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Article

Captopril oral solution for pediatric use: formulation, stability study and palatability assessment *in vivo*

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The aim of this work was to develop an oral solution of captopril at 5 mg/mL preservative-free. Two formulations were prepared, one containing sweetener (formulation 1) and the other without this excipient (formulation 2). The results found of validation parameters from analytical method performed by HPLC for captopril were, linearity 0.9998, the limit of detection 15.71 µg/mL, the limit of quantification 47.60 µg/mL, repeatability 1.05%, intermediate precision 2.42%, accuracy intraday 101,53%, accuracy inter-day 99.85%. Moreover, the results found for captopril disulfide were, linearity 0.9999, limit of detection 0.65 µg/mL, limit of quantification 1.96 µg/mL, repeatability 2.28%, intermediate precision 1.51%, accuracy intraday 101.36%, accuracy inter-day 100.29%. The appearance of formulations was clear and colorless, pH measures were 3.12 and 3.04, dosage of captopril and captopril disulfide were 99.45% and 99.82%, 0.24% and 0.12% for formulation 1 and formulation 2, respectively. The stability study demonstrated that the concentration of captopril and captopril disulfide in the formulations was > 90% and below 3%, respectively. The *in vivo* palatability study in animals and humans showed that Formulation 1 containing the sweetener had better acceptance. Thus, the sweetener was able to improve the unpleasant taste of the formulation.

Keywords: Captopril. Oral liquid. Pediatrics. Drug stability. Taste.

INTRODUCTION

Captopril is an antihypertensive drug of the class of angiotensin-converting enzyme inhibitors orally administered to adults and pediatric patients. Captopril is widely used to treat hypertension and pediatric heart failure condition (Pabari *et al*., 2012). It is necessary to observe the dose in infants and children, the initial dose of captopril may cause severe hypotension. An initial dose of 100 µg/kg is recommended and does not exceed 6.5 mg, along with 2 hours of blood pressure monitoring. If well tolerated, the drug will be administrated at doses of 100 to 300 µg/kg, 2 to 3 times daily, increasing if necessary, to a maximum of 6 mg/kg/day. In children from 1 month to 1 year, the maximum dose should not exceed 4 mg/kg/day (Martin *et al*., 2012).

Captopril is commercially available in the Brazilian market as a tablet in dosages 12.5, 25, and 50 mg, but not as an oral solution of captopril. To adjust the pediatric dose is necessary to break and crush the tablet, mix the tablet powder with a diluent and filler the capsules in a compounding pharmacy. The capsule containing the captopril powder is opened at the time of use and the powder is mixed with food, drink (fruit juice), or vehicle (water) prior to administration. The practice of opening a capsule and add the powder in an inappropriate vehicle (food or drink), normally causes a few problems such as

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difficulty to mask the unpleasant taste of the powder which may cause the patient to reject and decrease adherence to treatment. Furthermore, drug interaction with food may cause decrease absorption leading to low blood plasma concentration and compromise the pharmacological action; suspension formation when administered to patients with a nasogastric tube, may cause obstruction (Glass, Haywood, 2006; Méndez *et al*., 2006; Nahata, 1999).

Oral liquid solutions are ideal for administration in pediatric patients because of the following advantages: dose-easing, the possibility to mask unpleasant taste, simple preparation, ease of swallowing, prevention of drug-food interaction when food or beverage is used as a vehicle (Martin *et al*., 2012; Glass, Haywood, 2006; Silva *et al*., 2019). However, there are some restrictions on the use of some preservatives in oral solutions for pediatric use. Oral solution preservative-free is indicated to premature infants, newborns, and children under 6 months (Cuzzolin, 2018). Many preservatives cause problems in pediatric patients. Benzyl alcohol is a bacteriostatic preservative used in oral solutions. Mortality of newborns has been described by the use of an oral solution containing benzyl alcohol (Gershanik *et al*., 1982; LeBel *et al*., 1988). Thus, benzyl alcohol syndrome is a recognized event in newborns being contraindicated for premature infants and newborns. (Valeur, Holst, Allegaert, 2018; Graham, Turner, 2011) reported neurotoxicity according to the European Study of Neonatal Exposure to Excipients, for benzoic acid and sodium benzoate which causes metabolic acidosis. Propyl parabens as propyl hydroxybenzoate and propyl parahydroxybenzoate are a source of problems in oral solutions for pediatric use, due to the low elimination capacity. Cuzzolin, (2018) and Graham and Turner, (2011) described constant administration of oral solutions containing propyl parabens causes serious problems of hyperbilirubinemia and osteogenic effects in premature infants and newborns. Thus, the production of oral solution preservative-free is always recommended whenever possible. Preservatives-free formulations stored under refrigeration to limit microbial growth have a short shelf life. The maximum shelf life of 7 days at 2–8°C is assigned to the preservative-free oral solution unless a work involving microbiological and chemical stability studies has been carried out to guarantee the extended shelf life (Jackson, Lowey, 2010; Cuzzolin, 2018).

There is no commercially available oral liquid solutions of captopril in the Brazilian market due to problems of stability of this compound in aqueous solution. The development of a preservative-free oral solution of captopril for pediatric patients involves microbiological and chemical stability studies.

The palatability study is an important parameter in the development of formulation. Psychophysical evaluation by human taste panel is most widely used and is a standard method for measuring the extent of taste masking and individual acceptance of taste and flavor. However, concerns for human health, encouragement for tasting unpleasant compounds, drug toxicity, and individual taste perception may influence the study of palatability. Recruitment of volunteers is complex and slow and may not contain enough participants. An alternative for rapid assessment of palatability is taste-masking through the use of electronic tongue; however, such equipment is expensive and is not available in most analytical laboratories and hospitals (Noorjahan, Amrita, Kavita, 2014).

The evaluation of palatability using animal models preferably in rats has been accepted because the taste perception of rats is similar to humans (Anand *et al*., 2007). Rats can classify compounds with a similar, different, pleasant, or unpleasant taste. Rats exhibit repulsive responses when stimulated with unpleasant taste compounds (Winshaw, Kolb, 2004). In addition, the animal model assists in the selection of the best taste-masked formulation, reducing the number of samples for the in vivo palatability study in human volunteers (Noorjahan, Amrita, Kavita, 2014).

The aim of this work is to develop an oral solution of captopril at 5 mg/mL preservative-free and evaluate the presence and absence of the sweetener. In addition, standardizing the analytical method for captopril and captopril disulfide by High-Performance Liquid Chromatography (HPLC), characterize the formulations (appearance, pH, captopril and its main degradation product), perform microbiological and chemical stability study and evaluate *in vivo* palatability.

