

RECYT

Year 26 / Nº 42 / 2024 / 80–85

DOI: <https://doi.org/10.36995/j.recyt.2024.42.009>

# Metronidazole Delivery: An analysis of enhancers agents and their toxicity

## Liberación de metronidazol: Un análisis de agentes potenciadores y su toxicidad

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Received: 26/12/2023; Accepted: 24/09/2024

### Abstract

The objective of the present work was to evaluate the delivery of Metronidazole through cellulose acetate membranes, by means of the determination of parameters that quantify the delivery process. Propylene glycol (PG) and tween 20 were selected as delivery enhancers based on their hydrophilic properties, and urea for its lipophilicity, since they increase skin permeability affecting the resistance offered by the stratum corneum. The toxicity of the combination of the active ingredient and the promoters was also evaluated, taking into account that it is a critical aspect of any potential pharmaceutical agent for therapeutic use. Phosphate buffered saline (pH 7.4) was used in the receptor compartment. The physicochemical parameters calculated were the permeation and diffusion coefficients (P and D respectively). To obtain comparative results, all the enhancers were studied at the same concentration (10%). Combinations were previously evaluated at concentrations of 0.5, 5.0 and 10.0% and it was found that the optimal concentration was 10%. The best result was obtained when urea was used as a delivery enhancer. Urea significantly increases the release of MTZ (approximately 16 times more than the control), indicating that it is an effective enhancer of the therapeutic system under study.

Keywords: Metronidazole; Enhancer; Delivery; Toxicity; Urea.

### Resumen

El objetivo del presente trabajo fue evaluar la liberación de Metronidazol a través de membranas de acetato de celulosa, mediante la determinación de parámetros que cuantifiquen el proceso de liberación. Se seleccionaron propilenglicol (PG) y tween 20 como agentes potenciadores de la administración, según sus propiedades hidrofílicas y urea por su lipofílicidad, ya que incrementan la permeabilidad de la piel afectando a la resistencia que ofrece el estrato córneo. También se evaluó la toxicidad de la combinación del principio activo y los promotores, teniendo en cuenta que es un aspecto crítico de cualquier potencial agente farmacéutico para su uso terapéutico. En el compartimento receptor se utilizó tampón salino de fosfato (pH 7,4). Los parámetros fisicoquímicos calculados fueron los coeficientes de permeación y difusión (P y D respectivamente). Para obtener resultados comparativos, todos los potenciadores se estudiaron a la misma concentración (10%). Previamente se evaluaron combinaciones en concentraciones de 0,5, 5,0 y 10,0 % y se comprobó que la óptima concentración correspondía al 10%. El mejor resultado se obtuvo cuando se utilizó urea como potenciador de la administración. La urea aumenta significativamente la liberación de MTZ (aproximadamente 16 veces más que el control), lo que indica que es un potenciador eficaz del sistema terapéutico en estudio.

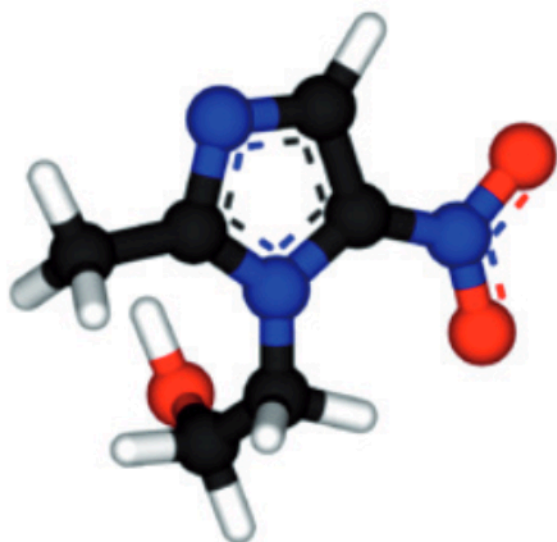
Palabras clave: Metronidazol; Potenciador; Liberación; Toxicidad; Urea.

