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Original Article

Pharmacokinetic disposition of erythraline in rats after intravenous administration

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ABSTRACT

Erythraline is the major alkaloid produced by *Erythrina verna* Vell., Fabaceae, a plant used in folk medicine and phytotherapeutic products to treat anxiety and sleep disorders. This study aimed to investigate the pharmacokinetic parameters of erythraline after intravenous administration in rats. For the analysis of erythraline in rat plasma, a method was developed and validated using UHPLC-MS/MS. Pharmacokinetic parameters were estimated by non-compartmental analysis. The metabolite 8-oxo-erythraline observed in previous biomimetic model studies was monitored during *in vivo* experiments. The quantification limit was 5 ng/ml within a linear range of 5–2000 ng/ml. After an intravenous dose of 1 mg/kg, the following pharmacokinetic parameters were observed: elimination half-life of 44.2 min; total clearance of 42.1 ml/min/kg and volume of distribution of 2085 ml/kg. In summary, a precise, accurate and selective UHPLC-MS/MS method was developed and successfully applied to investigate the pharmacokinetics of erythraline in rats. The metabolite 8-oxo-erythraline was observed in rat plasma after 20 min of erythraline administration.

Introduction

Erythrina genus comprises around 120 species widely distributed in tropical and subtropical regions (Lorenzi and Matos, 2008; da Silva et al., 2013). In Brazil, about twelve different species from this genus are known, including is *Erythrina verna* Vell., Fabaceae, which is popularly known as mulungu (De Albuquerque et al., 2007). *Erythrina verna* is important in medicine, which extracts from its bark are used in folk medicine as a sedative and to treat anxiety (Decker et al., 1995; Daly, 2005; Flausino et al., 2007a, 2007b; Dias et al., 2013). Commercial herbal medicines containing *E. verna* to treat anxiety are available internationally.

The ethnopharmacological use of *E. verna* is justified by biologically active alkaloids produced by this genus (Serrano et al., 2011; Rosa et al., 2012; Setti-Perdigao et al., 2013; Zarev et al., 2017). In fact, the alkaloids isolated from *Erythrina* sp. can induce effects on the central nervous system (CNS), promoting antifeedant (Cornelius et al., 2009), anticonvulsant (Faggion et al., 2011), and other neuronal properties depending on nicotinic receptors modulation (Decker et al., 1995; Dwoskin and Crooks, 2001). Garin-Aguilar et al. (2000) verified tranquilizer and sedative effects of alkaloids fractions from *E. americana* comparable to diazepam. *E. verna*, which is composed mostly by erythraline spicocyclic alkaloid, has presented sedative effects in studies performed on animals (Onusic et al., 2002, 2003; Vasconcelos et al., 2003, 2004; Viana et al., 2005; Ribeiro et al., 2006; Flausino et al., 2007a, 2007b; Faggion et al., 2011; Silveira-Souto et al., 2014).

Safety and effectiveness of natural bioactive compounds are crucial to new drug development. Although previous reports have demonstrated that *E. verna* extract and erythrinian alkaloid show relevant CNS effects, few studies have evaluated the metabolic or pharmacokinetic parameters of these alkaloids or extracts. In a previous study, our group demonstrated that *in vitro* biomimetic models with erythraline alkaloid produce 8-oxo-erythraline, a potential phase I metabolites that induces the CNS effects (Guaratini et al., 2014). In this context, this work aimed to

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develop and validate a chromatographic method to analyze erythraline in rat plasma using UHPLC-MS/MS in order to assess the pharmacokinetics in rats. The presence of the previously observed metabolite 8-oxo-erythraline was evaluated to correlate the *in vitro* metabolism with *in vivo* pharmacokinetics.

Material and methods

Animals

Five male Wistar rats (220–250 g) were housed in a temperature-controlled room (27 ± 2 °C) with a 12-h light/dark cycle and free access to water and food. The study was conducted in compliance with the ethical committee of the University of São Paulo Animal Care and Use Committee (Protocol number 14.1.765.53.3). All rats fasted for 8–12 h before the experiment. The immobilization was achieved by wrapping the animal in a felt cloth. After going through the experimental protocol, the animals were euthanized by anesthesia overdose (150 mg/kg thiopental, intravenous).

Chemicals

All chemicals used in UPLC were spectroscopy grade (J.T. Baker) and the water was obtained from Milli-Q system (de-ionized, 18 mΩ, Direct-Q, Millipore). Erythraline was isolated from *Erythrina verna* Vell., Fabaceae, as previously described by Callejon et al. (2014).

Intravenous administration of erythraline

Erythraline dissolved in saline containing no more than 5% DMSO was administered to rats (n = 5) as intravenous (*i.v.*) bolus dose of 1 mg/kg through the lateral tail vein. The total volume of drug administration varied from 100 to 120 μl. Blood samples (200–250 μl) were collected from lateral tail vein into heparinized tubes at 5; 10; 20; 40; 60; 90; 120; 180 and 240 min. Plasma was obtained by centrifuging blood samples at 2000 × *g*, at 4 °C for 10 min and stored at −80 °C until analysis.

UHPLC-MS/MS conditions

The analytical methodology was developed in a UHPLC-DAD-MS/MS, from Acquity UPLC Waters Corporation, Milford, USA. The stationary phase was a reversed phase column Acquity BEH (*Ethylene Bridged Hybrid*) (C18, 2.1 mm × 50 mm, 1.7 μm particle size) and pre-column with the same material (Vanguard 2.1 mm × 5 mm). The software used for data acquisition and analysis was MassLynx v.4.1., Waters (Micromass, Manchester, UK). Acetonitrile (B) and water (A), both containing with 0.1% formic acid (v/v), were used as mobile phase. The samples were kept at 10 °C in the auto-sampler and the column temperature was set at 35 °C. The flow rate was 0.3 ml/min and the volume of injection was 5 μl. The gradient elution was: 0–3 min: 5–40% B; 3–3.5 m in. 40–80% and 2.5 min for wash and column equilibration.

