NORMATIVE INSTRUCTION – IN No. 182 OF 5 SEPTEMBER 2022

Provides for permeability tests with Caco-2 cells and the validation of such assays.

The Collegiate Board of Directors of the Brazilian Health Regulatory Agency, in the use of the attributions vested in it under Article 7, item III, and Article 15, items III and IV of Law no. 9,782 of 26 January 1999, and Article 187, item VII, Paragraph 1 of the Internal Regulation approved by Collegiate Board Resolution – RDC no. 585 of 10 December 2021, adopts the following Normative Instruction, as decided upon in a meeting held on 31 August and 1 September 2022 and I, Director-President, determine its publication.

CHAPTER I

INITIAL PROVISIONS

Section I

Objective

Article 1. This Normative Instruction provides for permeability tests using Caco-2 cells and their validation, in accordance with Collegiate Board Resolution – RDC No. 749 of 5 September 2022, which provides for the waiver of bioequivalence/relative bioavailability studies, or any other resolution that may replace it.

Sole paragraph. When they are the only proof of classification as highly permeable for classification purposes by the Biopharmaceutical Classification System (BPS), tests with Caco-2 cells are limited to passively transported drugs.

Section II

Definitions

Article 2. For the purposes of this Normative Instruction, the following definitions are adopted:

I – Caco-2 cells: cells isolated and cultured in vitro, originating from human colon adenocarcinoma, which differentiate into structures similar to enterocytes, forming a monolayer of polarized cylindrical cells with microvilli at the apical edge;

II – Extent of absorption: total amount of drug absorbed by the body. It is measured by the pharmacokinetic parameter area under the curve (AUC);

III – Marker drug: drug with a known permeability value used as a control to demonstrate method consistency;

VI – Efflux transporter substrate drug: drug capable of bidirectional transport in the intestinal membrane and demonstrating the functional expression of efflux transporters;

V – Test drug: drug that is the subject of the analysis;

VI – Internal standards: marker drugs of high and moderate permeability, defined during method validation, which must be included in the donor fluid along with the test drug in the permeability assay to demonstrate method consistency;

VII – Intestinal permeability: property of the intestinal epithelial membrane to allow the transit of molecules through this biological barrier;

VIII – Transepithelial electrical resistance (TER): measurement of the electrical resistance of the cell monolayer used as a method for determining the integrity and permeability of the monolayer;

IX – Passive transport: transport of molecules across the plasma membrane by passive diffusion along a concentration gradient determined by their solubility in the lipid bilayer.

CHAPTER II

PERMEABILITY ESSAY VALIDATION

Article 3. Validation of the Caco-2 cell assay for determining permeability for classification by the BPS must be demonstrated by a ranked relationship between the experimental permeability values and the extent of absorption in humans using marker drugs of zero, low (<50%), moderate (50-84%), and high (³85%) permeability.

Paragraph 1. At least five marker drugs must be used for each of the high, moderate, and low permeability categories, as per the examples in Annex I of this Normative Instruction.

Paragraph 2. A zero-permeability marker must be included, and at least three replicates of the cell assay must be performed to provide a reliable estimate of each drug's permeability.

Paragraph 3. The assays must allow differentiation between drugs with low, moderate, and high permeability.

Paragraph 4. The method must be properly validated.

Article 4. The integrity of the Caco-2 cell monolayer must also be confirmed by comparing transepithelial electrical resistance (TER) measurements and other appropriate indicators before and after the experiment.

Sole paragraph. Additionally, the integrity of the cell monolayer must be demonstrated using zero-permeability drugs, as per the examples in Annex I of this Resolution.

Article 5. The method validation report must include:

I – a list of marker drugs with data on the extent of absorption in humans (mean, standard deviation, and coefficient of variation) selected to establish the suitability of the method;

II – permeability values for each marker drug (mean, standard deviation, and coefficient of variation);

III – permeability class for each marker drug;

IV – graph of extent of absorption as a function of permeability (mean ± standard deviation or 95% confidence interval), identifying the threshold for high permeability classification and the high permeability marker drug selected to classify the test drug;

V – description of the study method;

VI – concentration of the drugs (markers and test) in the donor fluid;

VII – description of the analytical method;

VIII – equation used to calculate permeability;

IX – information on potential efflux (data on bidirectional transport for a known substrate);

X – Caco-2 cell certificate; and

XI – information on Caco-2 cell bank maintenance.

