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Original Article

A newly validated HPLC–DAD-UV method to study the effects of medicinal plants extracts, fractions and isolate compounds on gastric emptying in rodents

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ABSTRACT

In this study, we developed and validated a chromatographic method by High-performance Liquid Chromatography coupled to Diode-Array Ultraviolet Detector for quantification of acetaminophen in small volumes of plasma. This analytical method is particularly suitable for studies of gastric emptying time and pharmacokinetics in small rodents. Orally administered acetaminophen is promptly and completely absorbed in the intestines, with negligible absorption from the stomach. Owing to these kinetic features, acetaminophen can be used to study gastric emptying. The newly-validated analytical method was employed to investigate whether plants used as anti-dyspeptics in the Brazilian traditional medicine affect the gastric emptying. Lyophilized aqueous extracts from leaves of *Baccharis trimera* (Less) DC., Asteraceae (carqueja), *Maytenus ilicifolia* Mart. Ex Reissek, Celastraceae (espinheira-santa) and *Lippia alba* (Mill.) N.E.Br. ex Britton & P. Wilson, Verbenaceae (erva-cidreira) were administered by gavage to female Wistar rats 30 min before a single oral dose of acetaminophen (50 mg/kg). *L. alba* extract (50 mg/kg) did not alter acetaminophen plasma concentration *versus* time curve an indication that it has no effect on gastric emptying. Extracts of *B. trimera* (50 mg/kg) and *M. ilicifolia* (30 and 50 mg/kg, but not 10 mg/kg), however, slightly delayed gastric emptying. *M. ilicifolia* given by intraperitoneal route (30 mg/kg) also retarded gastric emptying. In conclusion, the newly-validated analytical method is adequate for studying the effects of medicinal plants on gastric emptying.

Introduction

Gastric emptying (GE) time is the time taken by the stomach to deliver the chyme and other gastric contents – through the pyloric sphincter – to the duodenum. An abnormally long GE time (delayed gastric emptying) may give rise to vomiting, nausea, abdominal bloating and pain, erratic blood glucose levels, and other signs and symptoms (Hellström et al., 2006; Szarka and Camilleri, 2009; Varón and Zuleta, 2010). A rapid stomach emptying after a meal rich in sucrose, fructose and or fat, on the other hand, may cause diarrhea, vomiting, nausea, abdominal cramps, dizziness and lightheadedness, headache, sweating, accelerated heart rate, low blood glucose, and others (dumping syndrome) (Berg and McCallum, 2016). Disturbance of gastric emptying the underlying pathophysiological alteration leading to functional dyspepsia (or indigestion), one of the most common gastrointestinal complaints of patients (Black et al., 2018).

GE is a physiological process carefully regulated by gastrointestinal peptide hormones (*e.g.*, ghrelin, glucagon-like peptide-1, cholecystokinin and others) and autonomic nervous system function. Several diseases (*e.g.*, cancer, diabetes, gastric ulcers, autonomic neuropathy, and others) and exogenous factors, such as meal and chyme volumes, stress, alcohol consumption, tobacco smoking and medicines, can markedly affect GE time (Varón and Zuleta, 2010). Opioids, anticholinergic drugs, proton pump inhibitors, tricyclic antidepressants, histamine H2 receptor blockers and several other medications delay GE, whereas metoclopramide, erythromycin, cisapride, domperidone, and some other drugs accelerate it (Varón and Zuleta, 2010).

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GE time may modulate the rate and extent of drug absorption and its bioavailability as well. Drugs are as a rule only minimally absorbed in the stomach. Most medicines, even those undissociated in the stomach, and fully ionized in the small bowel, are predominantly absorbed in the intestines because of their far larger absorption surface area (Golub et al., 1986). Time for GE thus plays a key role in the rate of absorption and the extent of bioavailability of most orally administered drugs and, owing to this fact, a retarded GE may result in therapeutic failures. If a medicine, be it an isolate molecule, an herbal drug or a phytotherapic product, interferes with GE time, it is likely to alter the absorption and pharmacokinetics of another drug administered by the oral route.

Based on the foregoing, we validated a new Liquid Chromatography (HPLC) coupled to Diode-Array Ultraviolet Detector method (HPLC–DAD-UV) suitable to test effects of medicinal plant extracts, their fractions or isolate compounds on the GE time in small rodents. Since acetaminophen is a drug promptly and fully absorbed in the small intestines after oral administration, it was chosen as a probe molecule to determine GE time in rodents. This relatively fast, inexpensive, sensitive and reproducible analytical method is a procedure suitable for measuring plasma levels of acetaminophen in relatively small volumes of blood taken from rodents. It can also be used for larger animals and humans. Small rodents, however, are the preferred species in the context of preclinical studies and the newly validated method could be useful for screening of medicinal plants and their fractions for effects on GE before any human testing. In humans, GE time is generally determined using radioactive tracers (*e.g.* ^{99m}Tc) added to a meal (Szarka and Camilleri, 2009). Use of non-radioactive materials is also a potential advantage of this method. Radionuclide imaging methods require special handling and radioactive waste disposal procedures.