### Introduction

Metronidazole (MTZ), 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole (Figure 1), is a nitroimidazole compound active in the treatment of anaerobic protozoan and bacterial infections (Löfmark *et al.* 2010; Mc Evoy 2010; Naveed and Qamar 2014; National Center for Biotechnology

Information 2020). This compound is a cytostatic drug, well-known for its effect on trichomonas, anaerobic bacteria, giardiasis, and amoebiasis. (Upadhyay *et al.* 2019).

The percutaneous absorption involves drug passage from the surface of the skin, crossing the stratum corneum under influence of a concentration gradient and diffusing



**FIGURE 1. MTZ structure.** Molecular formula: C<sub>6</sub>H<sub>9</sub>N<sub>3</sub>O<sub>3</sub>. Molecular weight: 171.2. [https://www.anmat.gob.ar/webanmat/fna/flip\\_pages/Farmacopea\\_Vol\\_III/files/assets/basic-html/page298.html](https://www.anmat.gob.ar/webanmat/fna/flip_pages/Farmacopea_Vol_III/files/assets/basic-html/page298.html)

to the epidermis and dermis from where it passes into the blood circulation. This barrier can be more permeable to solutes, including skin delivery enhancers used for topical drug formulation (Aulton 2004; Nongkhlawa *et al.* 2020; Qindeel *et al.* 2020). These enhancers can be added to pharmaceutical formulations to reduce diffusional penetration barrier, various strategies are used, such as increasing the diffusion coefficient of the drug, increasing its solubility in the lipids that make up the skin and/or increasing the degree of saturation of the active ingredient in the vehicle. The first of these alternatives has been widely studied and a large number of substances are known that can increase the absorption of pharmacologically active ingredients (API) by different mechanisms, depending on their chemical structure. Ideally, they are pharmacologically inert, and interact with skin constituents, inducing a temporary reversible increase on its permeability (Zhao and Singh 2000; Vavrova *et al.* 2005; Olivella *et al.* 2006, 2007; Zhang *et al.* 2006).

The use of Franz diffusion cells to evaluate the delivery of the active principle has become an important methodology of investigation that provides key information on the relationships among the membrane, the drug under study and the formulation (Estévez *et al.* 2000; Baena *et al.* 2011). Not only these tests are very useful in the design and development of new formulations, but also for the toxicity detection (Roberts *et al.* 2002) and the control of quality (Fu and Kao 2010). The Franz diffusion cells are usually used with human skin or from extirpated animals. However, when the biological skin is not available, synthetic membranes can be used. One of the functions of the synthetic membranes employed in the studies of drug diffusion is simulation of the skin (Chen Y *et al.* 2014; Chen J *et al.* 2015). The Food and Drugs Administration has suggested that the simple porous

synthetic membranes are adapted to evaluate the yield of the topical formulations, since they act like a support, but they are not restrictive barriers of the speed (Shiow-Fern *et al.* 2010).

Nylon membranes, obtained from GE Osmonics, were studied as a candidate for artificial bilayer lipid membranes to also study the effect of non-uniformity of the pore geometry, surface roughness and uneven membrane thickness on the formation and stabilization of artificial bilayer lipid membrane (BLM) (Tien H *et al.* 2003).

In this work a synthetic membrane was used to simulate the behavior of a biological membrane, in order to measure the physical-chemical parameters controlling modulation and liberation of MTZ in association with different enhancers. Diffusion processes were studied applied to the delivery of pharmaceutical used compounds whose therapeutic dose is well-known (Barry 1983). The *in vitro* system allowed to establish the physical-chemical parameters for development of a possible transdermal formulation.

To ensure that a formulation is safe, toxicity studies were carried out at different doses and times of exhibition using laboratory animals. The measured answer is the death of the organisms in study and the results are expressed as lethal dose 50, the dose that produces the death of 50% of the animals. For these studies, animals are used to perform experiments whose results can be extrapolated to humans. Experiments differ in duration and frequency of the exhibition, administration road, used species of animals, period of observation, among other variables (Johnson and Finley 1980; Jover *et al.* 1992).

Generally, the most significant result obtained from a toxicity assay, is the percentage of tested organisms specifically affected by each treatment. The result obtained in this way, it is a measure of the toxicity of the agent on the test organisms under the conditions of the bioassay, in other words, a measure of the susceptibility of the tested organisms to the toxic agent (Gutiérrez *et al.* 2007; Alvarez *et al.* 2012).