MATERIAL AND METHODS

Material

The raw materials ascorbic acid and disodium ethylenediaminetetraacetic acid (EDTA) were obtained from Henrifarma (Brazil). Saccharin sodium was obtained from Farmos (Brazil). Captopril (98% by HPLC) was obtained from Sigma-Aldrich. Water for injection used in the preparation of liquid formulations was obtained from JP Farma (Brazil).

In the analytical method by HPLC, the following reagents (Analytical Grade) were used: hydrochloric acid, phosphoric acid, sodium hydroxide, methanol, hydrogen peroxide, copper sulfate that were obtained from Tedia (Brazil). Captopril disulfide (code: 1091221), United States Pharmacopeia (USP Reference Standard) was obtained from Sigma-Aldrich. Ultrapure water (Milli-Q purification system, Millipore, USA) was used for the preparation of the standard and mobile phase. The mobile phase was filtered on a cellulose membrane (0.45 µm, Sartorius). Analytical curve solutions were filtered on 0.20 µm filters (Cromafil Xtra PES).

METHODS

Preparation of formulations

Table I contains the composition of the formulations. Captopril, ethylenediaminetetraacetic acid (EDTA), and saccharin sodium were weighed separately on an analytical balance. Captopril and the pharmaceutical excipients were dissolved using water for injection (JP Farma, Brazil). Immediately after preparation, Formulation 1 and Formulation 2 were filtered directly into the sterile amber glass vials using sterling filters (pore = 0.22 µm, 33 mm, Millex® Syringe Filters, Durapore®, PVDF) in a laminar flow hood Class II B2l, AB2-3S1, Airstream®). The flasks were closed for use in chemical characterization, chemical and microbiological stability, and evaluation of *in vivo* palatability.

TABLE I - Composition of the captopril formulations

Formulation	Captopril (g)	EDTA (g)	Saccharin sodium (g)	Water (mL)
1	0.5	0.1	0.1	qs 100
2	0.5	0.1	-	qs 100

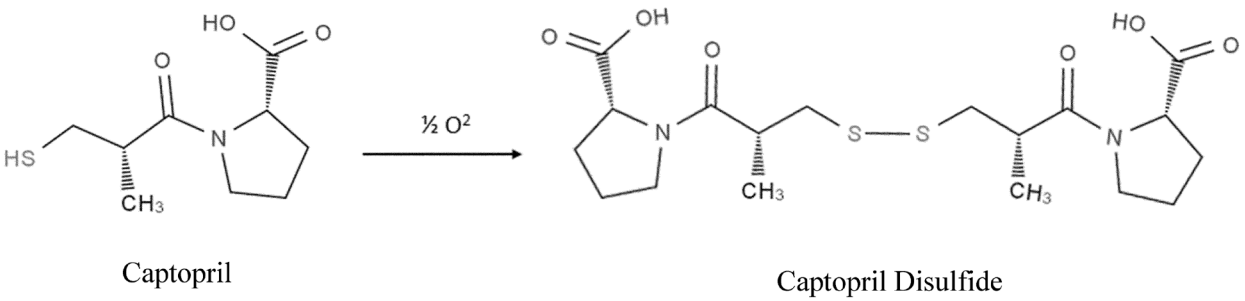
pH

Samples were analyzed in a potentiometer Meter Model 922, previously calibrated with standard buffer solutions. The pH measurements were performed in triplicate by inserting the electrode directly into the sample at a temperature of 25°C without prior dilution.

Analytical methodology
HPLC

The quantification of captopril in oral solutions was performed by HPLC based on a method validated and described in the Brazilian Pharmacopeia (5th ed., 2010) for the analysis of captopril raw material and captopril tablet. The quantification of the captopril disulfide (degradation product), (Figure 1) was also performed on the captopril solutions developed and submitted to the stability study. The limit established by the Brazilian Pharmacopeia (5th ed., 2010) and United

States Pharmacopeia (USP 35th ed., 2012) for captopril is 90-110%. In addition, the content of the captopril disulfide must be < 3% according to the United States Pharmacopeia (USP 35th ed., 2012)



Captopril

Captopril Disulfide

Figure 1 - Oxidative degradation of captopril in captopril disulfide.

The equipment used in the quantification of captopril and captopril disulfide was a Gilson chromatograph (United States), consisting of a pump model 321, UV-Vis 152 detector, 506C interface, and Rheodyne injector (model 7725) of 50 µL. The program used to control the functions of the liquid chromatography was UniPoint version 3.0. The chromatographic column used was an octadecyl (C18) HPLC column (Supelco, 300 × 3.9 mm, 5 µm particles; Sigma-Aldrich). The composition of the mobile phase was 50% (v/v) 0.1% phosphoric acid and 50% (v/v) methanol. The mobile phase was filtered using a cellulose membrane (0.45 µm, Sartorius) prior to use. The ratio of isocratic flow was 1.0 mL/min. The temperature used was ambient (25°C). Detection was performed with a UV-Vis detector at the wavelength of 220 nm. The volume of injection was 50 µL and injected manually.

Standardization of the quantification method

The validation of the method shall ensure that the results obtained are reliable and reproducible. The analytical methodology for quantification of the captopril was developed and validated by Brazilian Pharmacopeia (5th ed., 2010) and United States Pharmacopeia (USP 35th ed., 2012). Thus, standardization of an analytical method for captopril and captopril disulfide was based on criteria established by Resolution nº. 166 (Agência Nacional de Vigilância Sanitária, 2017). The parameters analyzed were selectivity, linearity, limits of detection and quantification, repeatability, intermediate precision, accuracy, and robustness.

Selectivity

A sample of captopril standard at a concentration of 1,000 µg/mL in the mobile phase was compared with a sample of captopril raw material at the same concentration. In addition, the captopril 1,000 µg/mL standard solution was contaminated with a 30 µg/mL of captopril disulfide solution and compared to a sample containing only captopril disulfide. In addition, a sample containing captopril, captopril disulfide, and the vehicle of formulation 1 was analyzed to verify the interference of components.

Linearity

The minimum acceptable criteria of the correlation coefficient (r) should be greater than 0.990. In order to check the linearity of captopril, three analytical curves with six different concentrations were determined. A stock solution containing 2 mg/mL of captopril was prepared using mobile phase as diluent. Then six solutions were prepared in the concentrations of 400, 600, 800, 1.000, 1.200, and 1.400 µg/mL. To check the linearity for captopril disulfide were prepared three analytical curves with six different concentrations and were determined. A stock solution containing 0.5 mg/

mL of captopril disulfide was prepared using mobile phase as diluent. Then six solutions were prepared in the concentrations of 12, 18, 24, 30, 42, and 54 µg/mL were prepared. In addition, were obtained correlation coefficient (r), angular coefficient (a), and coefficient of determination (R^2). The linearity results were verified by analysis of variance one-way ANOVA with the post-test Tukey and a significance level of α=0.05 (95% of the confidence interval). The analysis of variance (ANOVA) allowed analyzing the linearity of the method and validity of the linear regression. Residue analysis was performed for evaluation of the homoscedasticity. The statistical tests mentioned were applied to values with a normal distribution. The limit of detection (LD) and quantification (LQ) was estimated based on the following equations:

$LD = 3.3 \times \alpha / IC$
$LQ = 10 \times \alpha / IC$

Where α is the standard deviation of the y-intercept of the calibration curves; IC is the mean slope of three calibration curves.

