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Miniaturized Extraction Techniques in Drug Biotransformation Studies by using Endophytic Fungi *Cunninghamella elegans*

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In addition to predicting biotransformation in humans, drug biotransformation studies are important because they can generate active metabolites or new intermediates with possible use by the pharmaceutical industry. Endophytic fungi of the genus *Cunninghamella* can metabolize many drugs in a similar way to humans. The analysis of these metabolites requires prior treatment of the samples in order to obtain compatibility with the detection system and the separation technique. This work aimed to study the biotransformation of the drugs duloxetine (DLX), citalopram (CIT) and amlodipine (ANL) by endophytic fungi *Cunninghamella elegans* ATCC 9245, the development of microextraction methods in the context of green chemistry, and the validation of the analytical methods for drugs and their respective metabolites. The bioanalytical method by HPLC was successfully developed and validated for both drugs (DLX, CIT and ANL). The metabolites of DLX and CIT obtained by biotransformation studies were not detected in the conditions of this study, but it was possible to visualize one additional peak in the chromatogram analysis of ANL indicate that this drug has been metabolized by *Cunninghamella elegans*. Furthermore, it has been developed two liquid-liquid microextraction methods achieving success by removing drugs in both techniques, with recovery greater than 90%. The use of Quality by Design (QbD) through the experimental design provided the best conditions for carrying out biotransformation and micro extraction studies of drugs from the fungal matrix.

Keywords: biotransformation; endophytic fungi; miniaturized techniques; green chemistry; metabolites.

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Introduction

Biotransformation studies are important, especially in biological systems, for the synthesis of chemical compounds, such as drugs and metabolites, through biotechnological and analytical methodologies (1).

Drug biotransformation studies are important because, in addition to predicting biotransformation in humans, they can generate active metabolites or new intermediates with possible use by the pharmaceutical industry. Considering that metabolites are also therapeutically active, but specific stereoisomers can cause undesirable side effects, the study of their biotransformation by endophytic fungi can be useful in obtaining metabolites in enantiomerically pure form to be used in clinical and toxicological studies (2).

Fungi of the genus *Cunninghamella* can metabolize many drugs in a similar way to humans, especially those belonging to the classes of antidepressants, antibiotics, steroids, alkaloids, anti-inflammatories and some related substances (3-6).

In parallel, biotransformation reactions have great potential due to their ability to degrade polluting compounds and recover chemically compromised environments and are therefore also considered in the context of "green chemistry" (7,8).

In this context, the miniaturization is used both in the treatment of samples and in the simplification of the entire analytical process and it can be achieved by improving already established conventional systems such as liquid-liquid extraction or solid phase extraction or by completely developing new techniques (9,10,11).

Therefore, the use of techniques focused on miniaturization strongly influences the development of green analytical methodologies and procedures that contribute to reducing the use of toxic solvents and reagents, the amount of waste generated, as well as reducing energy consumption, through reducing the time spent on analyzes (12,13).

In the present study, the drugs duloxetine (DLX), citalopram (CIT) and amlodipine (ANL) were chosen because they are metabolized via cytochrome P450, have been used for a long time in Brazil and have metabolites identified by in vivo studies, but not in in vitro biotransformation studies using fungi.

Experimental

Chemicals and reagents

Purchase of duloxetine hydrochloride and amlodipine besylate (standard chemical reference substances) were from the American Pharmacopoeia (USP) - purity declared of 100.0%. Laboratório Cristália (Brazil) kindly provide a standard chemical reference (purity declared of 100.1%) of Citalopram hydrobromide (raw material). Sample preparation was carried out with methanol purchased from

Vetec® (Rio de Janeiro, Brazil). We used the solvents acetonitrile (ACN) HPLC grade (Tedia®, Brazil) and ultrapure water (Millipore®, France) to prepare mobile phase and the pH adjustment was done with 85% phosphoric acid and trimethylamine (Merck® - Darmstadt, Germany).
When executing and conducting the microextraction tests, we used the following solvents: dichloromethane (Dinâmica®, Brazil) chloroform and hexane (Shynt®, Brazil).
The composition of the mobile phase, optimized for the analysis of the drugs in this study, was: Triethylamine 0.5% pH 3.0: Acetonitrile (60:40 v/v), flow rate 0.8 $mL.min^{-1}$ in isocratic mode, temperature 25 °C and sample injection volume of 10 µL.