Therefore, this study evaluated the possible acute toxicity of the enhancers in order to establish its real scope and limitations as well as an eventual therapeutic window. (Mascotti *et al.* 2008; Jofré *et al.* 2013).

## Methods and materials

### Delivery studies

**Materials:** Metronidazol Parafarm®, Propyleneglycol Parafarm®, Urea Parafarm®, Tween 20 Parafarm®, anhydrous monoacid potassium phosphate (Biopack)\*, diacid potassium phosphate (MERCK)\*, sodium chloride (Biopack)\*, cellulose acetate membrane Osmonics INC, automatic sampler Microette System (Hanson- Research),

spectrophotometer UV-Vis (Shimadzu UV-160). \* Indicates that they are ingredients of phosphate buffer saline (PBS) pH: 7.4.

**Methods:** The control formulation was prepared by dissolving 1% MTZ in water as a vehicle. The different enhancers were added to the control solution, all at a concentration of 10%.

Delivery experiments were performed, by sextuplicate (n=6), by using automatic sampler Microette System (Hanson- Research) with 1.767 cm<sup>2</sup> area Franz-type diffusion cells (Figure 2). Cellulose acetate membranes were mounted between the donor and receptor compartments of the diffusion cells. Membranes was pre-treated with PBS for 12 h, with the aim of reducing the latency time and favoring the maximum diffusion of the active principle. Then, 0.4 mL of formulation containing MTZ (1%) was placed in the donor compartment. PBS, pH=7.4, was used as the receptor phase, to simulate the internal environment. All the system was maintained at 32 ± 0.5°C with a circulating water jacket and magnetic stirring (180 rpm). At predetermined intervals, 100 µL of receptor phase were removed and replaced with an equal volume of fresh receptor solution, keeping the sink conditions. 100 µL of these samples were brought to 2.6 mL of final volume and were analyzed by UV-VIS spectrophotometry at 320 nm, making use of a calibration curve previously constructed with solutions of increasing concentrations of MTZ in PBS. At the working concentrations, compliance with Beer's Law, was verified by determining the molar absorptivity of the drug ( $\epsilon = 8115.55 \text{ L mol}^{-1}\text{cm}^{-1}$ ). Cumulative corrections were made to determine the total amount of drug permeated at each time interval.

## Toxicity assay

**Acute toxicity assay:** it was used the technique recommended by the US Fish and WildLife Service by Johnson and Finley (1980) which was modified to use a smaller amount of test compounds, as was reported by Mascotti *et al.* (2008). *Poecilia reticulata* fish were obtained in our laboratory, reproduced from adult specimens purchased in commercial establishments.

Experiments were carried out using solutions of MTZ (Concentrations from 250 to 1000 mg/L) and of the different enhancers at the same concentrations used in the release tests, alone and associated with the active principle, to the mentioned concentrations.

For the experiments, specimens to 1 – 1,5 cm in length were selected, which showed favourable signs of vitality considering mobility and general external morphology. Ten specimens of *P. reticulata* were exposed for a period of 96 hours to each concentration of MTZ (Concentrations from 250 to 1000 mg L<sup>-1</sup>) and of the different enhancers. Solutions and specimens were placed in a 2 L vessel (ratio of 1 specimen per 200 mL of water) where they were kept until the end of the evaluations. The numbers of dead specimens in each container were removed every 24 h. The percentage of mortality was evaluated at 96 h. The minimum concentration of formulas that produced 100% mortality (MC 100% M) and the maximum concentration that did not cause mortality (MC 0% M) were determined.

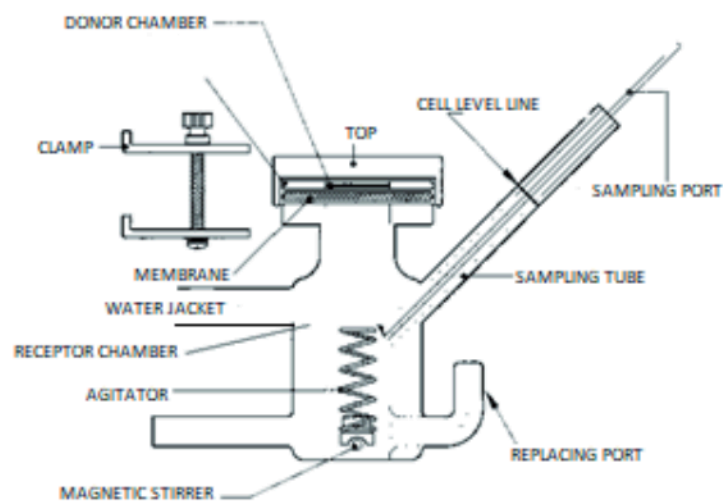


Figure 2: Franz-type diffusion cell.

