TEER values expressed in ohm*cm^2 at the end of 2h exposures are not modified compared to values recorded before the treatment nor to the values reported for the Negative Control (NC - tissue treated with saline solution), suggesting that in the defined experimental conditions tissue integrity and barrier function are perfectly preserved. The same mean LY Flux % of 11 has been found after 2h treatment with saline solution and 2% caffeine suggesting that HOE relative permeability has not been modified after treatment with 2% caffeine compared to the NC (**figure 2** right).

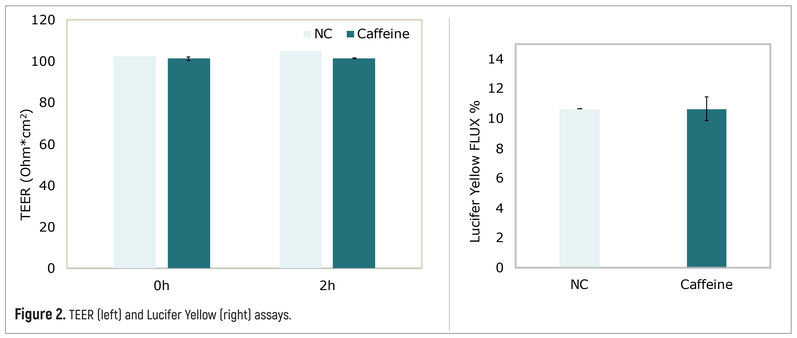

Figure 2. TEER (left) and Lucifer Yellow (right) assays.

HISTOMORPHOLOGICAL ANALYSIS

Images of haematoxylin and eosin (H&E) stained HOE histological sections after 2h treatment with saline solution (A) and 2% caffeine (B) are presented in **figure 3**. No differences are visible between the two tissue sections confirming that no modifications occur during the epithelial penetration study.

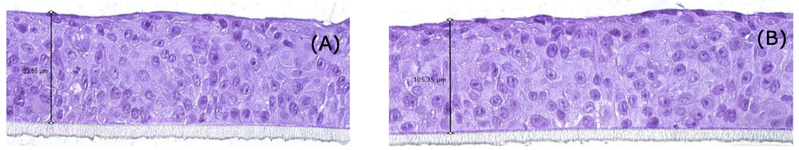

Figure 3. H&E staining of HOE tissue sections after 2h treatment with saline solution (A) and 2% caffeine (B). Magnification x40.

RESULTS

In **Table I** are reported the mean amount of caffeine quantified in the different biological samples for each of the three testing runs (series) and expressed as µg (total amount applied was = 868 µg). The Total Mass Balance (96% for each series) meets the reference acceptance criteria of OECD TG 428 (100 ±10%).
The standard deviation calculated for each run is low and the CV% among the 3 series is found to be < 15% except for the homogenate samples. These data indicate low variability of the results within and between the three independent series supporting a satisfying experimental reproducibility.

Table I. Caffeine quantification in the different compartments for the three series.

	Quantity Applied							TOTAL (µg)	MASS BALANCE %
	Caffeine (µg)		RECEPTOR FLUID				RESIDUAL	HOMOGENATE		
			15min	30min	1h	2h	2h	2h		
Series I	868	Mean	**305.79**	**204.33**	**196.68**	**87.76**	**37.97**	**1.33**	**833.87**	**96.07**
		St. dev.	2.66	3.95	3.36	1.66	1.40	0.75	5.55	0.64
		CV%	0.87	1.93	1.71	1.89	3.70	56.29	0.67	0.67
Series II	868	Mean	**341.98**	**200.98**	**182.78**	**92.06**	**19.30**	**0.93**	**838.03**	**96.55**
		St. dev.	5.37	3.50	3.41	3.83	2.36	0.33	10.34	1.19
		CV%	1.57	1.74	1.87	4.16	12.23	35.12	1.23	1.23
Series III	868	Mean	**359.07**	**194.18**	**185.87**	**77.78**	**19.51**	**0.79**	**837.20**	**96.45**
		St. dev.	3.54	10.09	12.01	1.48	0.47	0.20	16.62	1.91
		CV%	0.99	5.20	6.46	1.90	2.42	24.88	1.99	1.99

In **Table II** the distribution % within the different compartments is reported confirming the high rate of caffeine permeation through the oral mucosa in the experimental conditions applied.

TableII. Caffeine distribution within the different compartments for the three series.

	RECEPTOR FLUID				HOMOGENATE	RESIDUAL
Caffeine %	15 min	30 min	1 h	2 h	2 h	2 h
Series I	36.7	24.5	23.6	10.5	4.6	0.2
	95.3					
Series II	40.8	20.4	21.8	11.0	2.3	0.1
	97.6					
Series III	42.7	23.2	22.2	9.3	2.3	0.1
	97.3				**ABSORBABLE DOSE**	**NOT ABSORBED DOSE**
	ABSORBED DOSE					

CONCLUSION

The results of caffein permeation through HOE suggest that the adopted procedure on the 3D tissue is highly reproducible within the same batch and with the same trained operator. The complementary parameters investigated confirmed that the integrity and the barrier properties of the biological model have been preserved during the penetration study which resulted performed in homeostatic and physiological conditions. The results fully support the use of 3D human reconstructed epithelia in penetration studies as ethical, relevant and predictive models to assess the permeability and penetration kinetics of drugs and substance based medical devices delivered through mucosal tissues by defining flexible protocols which mimic realistic dose and exposure conditions. Furthermore, the MEA approach demonstrated the need to measure tissue integrity and barrier function during the penetration study in separate tissues to guarantee the relevance and robustness of the absorption results.
Further experiments have to be conducted to assess inter-batch reproducibility and demonstrate the relevance of the 3D epithelial models in the establishment of an *in vitro/in vivo* correlation.

References Massimo 3
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