Instrumentation

For the drug's biotransformation, an incubation oven (1298 Model, De Leo®) for fungal growth, an autoclave (C Model, Webeco®) for sterilization of materials, and a culture media orbital shaker (Innova 2300 Model, New Brunswick Scientific®) were used.
In the analytical method development, Shimadzu® model 20-A high-efficiency liquid chromatography equipment was used equipped with a CBM20A controller system, LC-20AT flow pump, SIL-20A/C automatic injector, oven and photo diode array. LCSolution software (version 1.25) performed control and data acquisition. The chromatographic column, used in the optimized and final condition of the experiments, was a C_{18} (150 x 2.5 mm; 5 µm particle diameter), Zorbax from Agilent®.

Biotransformation assays

Among the factors that can influence the growth and the drug biotransformation assays are the composition of the culture medium, the drug diluent, temperature and agitation of the reaction medium. Incubation of the strain under constant agitation of the biotransformation system determines fungal development in pellet format, resulting in a larger contact surface of the fungus with the medium (Figure 1). In this way, it is possible to provide greater exposure of the fungus to the drug, as well as to provide better oxygen supply in the reaction medium (14,15).
Fungal strains were maintained, through periodic maintenance subcultures, on Sabouraud agar and incubated at a controlled temperature of 27 °C ± 1 °C. The working inoculum was prepared from the maintenance strain and, we performed the inoculum standardization using a UV spectrophotometer at 570 nm with transmittance of 68 to 70% at approximately 10^7 $CFU.mL^{-1}$.
The assay incubation took place under controlled conditions of constant agitation (120 rpm) in Czapek liquid culture medium for 48 hours.

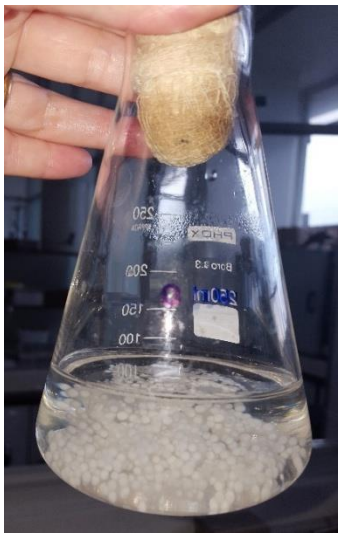
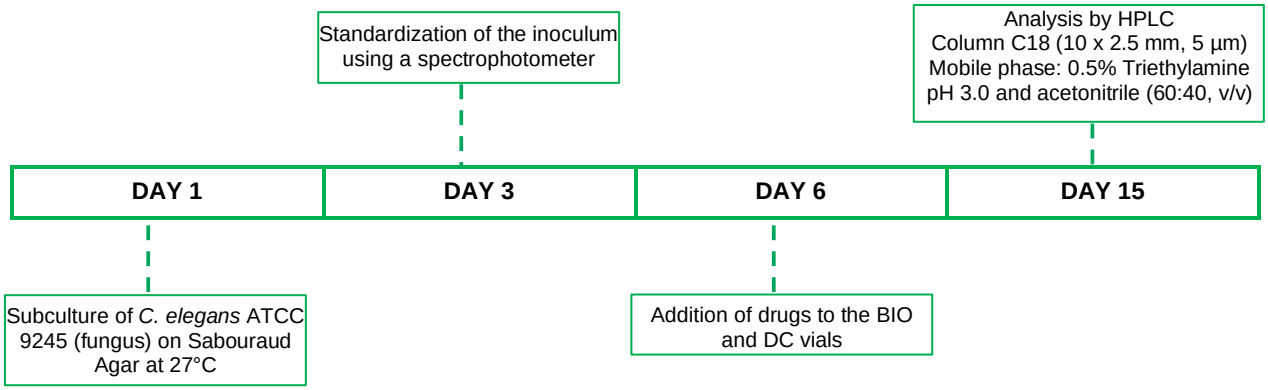


Figure 1. Demonstration of fungal growth in the form of pellets in the culture medium.