Medicinal plants and phytotherapeutic products with putative anti-dyspeptic actions are common medications for functional dyspepsia. The Herbal Compendium of the Brazilian Pharmacopeia (HCBF) (Anvisa, 2010) lists a number of medicinal plants commonly used to treat dyspepsia, including plants popularly known as "Brazilian boldo", "carqueja", "espinheira-santa", "gengibre" and "erva-cidreira". A PubMed literature search suggested that some plants listed by HCBF as anti-dyspeptics were studied regarding their effects on acid secretion and digestive enzymes, and healing of ulcerative gastric lesions. Nonetheless, there is a paucity of studies of the effects of these plants on GE. As far the authors are aware, except for a single study of "espinheira-santa" species (*Maytenus ilicifolia* Mart. Ex Reissek, Celastraceae) using the phenol red dye test (Baggio et al., 2009), the effects of putative anti-dyspeptic plants on GE remain virtually unstudied.

In this study, we used the newly validated HPLC–DAD-UV analytical method to quantify acetaminophen in plasma, to test the effects of plant extracts obtained from leaves of *Baccharis trimera* (Less) DC., Asteraceae (carqueja), *M. ilicifolia* Mart. ex Reissek (espinheira-santa) and *Lippia alba* (Mill.) N.E.Br. ex Britton & P. Wilson, Verbenaceae (erva-cidreira) on the rat GE time.

Materials and methods

Reagents

All chemicals were of analytical reagent grade or a higher purity. HPLC-grade methanol and acetonitrile were purchased from Tedia (Rio de Janeiro, Brazil); zinc sulfate heptahydrate ($ZnSO_4{\cdot}7H_2O$); acetic acid (CAS 64-19-7, CH_3COOH) were obtained from Merck Millipore. Sodium heparin (5000 IU/ml) was purchased from Hipolabor (Belo Horizonte, Brazil). Ultrapure water was provided daily by a Milli-Q® purifying water system (Merck-Millipore).

Plant collection

Baccharis trimera (Less.) DC., Asteraceae (voucher sample RB00641858, carqueja), *Lippia alba* (Mill.) N.E.Br. Ex Britton & P. Wilson, Verbenaceae (carvone chemotype) (voucher sample RB00528734, erva-cidreira) and *Maytenus ilicifolia* Mart. ex Reissek, Celastraceae (RB00648667, espinheira-santa) were collected on 5th March 2018 by MSc Ygor Jessé Ramos in the Socio-Environmental Responsibility Center of the Rio de Janeiro Botanical Garden, where they were grown (GPS S22°58′08″ O43°13′03″). These species were chosen for the study because they are listed in the HCBF as anti-dyspeptics, and are easy to cultivate. The studies in progress with *B. trimera*, *L. alba* and *M. ilicifolia* were registered in the National System of Genetic Resource Management and Associated Traditional Knowledge (SisGen) and were assigned with de identification A3CAO14, A27B5F7 and A1D1D4F, respectively.

Extract preparation

The fresh plant material was frozen in liquid nitrogen, fragmented and subjected to extraction by infusion with ultrapure water (Milli-Q®-Merck-Millipore), weight/volume ratio of 3%, as recommended by the HCBP (Anvisa, 2010). Aqueous extracts from *B. trimera*, *L. alba* (carvone chemotype) and *M. ilicifolia* were freeze-dried (lyophilized extracts) and stored in amber flasks at −20 °C until further use. Immediately before their administration to the animals, lyophilized extracts were solubilized in ultrapure water.

Solutions

On the day of use, working solutions of acetaminophen ($C_8H_9NO_2$; MW 151.17; m.p. 167 °C; lot number 124k0165 – Sigma-Aldrich) were freshly prepared by making dilutions from a stock solution (200 μg/ml) in ultrapure water. Chromatographic tests (HPLC–DAD-UV) showed that acetaminophen stock solutions kept protected from light and at −20 °C, are stable for at least 60 days.

Animals and treatment

Female Wistar rats, weighing 220–260 g, from the Laboratory Animal Breeding Center (CECAL) of Oswaldo Cruz Foundation (FIOCRUZ), were used. Animals were housed (five per cage) in standard plastic rat cages with stainless steel cover lids and white pine havings as bedding. Upon arrival to the laboratory animal facilities and thereafter, rats were placed and kept under controlled temperature (22 ± 2 °C), humidity (about 70%) and 12 h light/dark cycle, with *ad libitum* access to filtered tap water and a commercial pellet diet for rats and mice (Nuvital®, Nuvilab, Curitiba, PR, Brazil). Experiments were conducted in accordance with Brazilian and international guidelines for ethical conduct in the care and use of animals and approved by CEUA Fiocruz (41/19-1). Rats were treated by oral (gavage) or parenteral (*s.c.* or *i.p.*) routes with aqueous solutions (Milli-Q® ultrapure water) of acetaminophen (Sigma-Aldrich) (gavage), morphine sulphate (sterile ampoules; Granado) (*s.c.*), or lyophilized plant extracts from *B. trimera* (gavage), *L. alba* (carvone chemotype) (gavage) and *M. ilicifolia* (gavage and *i.p.*). All solutions were prepared on the day of the treatment.