## Results and discussion

### Delivery studies

Many mathematical models have been used to describe the absorption kinetics through membranes. These models are based on the laws of diffusion and on the study of the compartments of the organism (Roberts *et al.* 1998; Roberts *et al.* 1999). Passive diffusion of a molecule through a membrane depends on its concentration gradient, from the outermost to the deepest layer. Under such conditions and in a first approximation, the transport characteristics through membranes can be deduced from the diffusion properties using Fick’s laws as the theoretical basis for the transport of a molecule.

Fick’s second law predicts how diffusion produces concentration to change with time:

$$\frac{\partial c}{\partial t} = D \cdot \frac{\partial^2 c}{\partial x^2} \quad \text{Equation (1)}$$

where:

C: is the concentration of substance per area unit ( $\mu\text{g m}^{-2}$ ).

t: time(s).

D: is the diffusion coefficient ( $\text{m}^2 \text{s}^{-1}$ ).

x: is the thickness of the membrane ( $\mu\text{m}$ ).

In the above description D is the mean diffusion coefficient, representative of an inert membrane.

Table 1 reports the results obtained for MTZ in water and with PG, Urea and Tween 20 enhancers. The delivery profiles were obtained by graphing the accumulated amounts of MTZ per unit area as a function of time.

**Table 1:** Experimental values of MTZ permeation through a membrane.

Time (min)	Accumulative amounts of MTZ per unit area $\mu\text{g cm}^2 \times 10^{-3}$			
	MTZ	MTZ + PG	MTZ + UREA	MTZ + TWEEN
0	0	0	0	0
60	0.70	3.57	5.46	4.30
120	1.02	6.39	6.14	7.61
180	1.50	7.96	7.07	8.23
240	2.10	9.57	9.91	9.57
300	3.03	10.33	11.21	10.38

From equation 1, is possible to obtain the amount of active principle accumulated per area unit (M) (Roberts *et al.* 1998; Roberts *et al.* 1999), using equation 2:

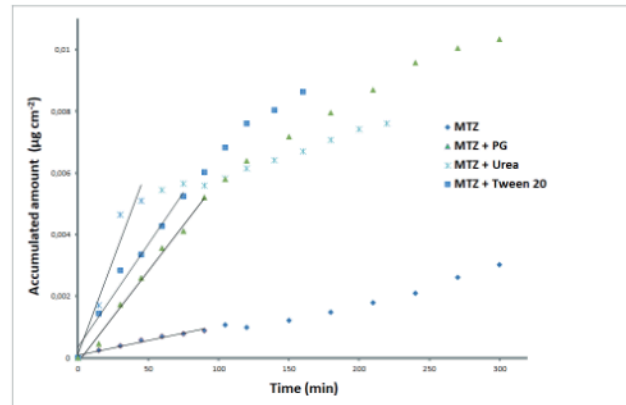
$$M = \frac{D \cdot K \cdot C_0}{x} \cdot t \quad \text{Equation (2)}$$

where:

K: partition coefficient

$C_0$ : Active principle concentration at the donor compartment

MTZ – water delivery profiles, and the profiles of the combination with studied enhancers. are shown in Figure 3.



**Figure 3:** Delivery profiles of MTZ in water through synthetic membranes without urea, tween 20 y PG as enhancers.

The permeability coefficients (P) was obtained from the slope of the straight part of Equation 2 representation,

$$P = \frac{C_0 \cdot K \cdot D}{x} \quad \text{Equation (3)}$$

Table 2 shows the values of permeability coefficients and linear regression obtained for the active principle, using different enhancers. It is observed that combinations with the different enhancers tested significantly increase the permeability coefficient.