After 48 hours of culturing the standardized inocula, the drugs solubilized in methanol were incorporated separately, at a concentration of 100.0 µg.mL-1, into the Czapek culture medium and the standardized inoculum.
After 3-day incubation period, it is possible to verify drug consumption and metabolite formation. 2.0 mL aliquots were collected in falcon tubes and frozen until analysis, 24 hours after adding the drugs.
The biotransformation process (BIO) was monitored with control samples taken simultaneously: drug (DC), microbiological (MC) and culture medium control (BL). The analyses were performed by HPLC. The biotransformation study for all drugs was carried out according to Figure 2 below.

Drug Microextraction

The microextraction of the analytes from the culture media preceded the chromatographic separation analysis and, in addition to eliminating possible interference, allowed concentration (increase in signal-to-noise ratio) of the analytes of interest. The extraction step was characterized by the development of two protocols: one for the Dispersive Liquid- Liquid Microextraction technique (DLLME), involving the use of disperser solvents miscible in the extracting solvent (organic phase) and the sample (aqueous phase), and another for the Hollow Fiber Liquid Phase Microextraction technique (HF-LPME) - employing hollow cylindrical membranes. Several commercially available organic solvents were evaluated as extracting solvents. Among them, were tested: dichloromethane, chloroform, and hexane (DLLME); diethyl ether, methyl-tert-butyl ether, and octanol (HF-LPME). The solvents tested as disperser solvents (favoring the interface of the organic solvent with the aqueous phase containing the drugs) were acetonitrile and methanol. After determining the ideal extraction conditions for each technique and each drug,
the analytes present in the culture medium were treated according to the established protocols.



VIAL	CZAPEK MEDIUM	DRUG	INOCULUM
BIOTRANSFORMATION (BIO)	✓	✓	✓
DRUG CONTROL (DC)	✓	✓	
MICROBIOLOGICAL CONTROL (MC)	✓		✓
BLANK (BL)	✓		

Figure 2. Schedule of the biotransformation studies for the drugs duloxetine, citalopram, and amlodipine.

DLLME

Separately, an aliquot of the stock solution of each drug was added to the Czapek culture medium, reaching a final concentration of 200 µg.mL^{-1}. The microextraction process comprised a biphasic system containing the organic (1.0 mL) and aqueous (2.0 mL of culture medium enriched with the drug standard) portions. The mixture was vigorously stirred for 1 minute (considered optimal agitation time). After stirring, phase separation of the biphasic system was observed, with the formation of the "cloud point" (Figure 3). The formation of this "cloud" is important to facilitate the separation and visualization of the organic and aqueous phases. Thus, the necessary amount of aqueous phase (containing the drugs) was collected with a micrometric pipette - lower phase in the falcon tube. After removing the aqueous phase, the drug recovery from the extraction was evaluated using HPLC analysis. The entire extraction process was carried out in a fume hood.

HF-LPME

The microextraction process was conducted using a three-phase system consisting of a donor phase (aqueous solution containing the drugs), an acceptor phase (an acidic or alkaline solution that promotes migration of the drugs through the hollow polypropylene membrane), and an organic solvent used to immobilize the membrane pores, facilitating the interface and migration of the analytes between the donor and receptor solutions. The three-phase system was assembled by removing 2.0 mL of the culture medium enriched with the drug standard and placing it in a test tube containing a magnetic stirrer. This solution was alkalized with a concentrated solution of sodium hydroxide. The hollow fiber was cut, immobilized by the most suitable solvent, and immersed in the donor solution present in the tube. Finally, the membrane lumen was acidified with hydrochloric acid to promote the migration of the analytes of interest from the donor solution into the membrane. After the stirring time, the acceptor solution was removed from inside the membrane with a micropipette, diluted appropriately in the mobile phase, and analyzed by HPLC.

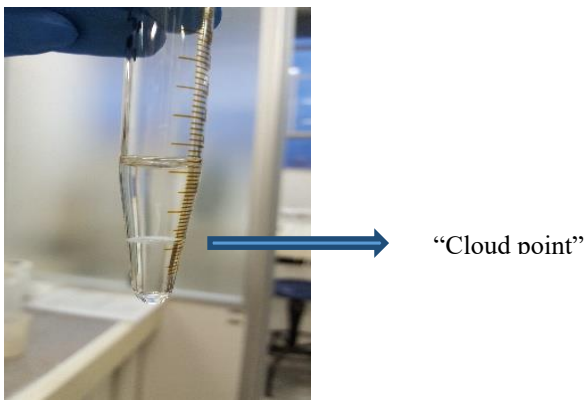


Figure 3. Visualization of the "cloud point" - interface between the aqueous phase and the organic phase - inside the glass falcon tube.