Plasma samples

Plasma was separated from the heparinized whole blood by centrifugation at 12,000 × *g* for 15 min. After separation, plasma samples were transferred to 1.5 ml Eppendorf tubes and stored at −20 °C until further use (<7 days).

Acetaminophen extraction procedure

Acidified acetonitrile (20 μl, 2% with acetic acid, w/v) was added to 1.5 ml Eppendorf tubes containing 50 μl of plasma. The tubes were carefully vortexed for 10 s avoiding any contact of the liquid mixture with the tube tip. The tubes received 12.5 μl of an aqueous solution of zinc sulfate 12.5% (w/v), and were vortexed again for 10 s. The mixture was then let stand for 30 min to ensure a complete precipitation of plasma proteins. The tubes were then centrifuged at 12,000 × *g* for 15 min and the supernatant was analyzed by HPLC–DAD-UV. All plasma samples were analyzed in triplicate.

Chromatographic analysis

HPLC–DAD-UV analyses were carried out using a Shimadzu Nexera XR® (liquid chromatographer coupled to a Shimadzu UV detector with the diode array SPDM20A, equipped with a CBM20A controller, DGU20A degasser, LC20AD binary pump, CTO20A oven, and SILA20A auto-injector). A Shimadzu LabSolutions Software (Shimadzu, Japan) was used to analyze chromatograms, and different combinations of acetonitrile and acidified water were tested as mobile phases. HPLC columns were silica-based C18 ACE® (250 mm × 4.6 mm i.d., 5 μm particle size). The injection volume was 20 μl for all analyses.

Validation

The method validation was based on a set of guidance documents, namely, manual for validation RE899/2003 issued by the Brazilian Health Surveillance Agency (Anvisa); guidance on the validation of analytical methods (DOQ-CGCRE-008/2007) of the National Institute of Metrology, Quality and Technology, Brazil (Inmetro) and ICH technical requirements for validation of analytical procedures (1995, 1996). The parameters used to evaluate analytical performance were (a) selectivity, (b) linearity, (c) intra-day precision, (d) inter-day precision, (e) accuracy, (f) recovery, (g) robustness, and (h) limits of detection (LOD) and quantification (LOQ). Calibration curves for eleven concentrations (0.10, 0.15, 0.22, 0.45, 0.89, 1.78, 3.57, 7.14, 10.71, 14.28 and 28.57 μg/ml) were generated using standard solutions of acetaminophen in plasma extract. Linearity was evaluated by three calibration curves obtained on three distinct days. Analyses for assessing the selectivity of the method were carried out with (a) ultrapure water, (b) blank rat plasma and (c) rat plasma spiked with an acetaminophen solution of 0.89 μg/ml. Differences between experimental values and theoretical values estimated by fitting a linear model to calibration data were used to determine homoscedasticity. Intra-day precision was evaluated using two acetaminophen concentrations (0.714 and 7.140 μg/ml) assayed three times within a single day. Inter-day precision was determined for these two concentrations of acetaminophen measured once a day on three distinct days. Accuracy was determined by comparing experimental and theoretical concentrations, that is, concentrations derived from the analytical curve. Recovery was determined by spiking the plasma sample with acetaminophen to achieve low, intermediate and high concentrations (0.22, 0.89 and 7.14 μg/ml). Robustness was determined for concentrations of 0.22 and 7.14 μg/ml (low and high) of the analytical curve and based on the influence of five analytical parameters (mobile phase flow rate, oven temperature, acetonitrile concentration in the mobile phase, pH of the aqueous phase and UV wavelength), and their respective variations. Limits of detection (LOD) and quantification (LOQ) of the method were obtained by evaluating the signal-to-noise ratio of known concentrations and by successive serial dilutions applied to a 200 μg/ml stock solution of acetaminophen.

GE activity study

The GE test consists of determining the plasma concentration *versus* time curve of acetaminophen after oral administration (Cohen et al., 2000; Kar et al., 2015). Since acetaminophen is promptly and fully absorbed in the small intestines, but not in the stomach, changes in GE time lead to alterations in pharmacokinetics parameters such as C_{max}, T_{max} and AUC.

Six rats were used per test, and repeated blood samples were collected from the lateral vein of the tail of each animal at 15, 30, 60, 90 and 120 min after acetaminophen administration. Table 1 summarizes the treatment scheme. In all GE tests, rats were administered by an intra-gastric cannula (gavage) with acetaminophen (50 mg/kg). Control rats did not receive any additional treatment whereas a positive control group was pretreated with a *s.c.* injection of morphine (10 mg/kg) 15 min earlier, and experimental groups were pretreated orally (gavage) with plant extracts (30 mg/kg or 10 mg/kg) by gavage 30 min prior to acetaminophen administration. The extract of *M. ilicifolia* (espinheira-santa) was also administered (30 mg/kg) by the intraperitoneal route 30 min before treatment with acetaminophen.

Statistical analysis

Data are means (±) standard errors. Means were compared by one-way ANOVA followed by Bonferroni *post hoc* test. Differences were considered statistically significant when $p < 0.05$. Statistical analyses were performed using Graphpad Prism6®.