Precision (repeatability and intermediate precision)

Repeatability was assessed on the same day with six different solutions of captopril (n=6 determinations) at a concentration of 1000 µg/mL and 30 µg/mL of captopril disulfide. Intermediate precision: samples were prepared in two different days, the first day with six different solutions of captopril (n=6 determinations) at a concentration of 1,000 µg/mL and 30 µg/mL captopril disulfide, and the second day the samples were prepared the same manner as for the first day and analyzed by different analysts. Precision was expressed as relative standard deviation (coefficient of variation).

$RSD=SDMCD \times 100$

Where RSD is the relative standard deviation, SD is the standard deviation and MCD is the mean concentration determined, not exceeding 5%.

Accuracy

The accuracy was performed using samples of captopril at concentrations of 800, 1000, and 1200 µg/mL, and of captopril disulfide at the concentrations of 24, 30, and 36 µg/mL were analyzed, representing levels of concentrations low (80%), medium (100%) and high (120%). For each concentration level, six different solutions (n=6 determinations) were injected.

The accuracy of an analytical method must be obtained by the degree of agreement between the individual results of the method under evaluation in comparison with a value accepted as true Resolution nº. 166 (Agência Nacional de Vigilância Sanitária, 2017).

Accuracy = experimental *mean concentration* / *Theoretical concentration* × 100

Robustness

For the determination of robustness, samples of standard captopril were analyzed in the concentration of 1 mg/mL and captopril disulfide 30 µg/ml. Variations in parameters of flow, temperature, and mobile phase concentration were evaluated for robustness. Table II shows the comparison between the conditions and factors considered.

TABLE II - Variations in analytical parameters for determining robustness

Parameters	Normal Condition	Robustness
Temperature (ºC)	28	40
Flow (mL/min)	1	1.1 – 0.9
Mobile phase: phosphoric acid (0.11%): methanol (% v/v)	50:50	45:55

Microbiological Analysis

The microbiological analyses of the formulations were carried out according to the procedures of the microbiological tests for non-sterile products described

in the Brazilian Pharmacopeia (5th ed., 2010). 10 mL of oral solution was transferred to 90 mL of sodium chloride-peptone buffer pH 7.0. Successive dilutions (1: 100 and 1: 1000) were prepared using the same diluent as described above. The soya bean agar and Sabouraud-dextrose agar media were prepared, sterilized and 20 mL were placed in Petri dishes. After solidification of the media, 0.1 mL of the diluted formulation was applied to the surface of each medium. The Petri dishes containing casein-soy agar were incubated at 32.5°C for 5 days, and the others with Sabouraud-dextrose agar were incubated at 22.5°C for 7 days for the determination of total aerobic microorganisms and yeasts and molds, respectively. The number of colonies forming units (CFU) was counted using a colony counter (CP-600 PLUS, PHOENIX).

Stability study

The stability was evaluated for 30 days, during which time the formulations 1 and 2 were stored under refrigeration at 5ºC. Samples were collected at times 0, 7, 14, and 30 days. Appearance, pH, and sildenafil citrate content were evaluated. Characterization, quantification of the captopril, and degradation product (captopril disulfide), pH, and microbiological analysis were carried out.

Forced degradation studies

The tests should promote degradation greater than 10% and less than the complete degradation of the drug (Agência Nacional de Vigilância Sanitária, 2015). Formulation 1 was submitted to a forced degradation in the following conditions: acid (HCl 0.1N), basic (NaOH 0.1N), presence of oxidant agent (H_2O_2 1% v/v), medium with the metallic ion (copper sulfate 0.25% w/v), and high temperature (80°C) for 2 hours. After exposure of formulation 1 to various stress conditions described above, the concentration of captopril and captopril disulfide were analyzed by HPLC.

In vivo palatability study

Human

The taste evaluation of the oral liquid formulation of captopril was carried out in order to determine the palatability and acceptability. The methodology of this test was adapted from the methodology described by Noorjahan, Amrita, Kavita, (2014) and approved by the Ethics Committee of the University Hospital Clementino Fraga Filho, with CAAE number 66723317.2.0000.5257. Fifteen health research participants were selected, with no history of diseases that interfere with the palate, both sexes, aged between 20 and 55 years, adequate relation weight-height, able to provide their free and enlightened consent (in writing) for voluntary participation in the study. Research participants with a history that during the study present signs or symptoms in the mouth, burning sensation, contact allergy associated with some component of the formula were excluded. Smoker patients, alcoholics, and severe disease sufferers were also excluded.

The test was carried out with formulation 1 containing sweetener and with formulation 2 without sweetener for the purpose. Volunteers received a sheet containing a Score Table (supplementary material) and asked to give notes on a gradual scale of 1-5. A score of 1 indicated a very bad taste formulation, or intolerable, and score of 5 indicated an excellent taste formulation. In Score Table, (supplementary material) participants classified the taste of captopril solutions in insipid, sweet, sour, bitter, metallic, astringent, and salty. In addition, participants reported whether after 1 minute of tasting of the samples felt or not any residual taste.

The test was performed in five steps: 1- rinse the oral cavity, 2- taste calibration with water, 3- tasting sample 1 with subsequent rinsing of the oral cavity and 15 minutes interval, 4- tasting sample 2. An interval of 15 minutes was applied between sample tasting. The method is not invasive and there is no need to ingest the samples during evaluation. Volunteers were not aware of the composition of samples 1 (Formulation 1) and 2 (Formulation 2) regarding respect to the ability to mask the unpleasant taste of the drug. Each volunteer was advised to refrain from eating and drinking for 1 hour

before the test. All material used was individualized and disposed of after the test. Each volunteer tested two samples, one containing a solution of the drug in water and another solution of the captopril in the vehicle with the ability to mask the unpleasant taste. The flowchart of the test steps is shown in Figure 2.