Application of Quality by Design (QbD)

To achieve better results for both the microextraction and biotransformation methods, the most suitable conditions

for conducting the studies were optimized using experimental modeling assays. The parameters that had the greatest influence on the evaluated methodologies were determined. This evaluation is essential because it can comprehensively understand the experiments and the critical attributes related to the studied factors (16). Experimental designs and statistical analysis were performed using the MODDE® 13 software (Sartorius Stedim Biotech, Umetrics, Sweden).

Results and Discussion

Bioanalytical validation by HPLC

This work focuses on developing a bioanalytical method to analyze the three drugs separately. After preliminary tests and optimization of the analytical methodology, the chromatographic conditions adopted for the analysis of the drugs and their metabolites were mobile phase containing 0.5% triethylamine (pH 3.0): ACN (60:40, v/v), C_{18} Zorbax Agilent® column (150 x 4.6 mm, 5 µm), injection volume of 10 µL, a flow rate of 0.8 mL.min^{-1}, and oven temperature of 25 °C.

Selectivity was confirmed through chromatographic analysis at 230 nm for duloxetine and 238 nm for citalopram and amlodipine of samples of Czapek culture medium enriched with drugs and medium with endophytic fungal growth. As shown in Figure 4, no interfering peaks appeared at the same retention times of the respective analytes.

The linearity of the method was evaluated through the construction of three analytical curves obtained by fortifying the Czapek culture medium to 100% with standard solutions of the drugs in the concentration range of 20 to 60 µg.mL^{-1}. Precision and accuracy parameters were determined by evaluating intraday (n = 9) and interday (n = 3) precision and accuracy. At concentrations 20, 40 and 60 µg.mL^{-1}, samples of 100% Czapek culture medium were fortified with standard drug solutions. The baseline noise value of the chromatograms of the standard curve points (triplicates) was the parameter used to calculate the limits of detection and quantification.

The validation results for each drug are in Table 1. All results found are within limits permitted to validate bioanalytical methods and attest to the analytical reliability of the method used.