CHAPTER III

PERMEABILITY ESSAY

Article 6. Passive transport of the test drug must be demonstrated using an appropriate system that expresses efflux transporters, demonstrating independence from the permeability measured *in vitro* at increasing drug concentrations (1%, 10%, and 100% of the highest dose dissolved in 250 mL), or by the direction of transport with an efflux ratio measurement of less than 2 (two) for the selected concentrations, according to the formula in Annex II of this Resolution.

Sole paragraph. The functional expression of efflux transporters must be verified using bidirectional transport studies demonstrating the asymmetric permeability of efflux transporter substrates, as per the examples in Annex I of this Resolution, selected at concentrations at which saturation does not occur.

Article 7. The concentration of the test drug selected for permeability testing must be justified.

Article 8. The test conditions defined during method validation must be used, and a marker drug with high and moderate permeability must be included in the donor fluid, along with the test drug, as an internal standard to demonstrate method consistency.

Article 9. The selection of drugs as internal standards must be based on their compatibility with the test drug; that is, they must not exhibit any physical, chemical, or permeation interactions.

Article 10. The permeability of internal standards can be determined by evaluating the test drug in the same monolayer or in other monolayers on the same plate, when it is not possible to include the internal standard in the same cell culture as the permeability assessment of the test drug.

Article 11. The permeability values of the internal standard must be consistent across different essays, including those conducted during method validation.

Article 12. Acceptance criteria must be defined for internal standards and efflux transporter substrate drugs.

Article 13. The average recovery of the test drug and internal standards must be defined at the end of the essay.

Article 14. For recovery of less than 80% (eighty percent), a mass balance evaluation must be conducted, including measurement of drug residuals in the cell monolayer and in the test apparatus.

Article 15. Permeability assessment for BPS classification purposes can be facilitated by selecting a high-permeability internal standard with permeability close to the limit between the moderate and high-permeability classes.

Sole paragraph. The test drug is considered highly permeable when its permeability is equal to or greater than that of the high-permeability internal standard.

Article 16. The data presented for the evaluation of the high permeability of the test drug (mean, standard deviation, coefficient of variation) must include permeability data for the test drug and internal standards, gastrointestinal stability information obtained *in vitro*, and information on the passive transport mechanism.

CHAPTER IV

FINAL PROVISIONS

Article 17. Failure to comply with the provisions contained in this Normative Instruction constitutes a health violation, in accordance with Law No. 6,437 of 20 August 1977, without prejudice to applicable civil and criminal liabilities.

Article 18. This Normative Instruction shall come into effect on 3 October 2022.

ANTONIO BARRA TORRES

Director-President

ANNEX I

Marker drugs/substances for validation of the permeability essay method

High permeability (f_a 285%)	Antipyrine Caffeine Ketoprofen Naproxen Theophylline Metoprolol Propranolol Carbamazepine Phenytoin Disopyramide Minoxidil
Moderate permeability (f_a = 50-84%)	Chlorpheniramine Creatinine Terbutaline Hydrochlorothiazide Enalapril Furosemide Metformin Amiloride Atenolol Ranitidine
Low permeability (f_a <50%)	Famotidine Nadolol Sulpiride Lisinopril Acyclovir Foscarnet Mannitol Chlorothiazide Macrogol 400 (Polyethylene glycol 400) Enalaprilat
Zero permeability	FITC-Dextran Macrogol 4000 (Polyethylene glycol 4000) Lucifer yellow (Lucifer yellow – LY) Inulin Lactulose
Efflux substrates	Digoxin Paclitaxel Quinidine Vinblastine

ANNEX II

Efflux ratio formula

$$\text{Efflux ratio} = P_{appBL->AP} / P_{appAP->BL}$$

where $P_{appBL->AP}$ is the apparent permeability in the basolateral-apical direction and $P_{appAP->BL}$ is the apparent permeability in the apical-basolateral direction.