**Table 2:** Permeation coefficient values and linear regression.

Formulation	Permeability coefficient ( $\mu\text{g s}^{-1}$ )	R <sup>2</sup>
MTZ	$9.66 \times 10^{-6}$	0.97
MTZ + PG	$5.89 \times 10^{-5}$	0.99
MTZ + TWEEN	$6.70 \times 10^{-5}$	0.97
MTZ + UREA	$1.53 \times 10^{-4}$	0.99

The delivery effect of penetration enhancers can be expressed in terms of an increase ratio (Equation 4):

$$RA = \frac{\text{coefficient of permeability in the presence of enhancer}}{\text{coefficient of permeability in the absence of enhancer}} \quad \text{Equation (4)}$$

The MTZ formulation added with 10% urea presented an RA of 15.84.

The values for each formulation tested are shown in Table 3.

**Table 3:** Relationship of MTZ release with enhancers.

Formulation	RA
MTZ	1
MTZ + PG	6.10
MTZ + TWEEN	6.94
MTZ + UREA	15.84

### Toxicity assay

MTZ concentrations from 250 to 1000 mg L<sup>-1</sup>, did not present acute toxicity in the experimental model used and under the indicated methodology.

Subsequently, toxicity for the different enhancers and associated with MTZ at a concentration of 500 mg L<sup>-1</sup> was determined; such results are shown in Table 4.

**Table 4:** Acute toxicity results of the evaluated compounds

Compounds	% of mortality			
	24 h	48 h	72 h	96 h
Urea (5 g L <sup>-1</sup> )	0	0	0	0
Tween 20 (5 g L <sup>-1</sup> )	100	-	-	100
Propyleneglycol (5 g L <sup>-1</sup> )	0	0	0	0
Urea (5 g L <sup>-1</sup> ) + MTZ (500 mg L <sup>-1</sup> )	0	0	0	0
Tween 20 (5 g L <sup>-1</sup> ) + MTZ (500 mg L <sup>-1</sup> )	100	-	-	100
Propyleneglycol (5 g L <sup>-1</sup> ) + MTZ (500 mg L <sup>-1</sup> )	0	0	0	0
Control	0	0	0	0

The toxicity test is standardized for a duration of 96 hours according to Johnson WW *et al.* United States Department of the Interior Fish and Wildlife Service. Washington D.C. Handbook of Acute Toxicity of Chemicals to Fish and Aquatic Invertebrates). In those cases, in which mortality occurs earlier, the time is reported and in the event that it is 100% after 96 hours, the data is repeated.

## Conclusions

*In vitro* methods control laboratory conditions and elucidate particular factors that modify drug penetration, although delivery may show variations *in vivo* tissue.

In this study, we use a Nylon membrane provided by Osmonic. Despite working in an aqueous medium with a hydrophilic solute, we chose a hydrophilic membrane taking special care that the concentration gradient was very favourable to the passage from the donor and receptor compartment in a Franz diffusion cell.

From the *in vitro* delivery studies of MTZ through a synthetic membrane, in the absence and presence of enhancers, it can be concluded that delivery is influenced by the intrinsic enhancer activity of the enhancer and the physicochemical compatibility between it and the active principle.

The values of permeability coefficients, calculated in the present study for MTZ using water as vehicle and different enhancer (PG, Urea, Tween 20) show that the different combinations with the tested enhancers considerably improve the permeability coefficient. The incorporation of urea increases the P value approximately 16 times that obtained with the solution of MTZ.

It is interesting to point out that aqueous MTZ itself shows no toxicity at all assayed concentrations.

Delivery through the synthetic membrane is influenced by the intrinsic promotion activity of the enhancer and the physical-chemistry compatibility between this and the active principle.

Although Tween 20 presents an increase in the permeability coefficient, its use as enhancer is not advisable due to its high toxicity. However, urea and PG enhance the permeability coefficient and did not show toxicity at the assayed concentrations.

These preliminary studies will guide towards solving some therapeutic problems for percutaneous administration and the development of pharmaceutical strategies to increase the adequate availability of active principle.

## Acknowledgment

Authors thank San Luis National University for financial support.

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