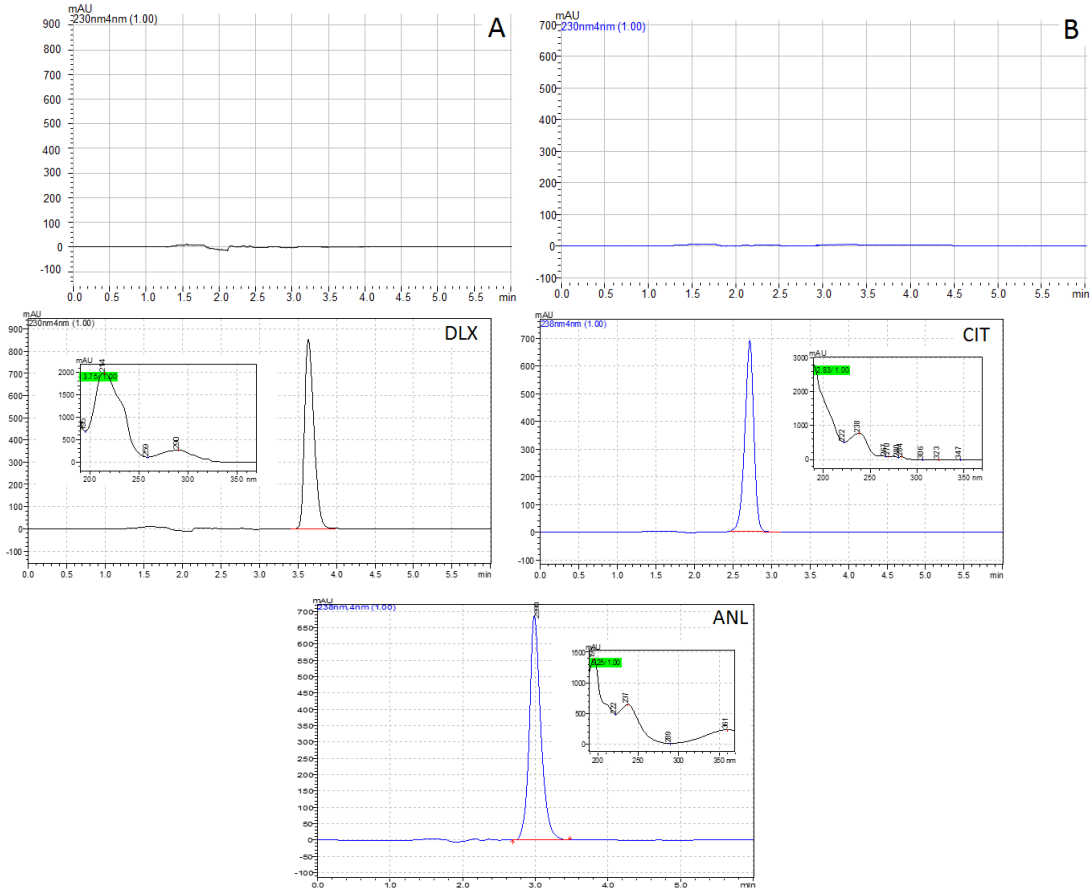


Figure 4. Chromatograms representing the vials containing Czapek culture medium (Blank) (A) and the microbiological control (medium + inoculum) (B). Chromatograms related to the analyzed drugs: duloxetine (DLX) at 230 nm, citalopram (CIT), and amlodipine (ANL) at 238 nm. Chromatographic conditions: mobile phase 0.5% TEA (pH 3.0): ACN (60:40, v/v), Agilent® Zorbax C18 column (150 x 4.6 mm, 5 µm), injection volume 10 µL, flow rate: 0.8 mL.min^{-1}, and oven temperature at 25°C.

Table 1. Validation Results of the HPLC analytical method for the drugs duloxetine (DLX), citalopram (CIT), and amlodipine (ANL).

		DLX	CIT	ANL
Linearity range (μg.mL^{-1})		20 – 60 μg.mL^{-1}	20 – 60 μg.mL^{-1}	20 – 60 μg.mL^{-1}
R^2		0.9983	0.9978	0.9965
RSD* Intraday (%)		2.84	3.30	3.59
RSD Interday (%)		3.00	2.79	3.24
Accuracy ± RSD (% recovery)	20 μg.mL^{-1}	99.62 ± 2.44	98.80 ± 3.58	97.20 ± 3.38
	40 μg.mL^{-1}	98.92 ± 3.51	98.22 ± 3.08	98.99 ± 4.25
	60 μg.mL^{-1}	98.05 ± 3.46	99.72 ± 2.85	98.97 ± 4.56
Detection limit (μg.mL^{-1})		0.02	0.05	0.03
Quantification limit (μg.mL^{-1})		0.18	0.32	0.35

* RSD = Relative Standard Deviation

Biotransformation study of the drugs duloxetine (DLX), amlodipine (ANL) and citalopram (CIT)

Preliminary biotransformation tests were conducted according to the methodology described in the Biotransformation Assays section. After numerous tests modifying the concentration of the culture medium between 50% and 100% and varying the volumes of each medium in 25 mL, 50 mL, 100 mL and 150 mL, we obtained the best conditions for carrying out the assays, for each of the drugs, according to below:

DULOXETINE: 100% Czapek, medium volume (50 mL)
ANLODIPINE: 100% Czapek medium volume (50 mL)
CITALOPRAM: Czapek medium 50%, medium volume (150 mL)

At the end of the incubation period for each drug with *C. elegans*, there was a reduction in its concentrations in the culture medium, demonstrating the consumption of the drugs by the microorganism; however, for the drugs duloxetine and citalopram, we did not find additional chromatographic peaks. As for amlodipine, there was the formation of an additional peak in the chromatogram (time of 2.7 minutes retention; resolution of 2.138), demonstrating that the drug underwent biotransformation by the filamentous fungus used, indicating a possible metabolite (Figure 5).