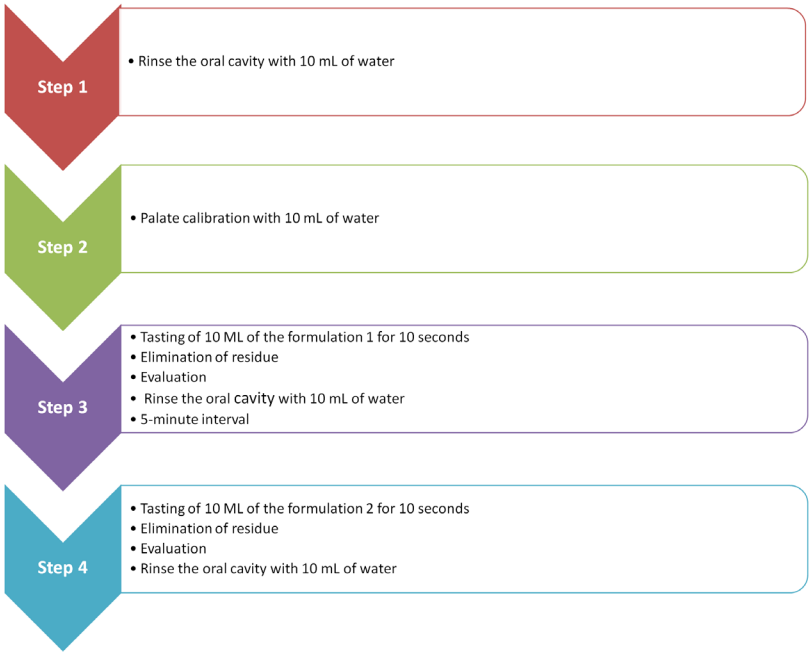


Figure 2 - Flowchart of human palatability test steps.

Each level of taste assessment (1- intolerable, 2- unpleasant, 3- acceptable, 4- good, 5- excellent) was converted to a percentage (%) whereas the total number of participants that were fifteen is equivalent to 100%. If the sum of the levels acceptable, good and excellent is, greater than 80% there is confirmation of the hypothesis that the sweetener containing saccharin was able to make the solution of the captopril palatable. The sum of the good and excellent levels to the acceptable level is possible because that is the lowest level.

Animals (rats)

The *in vivo* taste assessment using an animal model was adapted from previous studies described in the literature (Boughter *et al.*, 2002). The assessment of formulations acceptability involves measuring the response of the trained animal to a negative stimulus with exposure to an unpleasant or positive taste with exposure to a pleasant taste. The normal consumption of water should also be considered as a neutral or basal stimulus. The evaluation of the response to a positive, negative or neutral stimulus is based on the amount of volume of liquid consumed by the rat during the study (Boughter *et al.*, 2002; Breslin *et al.*, 1993). The condition of water deprivation is used to motivate rats to taste unpleasant taste compounds and thus obtain a measurable amount of formulation consumption. The consumption of water in mL can be considered the neutral stimulus for purposes of comparison with negative or positive stimuli, which use unpleasant taste solution or pleasant taste solution, respectively (Noorjahan, Amrita, Kavita, 2014).

The test was performed with 24 male Wistar rats weighing 300-350g and approved previously by the Ethics Committee of Experiments on Animals of the Fluminense Federal University (number 916). The experiments are in accordance with the Ethical Principles of Animal Experimentation of SBCAL. On the first day, the water

was taken out for liquid fast for 22 hours. On the second day, was offered 100 mL of water for 30 minutes, and then consumption was measured. Then, water was offered to all the animals in free demand for 90 minutes. After this period, the water was again withdrawn for a new fast of 22 hours. This procedure was repeated with water in another day, for gauging and training the animals, and the fasting of liquids remained among all the days of the experiment. On the fourth day, the animals were separated into 4 groups (n = 6) and 100 mL from different solutions were offered for each group: sucrose 40 mg/mL, caffeine 0.8 mg/mL, captopril 5 mg/mL (Formulation 2) and captopril 5 mg/mL (Formulation 1) consumption measured in volume after 30 minutes. The volumes of basal water consumption, sucrose solutions (positive stimulus), caffeine (negative stimulus), formulation 1 (taste-masked) and formulation 2 were measured in mL and compared by statistical analysis to verify the difference in the consumption of liquids in relation to taste, in order to evaluate the acceptability of taste-masked formulation.

Data Analysis

The experimental results were expressed as an average of standard deviation (SD), subjected to statistical analysis using the software GraphPad Prism 6 for Windows. The statistical analysis was performed by applying the Student T-Test for comparison of two groups or ANOVA (analysis of variance) for comparison of three or more groups ($p< 0.05$).

RESULTS AND DISCUSSION

Analytical Methods

The results of the analytical method validation are summarized in Table III.

TABLE III - Analytical Method Validation Results

Parameters	Captopril	Captopril Disulfide	Acceptance Criteria ANVISA (Brasil, 2017)
Selectivity	No interference	No interference	No interference
Linearity	0.9998	0.9999	$R^2 \geq 0.990$
Lq (µg/mL)	47.60	0.65	-
LD (µg/mL)	15.71	1.96	-
Precision (%) [a]	1.05	2.28	<5%
Precision (%) [b]	2.42	1.51	<5%
Accuracy (Recovery in %) [c]	101.53	101.36	100 ± 2%
Accuracy (Recovery in%) [d]	99.85	100.29	100 ± 2%
(80%) Accuracy (RSD) (%) [e] (100%) (120%)	1.05 1.16 0.74	0.95 0.76 0.88	<5%

r: Correlation coefficient
a Precision (RSD) (%) Intraday with n = 6 injections (n = 6 determinations)
b Precision (RSD) (%) Interday with n = 6 injections in 2 days (n = 12 determinations)
c Accuracy (Recovery) intraday com n=6 injections (n=6 determinations)
d Accuracy (Recovery) interday com n=6 injections in 2 days (n=12 determinations)
e Accuracy (RSD) (%) based on 3 concentration levels: low (80%) (800 µg/mL), medium (100%) (1000 µg/mL) and high (120%) (1200 µg/mL) and n=6 injections (n=6 determinations) for each concentration level

The chromatograms of captopril at concentration 1000 µg/mL and captopril disulfide (degradation product) at 30 µg/mL are shown in Figures 3A and 3B, respectively. The retention time for captopril was 4.28 minutes and captopril disulfide was 6.83 minutes. It was analyzed the interference from the components of formulations in the absence of captopril and captopril disulfide as showed in Figure 3C. The chromatogram from Figure 3D shows the captopril and captopril disulfide added to the components of formulations. Through the chromatogram, the profile was possible to conclude that the components from formulations did not interfere with the analysis of captopril and captopril disulfide.

Figure 3D shows the added vehicle of captopril and captopril disulfide. The components of formulation did not interfere with the analysis of captopril and captopril disulfide, as shown in the chromatogram profile.