Results

Validation of a new analytical HPLC–DAD-UV method for quantification acetaminophen in plasma

Three solutions, with different proportions of acetonitrile and acidified water, were tested as the mobile phase using a silica-based C18 column as the stationary phase. Optimal HPLC conditions for quantification of acetaminophen in blood plasma were obtained with silica-based C18 ACE® column (250 mm × 4.6 mm i.d., 5 μm particle size), and a solution of acetonitrile and acidified water (pH 3) 20:80 as the mobile phase. The flow rate was set at 1 ml/min, the temperature was kept at 50 °C, and UV absorbance was monitored at 264 nm (λ_{max} for acetaminophen quantification). The developed method was characterized by a short analysis time (tR 3.81 ± 0.20 min and a total analysis time of 6 min), a great signal symmetry (~1), a high retention factor ($\alpha = 1.9$), and low detection and quantification limits. Selectivity and specificity of the method were determined by analyses of blank plasma samples and plasma samples spiked with acetaminophen.

Fig. 1 shows (A) the acetaminophen signal obtained in ultrapure water; (B) signals in blank plasma extract; (C) acetaminophen signal in plasma extract (spiked at a final concentration of 28.57 μg/ml); and (D) overlapping of the chromatograms. No interference within the acetaminophen chromatographic window was detected, an indication of the selectivity and specificity of the method.

A technique for extracting acetaminophen from 50 μl samples of rat plasma was established. Full precipitation of plasma proteins was achieved by adding acetonitrile, and zinc sulfate (see Methods) with a recovery rate higher than 80% (for low, intermediate and high concentrations of acetaminophen spiked in plasma samples). The total time required for full precipitation of proteins and acetaminophen extraction was approximately 65 min.

Table 1
Design of the study of the effects of medicinal plant extracts on rat gastric emptying (GE). Female Wistar rats were treated orally (gavage) with acetaminophen (50 mg/kg) after having been treated with morphine (positive control) or a lyophilized plan extract.

Test sample (*n*)	Dose (mg/kg)	Time before acetaminophen (min)	Route of administration	Blood sampling (min)
Control (12)	–	–	–	15, 30, 60, 90 and 120
Morphine (6)	10	15	*s.c.*	15, 30, 60, 90 and 120
B. trimera (6)	50	30	Gavage	15, 30, 60, 90 and 120
L. alba (6)	50	30	Gavage	15, 30, 60, 90 and 120
M. ilicifolia (6)	50	30	Gavage	15, 30, 60, 90 and 120
M. ilicifolia (6)	30	30	Gavage	15, 30, 60, 90 and 120
M. ilicifolia (6)	10	30	Gavage	15, 30, 60, 90 and 120
M. ilicifolia (6)	30	30	*i.p.*	15, 30, 60, 90 and 120

s.c., subcutaneous; *i.p.*, intraperitoneal. Repeated blood samples were taken from each rat at different time intervals after administration of a single oral dose acetaminophen. Controls were treated with acetaminophen only.