Drug Microextraction

The selection of basic analysis conditions to achieve maximum selectivity with adequate efficiency was made from preliminary screening experiments. In these experiments, the critical parameters of the microextraction process were selected. Initially, for the evaluation of these critical parameters, experimentally designed analyzes were conducted from the D Optimal design containing 14 analyzes for each drug at two levels, where variations were tested in the following parameters: proportion of organic solvents, collection volume, extraction time, salt addition, medium volume, and medium concentration. After this screening, the biotransformation step was optimized using only variations in the proportion of the solvent dichloromethane and the volume of the culture medium by a central composite design factorial. Analyzing the recovery graphs "Contour Plot" - Figure 6 and "Design Space" (Figure 7), the volumes of medium and percentage of dichloromethane needed to obtain the best microextraction results were established. In Figure 6, the probability of failure (%) of the executed method is indicated, with the point signaled in the green region of each of the graphs representing the location where the chance of error is lower, and the model is then optimized. According to the presented graphs, the best conditions found for the drugs were as follows: duloxetine (100 mL of medium and 50% dichloromethane), citalopram (50 mL of medium and 70% dichloromethane), and amlodipine (100 mL of medium and 70% dichloromethane).

DLLME

A general protocol was established for the dispersive liquid-liquid microextraction procedure, with different solvent proportion conditions for each of the drugs as follows:

•DULOXETINE: dichloromethane: acetonitrile (50:50)
•CITALOPRAM: dichloromethane: acetonitrile (70:30)
•AMLODIPINE: dichloromethane: acetonitrile (70:30)

For all drugs, the extraction time was 2 minutes, centrifugation time was 5 minutes, and the total volume of the solvent mixture was 1.0 mL.

DLLME PROCEDURE PROTOCOL:
• Alkalization of 2.0 mL (drug + medium) with 2M NaOH (100 μL) - obtaining pH 13.0.
• Addition of the best solvent ratio (extractor:dispersant) totaling 1.0 mL.
• Vortex agitation for the specified time for each drug.
• Centrifugation for 5 minutes.
• Visualization of the cloud point and removal of the lower phase (bottom of the tube).

• Dilution in methanol and filtration through a 0.45 µm membrane for specific vials with subsequent analysis by HPLC.

HF-LPME

Similarly to DLLME, a general protocol was established for the microextraction procedure of the drugs from the culture medium, as follows.

HF-LPME PROCEDURE PROTOCOL:

• Cutting the membrane to the appropriate size for analysis (7 cm).
• Immobilization of the membrane for 30 seconds in octanol.
• Addition of the extraction solution (20 mM HCl) inside the membrane - 20 µL for a 7 cm membrane) - pH 2.0.
• Addition of the alkaline solution (20 mM NaOH) to the donor solution (sample) - 300 µL - pH 14.0.
• Assembly of the system in U (membrane) to start microextraction in a test tube with a magnetic stirrer.
• Total extraction time: 90 minutes.

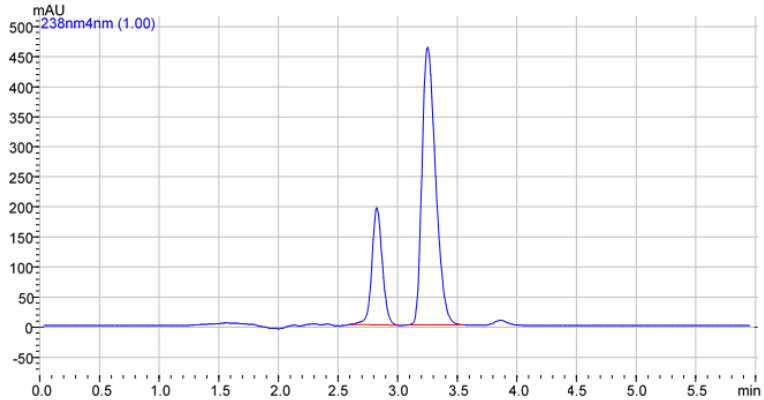


Figure 5. Chromatogram obtained after biotransformation and microextraction of the drug amlodipine. Retention time of ANL: 3.2 minutes; retention time of the obtained metabolite: 2.8 minutes. Resolution between peaks: 2.138.