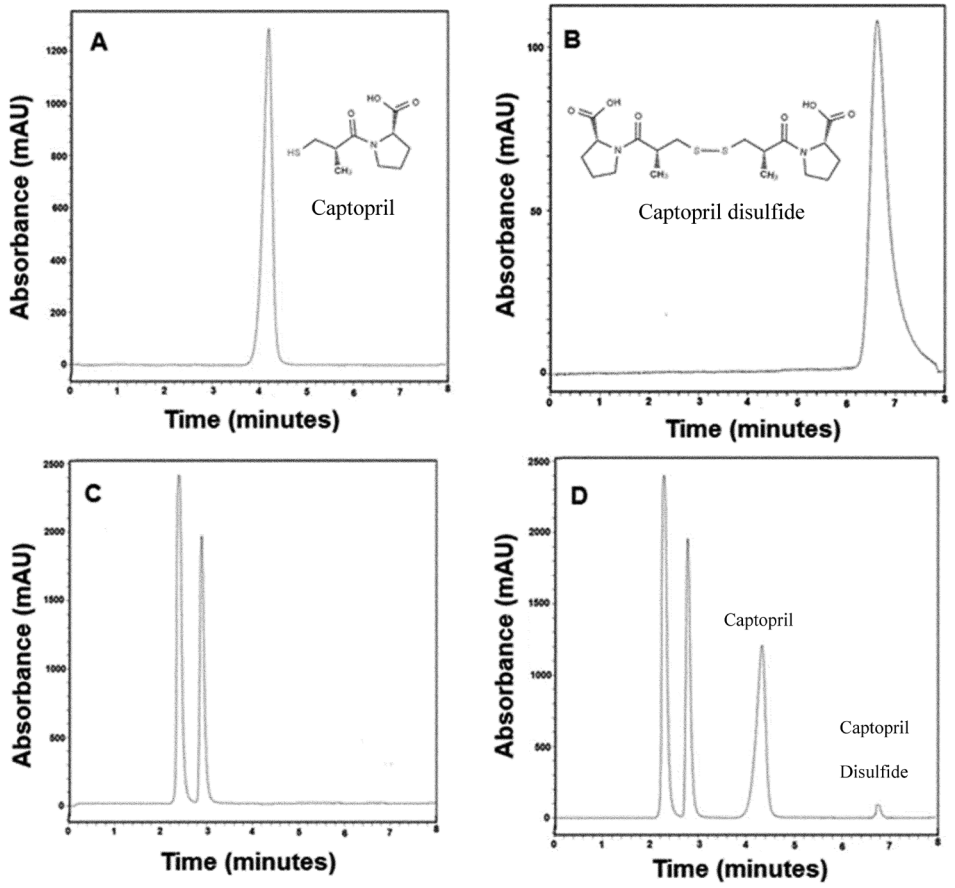


FIGURE 3 - Chromatograms of captopril 1000 µg/mL in the mobile phase (A), captopril disulfide 30 µg/mL in the mobile phase (B), a vehicle without captopril and captopril disulfide dissolved in the mobile phase (C), and vehicle added with captopril and captopril disulfide at concentrations of 1000 µg/mL and 30 µg/mL (D).

The analytical curves of captopril and captopril disulfide were linear in the range of 400 to 1400 µg/mL and 12 to 54 µg/mL, presenting a determination coefficient of 0.9998 and 0.9995, respectively (Table III and Figure 4A and 4B). The results of the determination coefficient are in accordance with the acceptance criteria which is equal or greater than 0.990 as recommended by Resolution nº. 166 (Agência Nacional de Vigilância Sanitária, 2017). The analysis of variance (ANOVA) allowed evaluating the linearity of the method and the

validity of the linear regression for captopril and captopril disulfide. The peak area was divided by the corresponding concentration, and then the values obtained were analyzed by the test one-way ANOVA with the post-test Tukey using a significant level of α=0.05 (95% of the confidence interval). In relation to captopril, the value found for $F_{(calculated)}$ (0.552) was lower than the $F_{(tabulated)}$ (3.239). Moreover, the p-value obtained was 0.65, indicating no significant difference between the results. The results of captopril disulfide showed that $F_{(calculated)}$ (0.293) was lower than $F_{(tabulated)}$ (3.239). In addition, the p-value obtained was 0.829, indicating no significant difference between the results.

Residue analysis was performed on the evaluation of the homoscedasticity. The residue analysis exhibited a random pattern; thus, the homoscedasticity assumption is satisfied indicating a good fit for the linear model (Figure 4C and D).