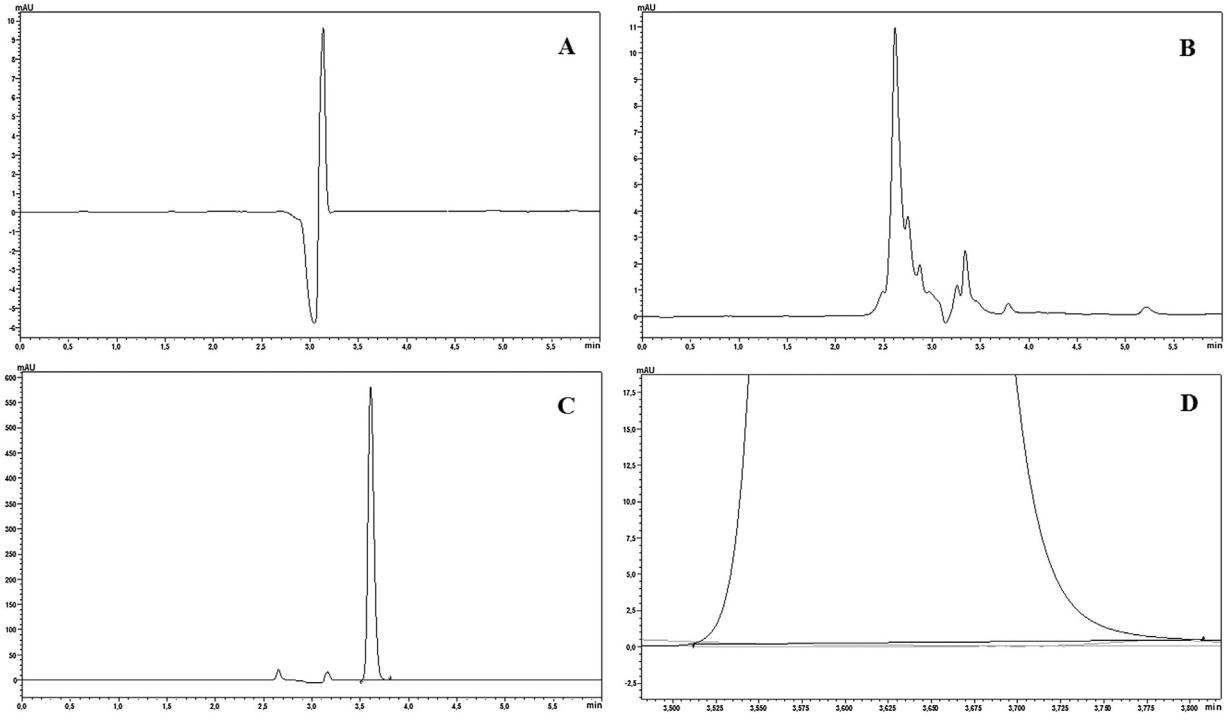

Fig. 1. HPLC–DAD-UV chromatograms used to determine method selectivity and specificity: (A) the acetaminophen signal in ultrapure water; (B) signals in blank plasma extract; and (C) acetaminophen signal in an extract of plasma spiked to achieve a final concentration of 28.57 μg/ml. (D) Expansion (tR ~ 3–4 min) of overlapping chromatograms from figure A, B and C.

The HPLC–DAD-UV method developed for measuring acetaminophen in plasma was validated for linearity, intra-day and inter-day precision, accuracy, recovery, robustness, and detection and quantification limits. The calibration curves obtained in the concentration range from 0.10 to 28.57 μg/ml showed excellent linearity ($r = 0.9996 \pm 0.0003$; average and standard deviation of three distinct calibration curves). The difference between experimental and estimated values in the concentration range of 0.10–28.57 μg/ml indicated that value distributions were homoscedastic. The linear regression equation for acetaminophen quantification in plasma samples was as follows: concentration = [(absorbance = 96,383 (±365)] × concentration − 6,678 (±555). The method LOQ and LOD were 45 and 30 ng/ml, respectively. Precision and accuracy values were within an acceptable range (85–115%). Regarding the evaluation of method robustness, the tested deviations of the standard had only a minor influence on the average of the peak areas for both tested concentrations, which indicates that the developed method is robust (Anvisa, 2003; Inmetro, 2007) (Table 2).

Gastric emptying study

A plasma concentration *versus* time curve of acetaminophen after oral administration to rats is shown in Fig. 2. The highest concentration (11–12 μg/ml) was recorded at the first sampling time (15 min) suggesting a prompt absorption. Afterwards, plasma concentrations showed a nearly linear decline attaining very low concentrations at 120 min after the treatment. This finding indicated that, control rats (not treated with morphine or plant extracts) eliminated most of the absorbed acetaminophen within 120 min. In rats pretreated with morphine (*s.c.*), however, absorption is much slower and a peak (C_{max}) concentration (1.41 μg/ml) was found in the rat plasma 30 min (T_{max}) after treatment. This C_{max} in morphine-pretreated rats is much lower than that the highest level recorded in control rats at 15 min (Fig. 3). Moreover, in contrast with the linear decline of plasma levels of acetaminophen in controls, in morphine-pretreated rats plasma levels remained low and practically unaltered for at least 120 min. Since acetaminophen is predominantly absorbed in the intestines, this plasma

Table 2
Evaluation of the robustness of the HPLC–DAD-UV method for acetaminophen quantification.

Parameters	Condition					
	0	1	2	3	4	5
Mobile phase flow	A	a	A	A	A	A
Oven temperature	B	B	b	B	B	B
Mobile phase composition	C	C	C	c	C	C
Aqueous phase pH	D	D	D	D	d	D
UV λ_{max}	E	E	E	E	E	e
High concentration sample: 7.14 μg/ml						
M (mAU)	699,383	660,504	700,258	700,882	698,945	675,518
SD	798	4,639	709	360	517	548
RSD%	0.11	0.71	0.11	0.05	0.07	0.08
Low concentration sample: 0.22 μg/ml						
M (mAU)	20,078	19,238	19,916	19,981	20,065	19,343
SD	261	116	333	50	213	291
RSD%	1.31	0.61	1.67	0.25	1.06	1.51

Condition 0, standard condition; 1–5, deviations of the standard condition; *M*, mean absorbance (mAU); SD, standard deviation; RSD%, relative standard deviation.

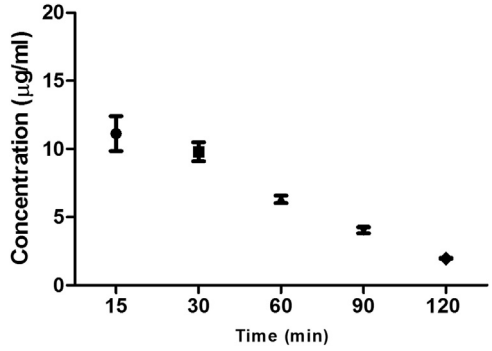

Fig. 2. Plasma concentration (μg/ml, mean ± standard error) *versus* time curve of acetaminophen after a single oral (gavage) dose of 50 mg/kg given to rats.