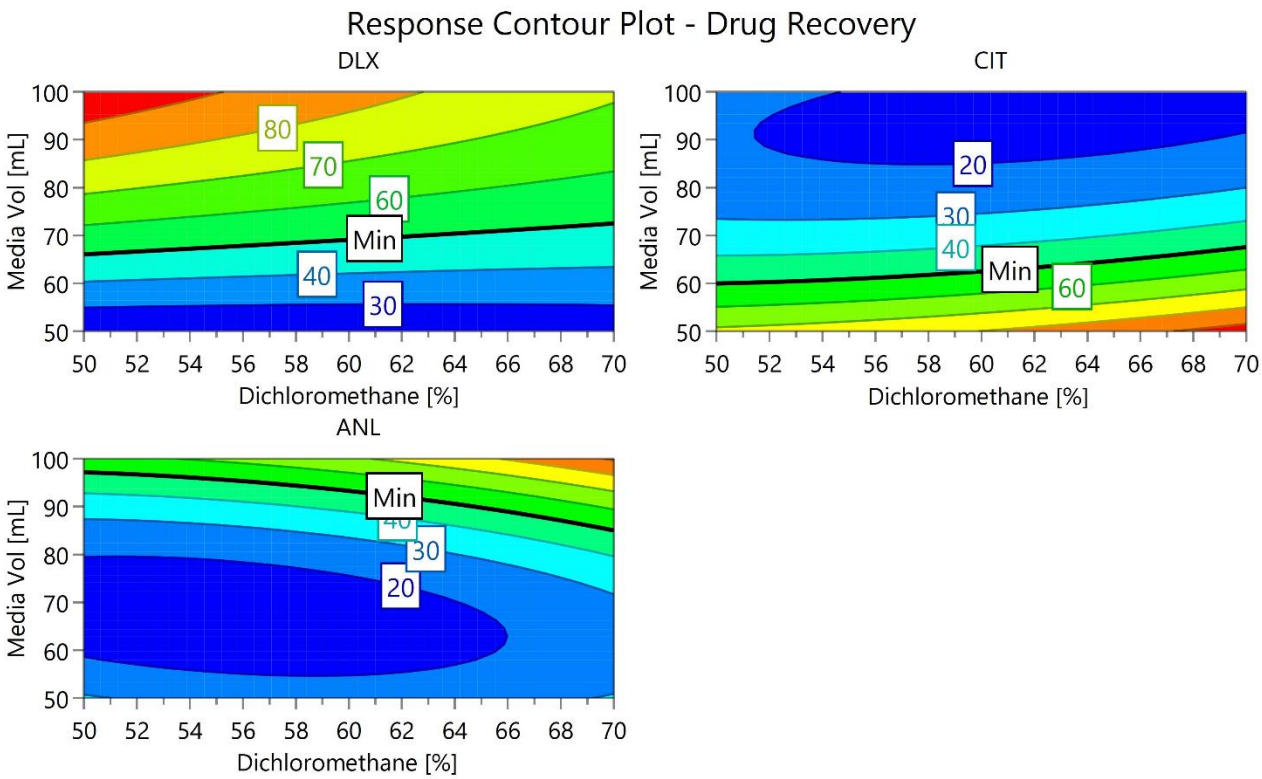


Figure 6. Evaluation of recovery percentages from the culture medium by Contour Plot. The graph shows the results found for the drugs DLX (duloxetine), CIT (citalopram), and ANL (amlodipine) when plotting the volume of medium versus the percentage of dichloromethane.