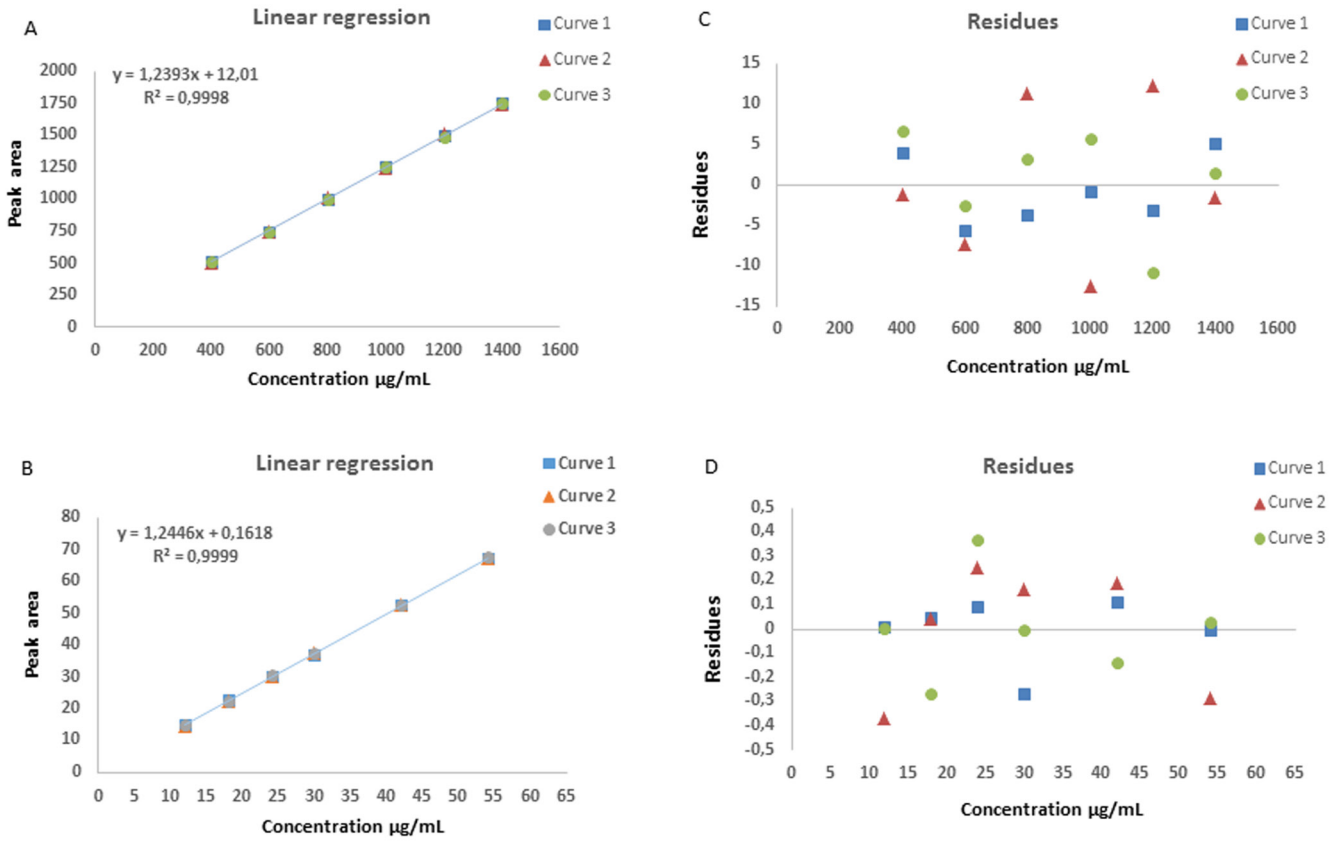


FIGURE 4 - Analytical curve for captopril (A); analytical curve for captopril disulfide (B), residue analysis for captopril (C), and residue analysis for captopril disulfide (D).

The values of the limit of detection (LD) and the limit of quantification (LQ) are shown in Table III. The LD value of captopril was 15.71 µg/mL, while the LQ was 47.60 µg/mL. The LD found for captopril disulfide was 0.65 µg/mL and the LQ was 1.96 µg/mL. The values found are adequate for the proposed method, since the concentration of drug and degradation product in the sample to be analyzed, were above the lower limit of quantification obtained.

The results of precision (repeatability and intermediate precision) and accuracy are shown in Table III. The results of repeatability and intermediate precision for captopril were 1.05% and 2.42% (RSD), respectively, and for captopril disulfide was 2.28% and 1.51% (RSD), respectively. The

results of intraday accuracy for captopril and captopril disulfide based on recovery were 101.53% and 101.36% (RSD), respectively. The values found of accuracy inter-day based on recovery were 99.85% and 100.29% (RSD) for captopril and captopril disulfide, respectively. The maximum value (RSD) found of accuracy based on three concentration levels was 1.16 in the medium level of 100% for captopril. In addition, for captopril disulfide was 0.95% (RSD) in the low level of 80%.

Thus, all values found of precision (repeatability and intermediate precision) and accuracy are in accordance with the acceptance criteria determined by Resolution nº. 166 (Agência Nacional de Vigilância Sanitária, 2017).

The results of the robustness are shown in Table IV. From the results obtained in the test, it was verified that the changes in flow, the composition of the mobile phase, and temperature did not show significant variations in the values obtained for both captopril and captopril disulfide. The RSD obtained was below 5%. The robustness values are in accordance with the acceptance criteria determined by Resolution nº. 166 (Agência Nacional de Vigilância Sanitária, 2017).

TABLE IV - Robustness results

Condition	Captopril		Captopril Disulfide	
	Assay (%)	Retention Time (min)	Assay (%)	Retention Time (min)
	101.04	4.21	101.37	6.89
Normal	100.34 100.48	4.22 4.21	99.96 100.15	6.89 6.86
	100.16	4.01	100.87	6.58
Flow - 0,1 mL/min	101.14	4.00	100.96	6.57
	100.97	4.02	101.25	6.58
Flow + 0,1 mL/min	100.46 100.14 98.99	3.69 3.69 3.72	100.46 100.37 99.95	5.84 5.84 5.88
MP + 5% Methanol	101.09 101.16 101.29	4.68 4.65 4.67	102.67 100.49 100.76	7.38 7.38 7.37
Temperature 40°C	100.39 100.66 99.07	4.20 4.17 4.19	98.32 98.60 100.31	6.19 6.16 6.17
Mean (%)	100.58		100.38	
RSD (%)	0.73		1.12	

RSD-Relative Standard Deviation in %, MP-Mobile phase

It was concluded that the analytical method for quantification of captopril and captopril disulfide indicated selectivity, linearity, precision, accuracy, and robustness within the evaluated parameters.

Formulation and stability study

Captopril solutions were prepared at a concentration of 5 mg/mL and immediately analyzed (See Table V, Time Zero). The water for injection used was to eliminate the

excess of metallic ions. EDTA has been added as a chelating agent to block metal ions that can degrade captopril by enhancing the oxidation reactions. Saccharin sodium was added to the formulation to improve palatability.

TABLE V - Characterization of formulations containing captopril and captopril disulfide

Formulation	Visual Aspect	pH	Assay (%)	
			Captopril	Captopril disulfide
1	Clear and colorless	3.12 ± 0.07	99.45 ± 0.12	0.24 ± 0.04
2	Clear and colorless	3.04 ± 0.05	99.82 ± 0.23	0.12 ± 0.07

The solutions presented a clear and colorless appearance with content close to 100%. The pH of the formulations was acidified close to 3, due to the presence of captopril. Kadin, (1982) reported the stability of captopril in solutions with pH < 3.5. The oxidation is catalyzed by transition metal ions through the recycling of oxygen free radicals. Thus, the presence of EDTA improves the stability of captopril in an aqueous solution. The acute oral toxicity LD_{50} of disodium EDTA is approximately 3.7 g/kg in male and female Winstar rats (Lanigan, Yamarik, 2002). Formulations 1 and 2 presented captopril content above 99% and captopril disulfide content below 3%, these values are in accordance with the requirements of the Brazilian Pharmacopeia (5th Edition). In the microbiological analysis, Formulations 1 and 2 did not present the growth of microorganisms. Preservative-free oral solutions were successfully produced in accordance with good handling practices.

The results of the stability study for Formulations 1 and 2 are shown in Table VI. The captopril content found was greater than 90% and captopril disulfide content below 3%, and no change in the appearance (color, clarity, and odor) was observed for both formulations. At the end of the stability study for 30 days, Formulation 1 exhibited a captopril content of 95.06 ± 0.65%, a captopril disulfide content of 1.09 ± 0.10, and a pH range of 3.05 ± 0.23. While Formulation 2 exhibited a captopril content of 94.89 ± 0.34%, captopril disulfide content of 1.61 ± 0.83, and pH a range of 3.21 ± 0.18. The profile of captopril content decreases and the profile of captopril disulfide increase throughout the time in both formulations. The final captopril content was similar for two formulations (> 90%). A higher concentration of captopril disulfide (degradation product) was found for Formulation 2. The results from both formulations meet with requirements by the United States Pharmacopeia (USP 35th ed., 2012).

Thus, it can be concluded that Formulation 1 with Saccharin and Formulation 2 without sweetener were stable for 30 days at 5°C when stored away from the light.