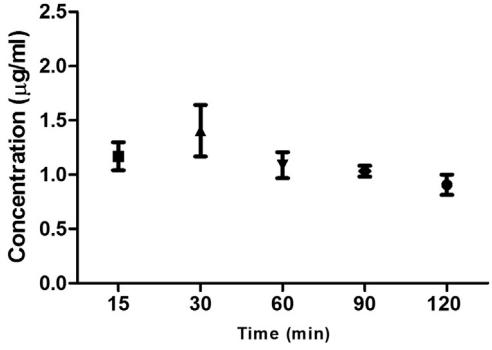

Fig. 3. Plasma concentration (μg/ml, mean ± standard error) *versus* time curve of acetaminophen (50 mg/kg) administered orally to rats pretreated with morphine (10 mg/kg, *s.c.*) 15 min earlier.

concentration *versus* time curve is consistent with a slow absorption rate due to a dramatic inhibitory effect of morphine on gastro-intestinal tract motility and GE process.

As shown in Fig. 4A, the curve of plasma concentration *versus* time of acetaminophen in rats pretreated with *L. alba* lyophilized extract (50 mg/kg) did not differ from the curve determined in control rats (Fig. 2). This finding indicated that, at the tested dose, *L. alba* extract did not alter GE time. Pretreatment with 50 mg/kg of *B. trimera* extract (Fig. 4B) or of *M. ilicifolia* extract (Fig. 4C), however, altered the curve of plasma concentration-time of acetaminophen. Plasma concentrations in rats pretreated with these plant extracts were lower than those found in controls at the same sampling times. Moreover, after pretreatment with *B. trimera* and *M. ilicifolia*, peak concentrations (C_{max}) were noted 30 min after administration of acetaminophen (Fig. 4B and C). These results showed that both *B. trimera* and *M. ilicifolia*, at the tested dose (50 mg/kg), delayed GE and, by doing this, slowed the absorption of acetaminophen. The effects of an oral dose of *M. ilicifolia* extract as low as 10 mg/kg on GE (Fig. 4F) were less marked that those of higher doses (50 and 30 mg/kg) (Fig. 4C and D) suggesting that the effect of the plant extract on GE was dose dependent. It is of note that the curves after pretreatment with *M. ilicifolia* extract (30 mg/kg) by oral (Fig. 4D) and intraperitoneal (Fig. 4E) routes were almost identical to each other, a finding suggestive plant-caused delay of GE is mediated systemically by one or more of extract constituents that was (were) promptly absorbed through both routes of administration.

Alterations of plasma concentration of acetaminophen *versus* time curves due to pretreatment-caused delays in GE are also apparent as decreases of the Area Under the Curve (AUC). Pretreatment with morphine (10 mg/kg *s.c.*) caused a drastic decrease of AUC15–120 min for acetaminophen plasma concentrations across time (Fig. 5). A less marked, yet statistically significant reduction of AUC15–120 min, was also observed in rats pretreated orally (30 min earlier) with *M. ilicifolia* extract (50 and 30 mg/kg). No other discernible alteration of AUC after pretreatment with plant extracts was found (Fig. 5).

Discussion

A new chromatographic (HPLC–DAD-UV) method for the quantification of acetaminophen in small volumes of plasma was developed and validated in this study. The absence of interferences in the retention time window for the analyte (tR = 3.80 ± 0.20) confirmed the selectivity of the method. It was also found that only adjustments in the composition of the mobile phase (acetonitrile/acidified ultrapure water) were sufficient to develop the new analytical method, and to obtain a chromatographic peak of acetaminophen with capacity factor or separation (α) calculated in 1.9 and signal symmetry of ~1. The total run time (6 min) is suitable for quantitative analyses. The proportion of ultrapure water in the mobile phase was higher than that of acetonitrile (80/20). The use of a modified C18 silica column is also an advantage of the developed method because this type of column can be purchased for a reasonable price. Owing to the mobile phase composition and the column used in the analysis, the newly validated method is cost-effective.

The technique of extraction of acetaminophen from the plasma matrix was very similar to an extraction technique for primaquine described by Carmo et al. (2017). The procedure employed to precipitate the plasma proteins allows a further recovery of the analyte from a liquid phase with characteristics similar to those of the