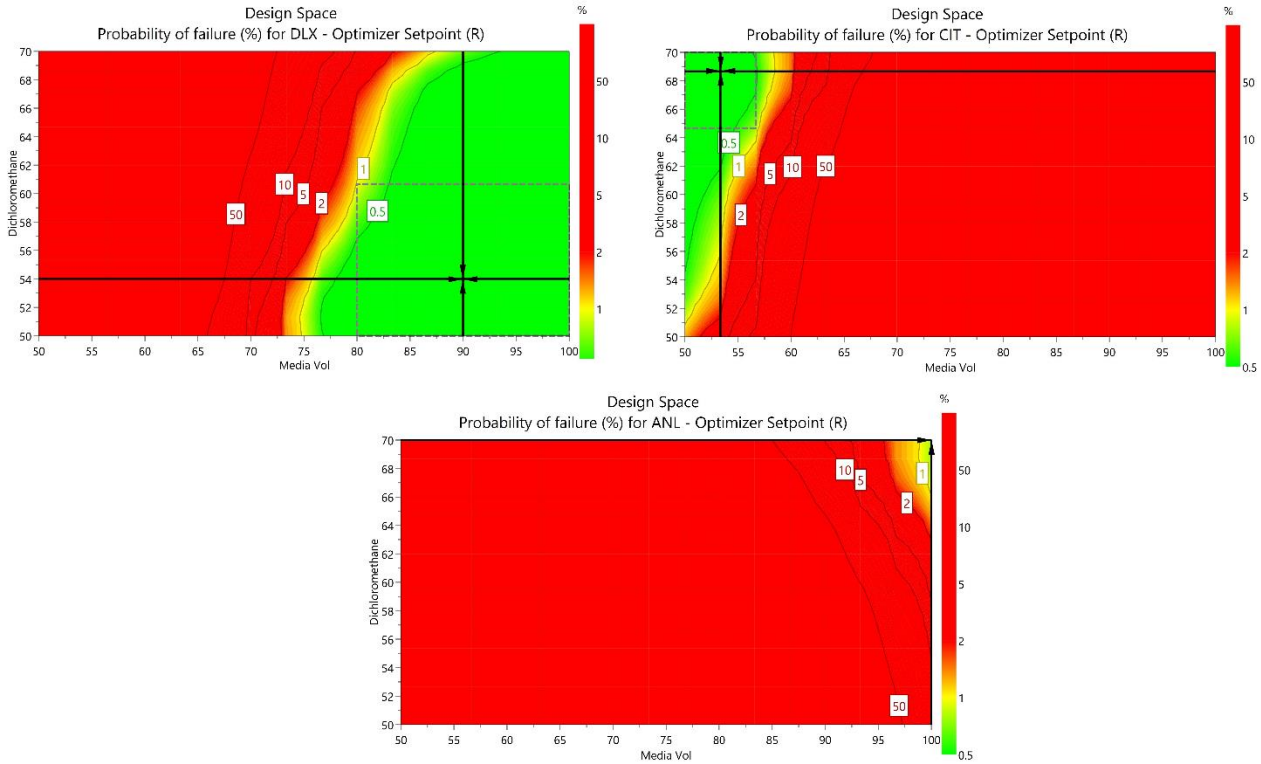


Figure 7. Evaluation by Design Space indicating the probability of failure of the experimental model for each drug (DLX, CIT, and ANL).

Conclusions

The bioanalytical method validated by HPLC proved to be specific, linear, sensitive, precise, and accurate for simultaneous analysis of the drugs duloxetine, citalopram, and amlodipine resulting from biotransformation by endophytic fungi of the genus *C. elegans* ATCC 9245 in Czapek culture medium. Additionally, the development of a single method for the evaluation of drugs and their potential metabolites minimized the amounts of solvents and residues in the context of green chemistry.

Although in this study, the metabolites of duloxetine and citalopram were not detected by biotransformation, enzymatic metabolism represented by CYP450 isoforms in microorganisms represents a potential possibility of using other fungal species to obtain derivatives of these drugs.

The results presented in the biotransformation study of amlodipine indicate that it was metabolized by *C. elegans*. There was a presence of a signal indicative of biotransformation in the chromatographic analysis performed by HPLC. However, further complementary assays are necessary for the structural elucidation and characterization of the metabolite found in the biotransformation of the drug amlodipine by *C. elegans*. Furthermore, analyses of drug control (DC) did not present these peaks, excluding the hypothesis of them originating from endogenous products of the fungus itself.

In the context of microextraction, protocols for two liquid-liquid extraction methods (DLLME and HF-LPME) were developed aiming to use small amounts of solvents, successfully extracting the drugs in both techniques, with recoveries exceeding 90%.

Finally, using Quality by Design (QbD) through experimental design enabled obtaining the best conditions for biotransformation and microextraction studies of drugs from the fungal matrix, allowing the successful development of the aforementioned methods for the drugs under study.

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Conflict of interest

The authors declare no conflicts of interest.

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