TABLE VI - Stability study of oral liquid Formulation 1 and Formulation 2 stored in refrigerator at 5oC

Days	Chemical Stability		Microbiological stability	
	Captopril (%)	Captopril Disulfide (%)	Casein-soy agar (CFU/mL)	Sabouraud-dextrose Agar (CFU/mL)
			Formulation 1	
0	99.70±0.84	0.39±0.01	0	0
7	97.21±0.19	0.56±0.19	0	0

(continues on the next page...)

TABLE VI - Stability study of oral liquid Formulation 1 and Formulation 2 stored in refrigerator at 5oC

Days	Chemical Stability		Microbiological stability	
	Captopril (%)	Captopril Disulfide (%)	Casein-soy agar (CFU/mL)	Sabouraud-dextrose Agar (CFU/mL)
15	96.93±0.06	0.61±0.02	0	0
30	95.06±0.65	1.09±0.10	0	0
Formulation 2				
0	99.56±0.34	0.43±0.12	0	0
7	98.10±0.11	0.76±0.89	0	0
15	96.51±0.13	0.88±0.14	0	0
30	94.89±0.34	1.61±0.83	0	0

Mean ± SD of n = 3 determinations. Solutions with remaining captopril content <90% are out of the specifications of 90 to 110% according to the Brazilian Pharmacopoeia (5th Edition) and Pharmacopoeia of the United States (USP 35th ed., 2012). The solutions must have captopril disulfide content <3%, according to the United States Pharmacopoeia (USP 35th ed., 2012). CFU=Colony Forming Units. Formulation 1: solution of captopril with saccharine
Formulation 2: solution of captopril without saccharine

Table VI shows microbiological analysis during stability the study of Formulations 1 and 2. The results did not show microbial growth in the casein-soy agar and Sabouraud-dextrose agar. The formulations stored at 5°C and pH around 3 helped in the preservation of the same. To solution oral preservative-free a maximum shelf-life of 7 days at 2–8°C should be assigned unless a validation work involving microbiological and chemical stability studies have been carried out to guarantee the security of the formulation with extended shelf-life (Jackson, Lowey, 2010; Cuzzolin, 2018). The microbiological stability study confirmed that the formulations when produced by sterile filtration to eliminate the microorganisms and stored for 30 days at 5°C are stable and safe.

Similar studies involving the development of captopril solutions were described in the literature. García *et al.*, (2005) developed a solution containing 1 mg/mL in distilled water suitable for oral administration. The solution in a PVC container, storage at 4°C protected from the light by 30 days was chemically and microbiologically stable. The final concentration of captopril and captopril disulfide found were around 98.1% and 2.8%, respectively. In another study, Allen and Erickson, (1996) prepared oral solutions of captopril 0.75 mg/mL from tablets. The vehicles used for the preparation of solutions were a 1:1 mixture of Ora-Sweet and Ora-Plus, a 1:1 mixture of Ora-Sweet SF, and Ora-Plus and Cherry syrup. The oral solutions stored at 5°C, protected from light, using a 1:1 mixture of Ora-Sweet and Ora-Plus, a 1:1 mixture of Ora-Sweet SF were chemically stable for 14 days. Moreover, in Cherry syrup was chemically stable for 2 days.

In our study, the concentration of captopril in the oral solution was 5 mg/mL, being superior in relation to the studies described. The chemical stability of 30 days found for oral solution in our study was similar to that found by García *et al.*, 2005. Although the captopril disulfide concentration found was higher in the García study (2.8%), almost reaching the 3% limit. Still, both studies did not evaluate the microbiological stability. In addition, our study evaluated the presence and absence of sweetener agent (saccharin sodium) in the formulations during the stability study. The results demonstrated that formulation 1 containing saccharin sodium and formulation 2 in absence of sweetener, both in amber glass vial were stable for 30 days when stored away from the light at 5°C.

Oral liquid solutions for pediatrics may or not contain preservatives depending on age and disease. In the preservative-free oral liquid solutions, microbiological stability studies must prove that the vehicle, container, and storage conditions at 5ºC are able to prevent microbiological growth. Merino-Bohórquez *et al*., 2019 developed an oral liquid solution of clonidine hydrochloride for pediatric patients preservative-free. The preservative-free solution stored at 5±3ºC was chemically and microbiologically stable for 90 days in closed containers and for 42 days after opening.

Forced degradation test

The remaining concentrations of captopril and captopril disulfide after forced degradation are shown in Table VII. The results of the forced degradation test showed the extreme stress conditions were the oxidant, basic and metallic conditions, demonstrating which the chromatographic method is capable of being an indicator of stability. Adverse conditions as acidic medium and high temperature were also able to degrade captopril with increased captopril disulfide.

TABLE VII - Remaining concentrations of captopril and disulfide of captopril in the formulation after the forced degradation test

Condition for degradation	Content (%)	
	Captopril	Captopril Disulfide
Acid medium	24.6 ± 0.5	7.9 ± 1.2
Basic medium	12.9 ± 1.7	17.3 ± 1.1
Medium with oxidant	5.7 ± 0.9	17.4 ± 1.5

(continues on the next page...)

TABLE VII - Remaining concentrations of captopril and disulfide of captopril in the formulation after the forced degradation test

Condition for degradation	Content (%)	
	Captopril	Captopril Disulfide
Medium with metal ions	8.2 ± 0.7	16.4 ± 0.8
Temperature increase	26.3 ± 1.1	8.7 ± 0.6

Mean ± S.D. of 3 determinations

In vivo Palatability test in humans

The objective of the test is to evaluate the palatability of an oral liquid solution of captopril. Samples 1 and 2 were tested and discarded by a non-invasive method, followed by evaluation of taste, type, acceptability, and residual sensation after 1 minute by 15 participants on the research. The results of the palatability test are presented in Figure 5. It was possible through the results to notice that Formulation 1 containing the sweetener was rated by major of the participants as acceptable, good and excellent reaching 93.3%. As the sum of the excellent, good, and acceptable levels is greater than 80%, there is confirmation of the hypothesis that a solution containing saccharin may produce a captopril solution palatable. In relation the Formulation 2, the majority of the participants (67%) classified it as intolerable and unpleasant. The sum of acceptable levels, good and excellent reached the value of 33%. If the sum of the excellent, good, and acceptable levels is less than 80%, there is confirmation of the hypothesis that the captopril solution is not palatable.