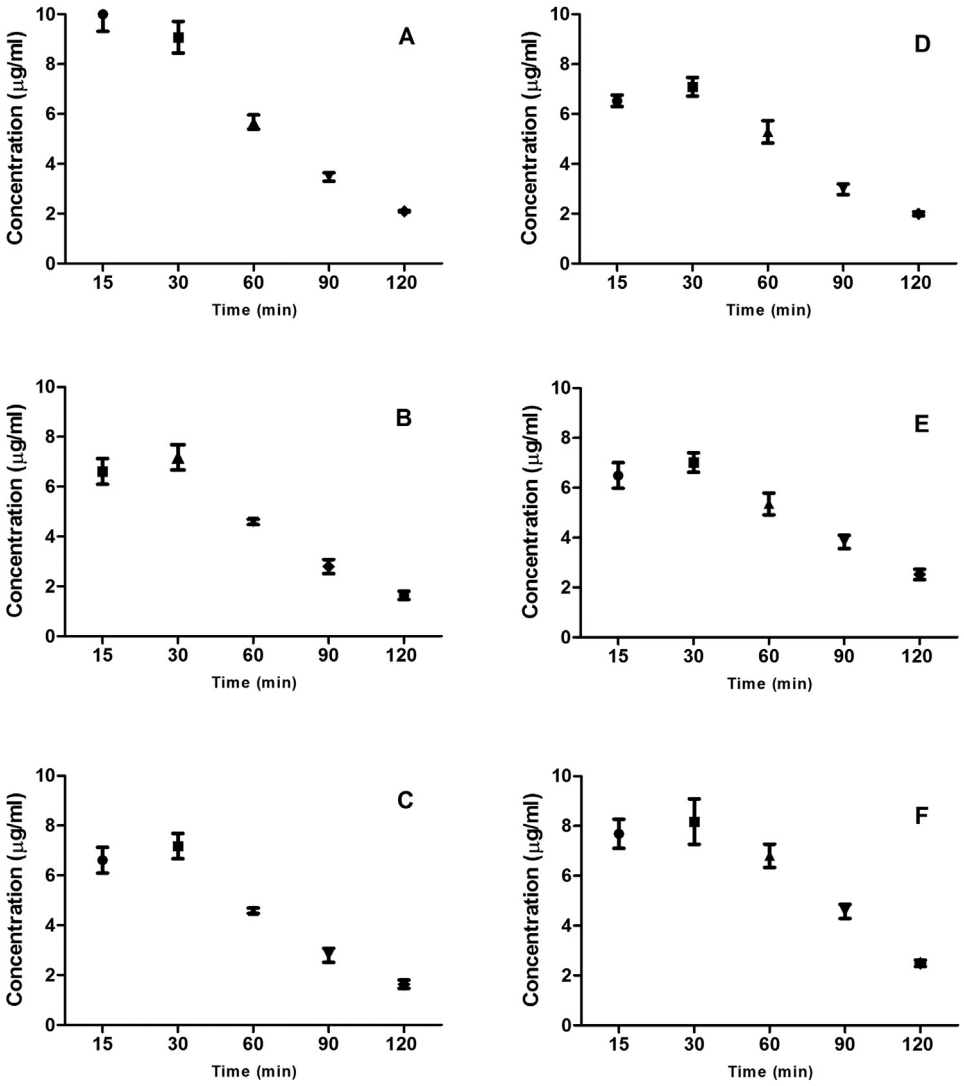

Fig. 4. Distribution profiles of acetaminophen in plasma of Wistar rats. (A) *Lippia alba* (50 mg/kg, oral route); (B) *Bacchari strimera* (50 mg/kg, oral route); (C) *Maytenus ilicifolia* (50 mg/kg, oral route); (D) *M. ilicifolia* (30 mg/kg, oral route); (E) *M. ilicifolia* (30 mg/kg, intraperitoneal route); (F) *M. ilicifolia* (10 mg/kg, oral route).

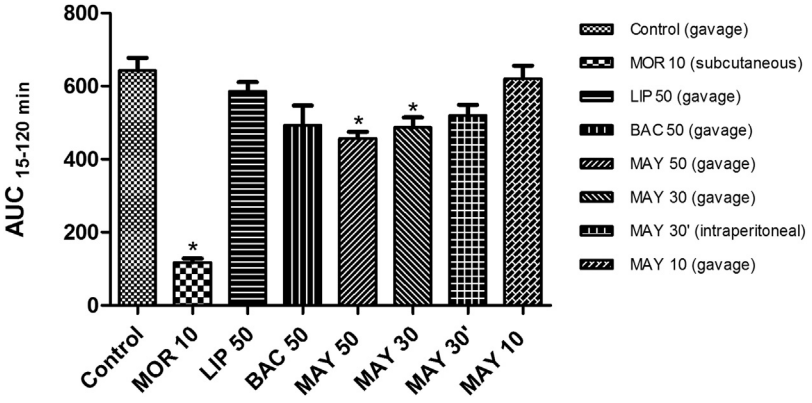

Fig. 5. Graphical representation of the area under the curve (AUC) calculated after oral administration of 50 mg/kg of pure acetaminophen (control) or in combination with the different lyophilized aqueous extracts (LIP 50: *Lippia alba*, 50 mg/kg; BAC 50: *Baccharis trimera*, 50 mg/kg; MAY 50: *Mautenus ilicifolia*, 30 mg/kg; MAY 30: *M. ilicifolia*, 30 mg/kg; MAY 30': *M. ilicifolia*, 30 mg/kg, intraperitoneal route; MAY 10: *M. ilicifolia*, 10 mg/kg) or with morphine sulphate (MOR 10: morphine sulphate, 10 mg/kg). Data were analyzed by one-way ANOVA followed by Bonferroni *post hoc* test with multiple comparisons. Differences from control AUC ($p < 0.05$) are indicated by an asterisk (*) on the top of the histogram bar.

mobile phase used to analyze acetaminophen by HPLC–DAD-UV (in this case, a mixture of acetonitrile/ultrapure acidified water). The recovery of the analyte was determined with concentrations of 0.22, 0.89 and 7.14 μg/ml in the rat plasma. Percentages of recovery were higher than 80% and, therefore, within the limits recommended by Anvisa (2003). The main advantages of the method of extraction of acetaminophen from the plasma are a good recovery, a relatively low cost and its suitability for analysis of small volumes of plasma (50 μl), making it a feasible method for preclinical studies with rodents.