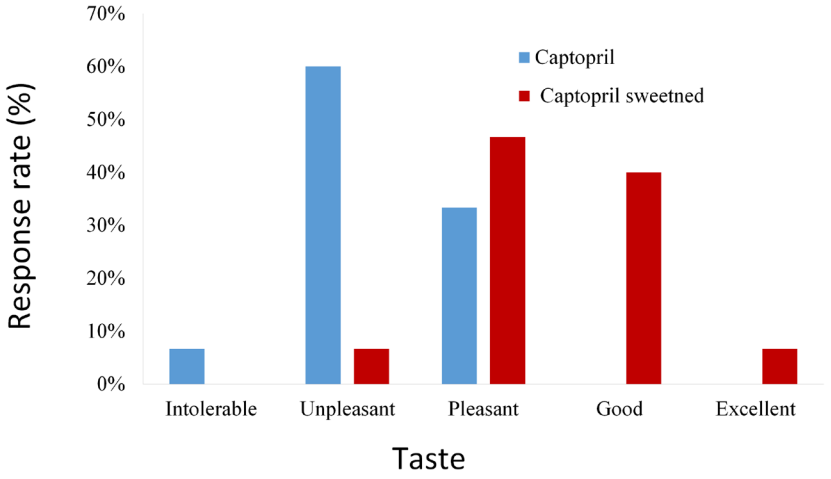


Figure 5 - Result of the palatability test. Formulation 1 corresponds to Captopril sweetener and Formulation 2 corresponds to captopril solution without sweetener. If the sum of the excellent, good, and acceptable levels is greater than 80%, there is confirmation of the hypothesis that the solution is palatable. Number of volunteers: 15.

The results of the taste type evaluation are shown in Figure 6. For Formulation 2, the participants identified mainly acid taste (66.7%). Whereas 13.3 and 6.7% felt bitter or metallic taste, respectively. One participant identified the salted taste (6.7%) and another astringent taste (6.7%). The participants identified residual taste after 1 minute of tasting, indicating the need for a vehicle capable of masking the unpleasant taste of the captopril solution.

An amount of 93.3% of participants rated Formulation 1 as sweet (Figure 6), demonstrating greater palatability and acceptability. For Formulation 1, 80.0% of the participants felt the residual sweet taste after 1 minute and 13.3% did not feel this residual taste. Only 6.7% of the participants classified Formulation 1 as bitter taste, furthermore, the residual taste was felt after 1 minute of tasting.

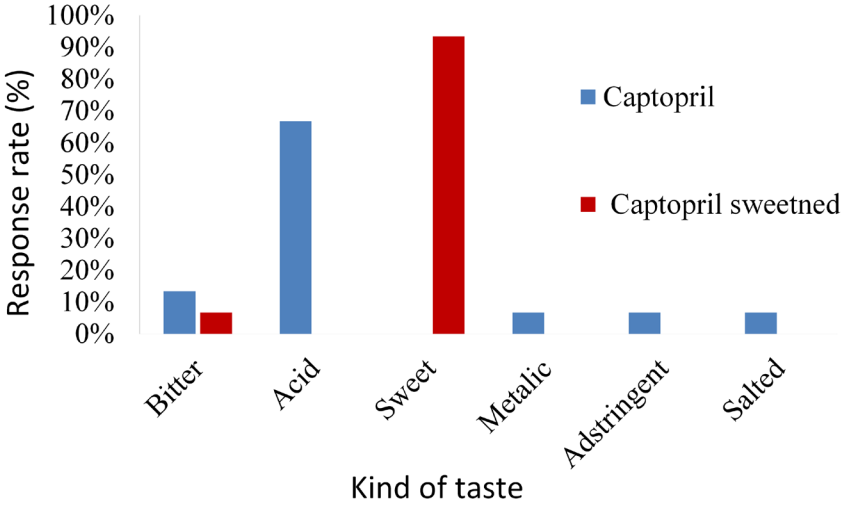


Figure 6 - Results of the taste evaluation. Formulation 1 corresponds to Captopril sweetener and Formulation 2 corresponds to captopril solution without sweetener. Number of volunteers: 15.

Palatability test *in vivo* in rats

The results of the *in vivo* palatability study in rats are shown in Figure 7. The volume of water consumed per day can be considered the basal volume, its taste is tasteless. The consumption of the sucrose solution considered the positive stimulus, was significantly higher than the water consumption ($p < 0.05$). On the other hand, the consumption of the caffeine solution considered the negative stimulus, was unpleasant and significantly lower than the water ($p < 0.05$). In this manner, the rats perceived distinct tastes as pleasant (sweet) and unpleasant taste (bitter).

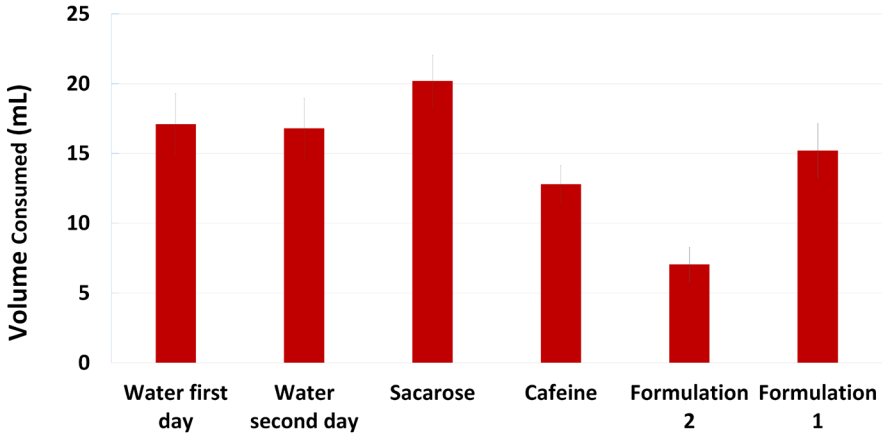


Figure 7 - Results of the *in vivo* palatability test in rats. The values are mean S.D. of 6 determinations.

Formulation 1 presented average consumption similar to the caffeine without a statistical difference ($p > 0.05$). The average consumptions of the Formulation 1 and water were 14±1.8 and 16.92±2.4 mL, respectively. One way-ANOVA proved there was not a significant difference between the average consumption of Formulation 1 in relation to the water ($p< 0.05$).

Formulation 2 presented lower average consumption (8.33±1.63 mL) than that observed for the caffeine (13.5±1.64 mL) and water (16.92±2.4 mL) ($p < 0.05$), showing that this formulation was unpleasant. Formulation 1 had significantly higher consumption than that observed for Formulation 2 ($p < 0.05$). It can be concluded that Formulation 1 has greater acceptability than Formulation 2, in relation to the taste perception of the trained rats.

CONCLUSIONS

It was concluded that the analytical method for quantification of captopril and captopril disulfide indicated selectivity, linearity, precision, accuracy, and robustness within the evaluated parameters. Formulations 1 and 2 were stable chemically and microbiologically for 30 days when stored at a temperature of 5 °C, protected from light with the content of captopril remaining > 90% and amount of captopril disulfide < 3%. *In vivo* palatability studies in humans and animals showed that Formulation 1 has a better taste than Formulation 2 without sweetener.

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