In summary, the newly validated analytical method for acetaminophen in plasma proved to be selective, linear, precise, accurate and robust (Anvisa, 2003; Brito et al., 2003; Inmetro, 2007; Roca et al., 2007). LOD and LOQ are excellent for determination of GE time and other kinetic studies in rodents.

The GE study based on the measurement of plasma levels (absorption rate) of acetaminophen compares favorably with other methods to evaluate gastrointestinal transit in small rodents, such as the phenol red dye, the active charcoal and the radioisotope-labeled polyethylene glycol (PEG) techniques (Goineau et al., 2015). A drawback common to these methods is the fact that they are rather laborious techniques that give rise to results with a large variability between animals and assays (Shaughnessy et al., 2013). Moreover, it is worth noting that the GE study based on the plasma concentrations of acetaminophen requires a small number of animals and causes less suffering and harm to them than other methods.

The extract of *B. trimera* (carqueja), tested at a concentration as high as 50 mg/kg, slightly delayed GE in rats. This finding is consistent with results from a previous study showing that aqueous extracts of "carqueja" slowed the active charcoal gastric transit measured by about 20% or so (Gamberini et al., 1991). This study associated the reduced gastrointestinal motility and delay of GE with a carqueja extract-induced decrease of the stomach acid secretion (Gamberini et al., 1991).

The delay of GE by *M. ilicifolia* (espinheira-santa) extract (30 and 50 mg/kg by gavage) was particularly noticeable. At a lower oral dose (10 mg/kg) the extract of *M. ilicifolia* caused no discernible delay of GE. The fact that pretreatment with *M. ilicifolia* extract (30 mg/kg), regardless of whether it was given by the oral or the intraperitoneal route, produced a similar delay of GE suggested that it is a systemic effect caused by one or more crude extract constituents.

Maytenus ilicifolia (espinheira-santa) is a medicinal plant widely used in Brazil to treat disorders such as gastric ulcers, dyspepsia and diarrhea. Its putative gastroprotective effect has been attributed to an inhibition of gastric acid secretion, through the blockade of the proton pump (H^+) and a reduction of nitric oxide release (Baggio et al., 2007). It is of note the omeprazole and proton pump inhibitors are the mainstay of treatment for gastroesophageal reflux disease and gastric ulcers. Proton pump inhibitors effectively suppress acidity in the stomach and retard GE after a solid meal (Benini et al., 1996; Tougas et al., 2005). Baggio et al. (2009), using the phenol red method, demonstrated that fractions of *M. ilicifolia* extracts reduced gastrointestinal peristalsis and delayed GE in Swiss female mice. According to Baggio et al.'s study, an *i.p.* injection of a flavonoid-enriched extract from *M. ilicifolia* prolong GE time (ED_{50} = 89 mg/kg). A subsequent purification of the fraction increased the concentration of flavonoids caused a 12–14 fold increase in the GE inhibitory activity (ED_{50} = 9.7 mg/kg). GE delay was reversed when the plant extract was given combined with bethanechol, but it remained unaltered (delayed) when the extract was co-administered with metoclopramide. These results suggested that flavonoids contribute to the GE delay caused by *M. ilicifolia* extracts with a mode of action involving not only a reduction of gastric acidity but also an anticholinergic (anti-muscarinic) activity (Baggio et al., 2009). It is of note that these results for lyophilized crude aqueous extracts administered by gavage to rats are not directly comparable to those reported by Baggio et al. (2009) with mice treated with purified fractions of the plant extract.

Conclusions

A sensitive, robust and cost-effective chromatographic (HPLC–DAD-UV) method for quantification of acetaminophen in small volumes of plasma was developed and validated. This analytical method proved to be particularly suitable for preclinical studies of gastric emptying (GE) time and pharmacokinetics in small rodents. A GE study using the newly validated analytical method, showed that lyophilized aqueous extracts of leaves from *B. trimera* (50 mg/kg) and *M. ilicifolia* (30 and 50 mg/kg) slightly delayed GE in rats. Extracts of *L. alba* (50 mg/kg), however, had no effect on GE. A retardation of GE is an unexpected finding for a putative anti-dyspeptic herbal medicine. Nonetheless, it is unlikely that such a short delay of GE (at the tested doses) is of any clinical relevance. At any rate, the validated method reported here seems to be a useful tool to study the effects of medicinal plants, theirs fractions and isolate compounds on GE.

Author's contributions

MRPS, method development and validation, as well as HPLCDAD-UV sample analysis; LCR and ROS, treatment of animals with plant extracts and pure substances; MPGSL, preparation and extraction of plant material, HPLC-DAD-UV analysis; FJRP, supervision of animal treatment, text revision and discussion of results; DLM; HPLC-DAD-UV analysis supervision, text review and contribution to results discussion.

Conflicts of interest

The authors declare no conflicts of interest.

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