The electrospray mass spectrometry detection was set at positive ionization mode. For MRM transitions the following parameters were performed: capillary energy 1.0 kV, source and desolvation temperature were 150 °C and 350 °C, respectively. The nitrogen was used as desolvation gas (600 l/h) and argon as collision gas (0.16 l/h, pressure in the collision cell: 3.02×10^{-3} mBar). For erythraline detection the MRM transition monitored was 298 > 266, with 15 V of cone energy, for 8-oxo-erythraline was 312 > 280 and 15 V of cone energy and for caffeine (internal standard) was 195 > 138, with 40 V of cone energy. For all transitions the collision energy was 20 eV.

Sample preparation

Protein precipitation of plasma samples was tested using methanol, acetonitrile or equal mixture of these solvents and monitoring the standard signal. Aliquots of 100 μl acetonitrile, which was the solvent used to dilute the internal standard caffeine, were added to 100 μl of plasma samples. The samples were centrifuged (10 °C, 15 min, 21,000 × *g*). The supernatants were separated, filtered (0.22 μm, Millex, Millipore) and analyzed in UPLC-MS/MS.

Method validation

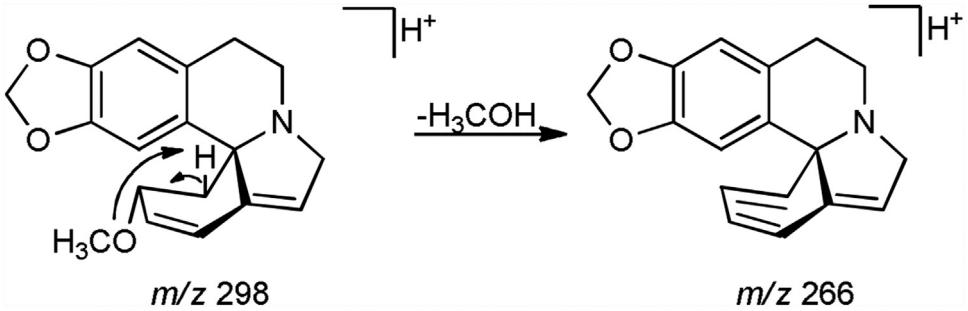
ICH guidelines (ICH, 1996) were followed to validate selectivity, linearity, precision, accuracy, and for quantification of limit parameters. The calibration curve was performed using 100 μl of blank plasma, which was added to 50 μl of internal standard caffeine (500 ng/ml) and 50 μl of erythraline solution in different concentrations, both in acetonitrile. Selectivity was evaluated using six blank plasma samples from different animals and monitoring interfering peaks in the MRM transitions for erythraline and internal standard. Linearity was tested analyzing the calibration standards in the range of 0.625–2000 ng/ml, in triplicates. The final concentrations used in analytical curve were: 2000; 1500; 1000; 750; 500; 250; 50; 15; 10; 5 ng/ml. Quality control samples at five different levels were prepared: lower limit of quantification (LLOQ, 5 ng/ml), lower quality control (LQC, 15 ng/ml), mid-quality control (MQC, 750 ng/ml), high quality control (HQC, 1750 ng/ml) and upper limit of quantification (HLOQ, 2000 ng/ml). Precision and accuracy were evaluated by the coefficient of variation (CV) and the relative error (RE) of quality control samples prepared in quintuplicates, respectively, at the same run (intra-assay precision and accuracy) and in three different runs (inter-assay precision and accuracy).

Pharmacokinetic analysis

Non-compartmental pharmacokinetic analysis was performed using the freely available Microsoft Excel add-in program PKSolver (Zhang et al., 2010). The pharmacokinetic parameters area under the curve plasma concentration vs time (AUC^{0-t}) extrapolated to infinity ($AUC^{0-\infty}$), half-life of elimination ($t_{½}$), mean residence time (MRT), volume of distribution (Vd) and total drug clearance (CL) were estimated using the trapezoidal method with intravenous bolus input. Descriptive statistics of pharmacokinetic data were presented as medians and percentiles since they do not follow normal distribution.

Results and discussion

Erythrina verna extract is a raw material of several herbal medicines sold around the world, and species of the *Erythrina* genus are used in folk medicine to treat anxiety, insomnia and pain (Rodrigues and Carvalho, 2001). The scientific evidence of its effectiveness can be verified by an increasing number of studies that demonstrate the activity of alkaloids from these species acting on the CNS (Ribeiro et al., 2006; Serrano et al., 2011). In fact, previous reports demonstrate that hydroalcoholic extracts of the bark, flowers and leaves of *E. verna* reduced agitation, anxiety and depression in animal models. The effects of these extracts seen in these studies were very similar to diazepam, which acts on the GABAergic receptors and is used as anxiolytic, anticonvulsant and sedatives drugs. In addition, erythrine alkaloids have been shown to induce effects on the CNS by modulation of nicotinic receptors, suggesting that the antagonist effect may be responsible for anticonvulsant



Scheme 1. Fragmentation pathway proposed for erythraline.

and anxiolytic effects reported in previous studies (Decker et al., 1995; Iturriaga-Vasquez et al., 2010; Setti-Perdigao et al., 2013).

Chromatographic methods to analyze erythraline and other alkaloids of *Erythrina* in plant materials have been developed previously (Garín-Aguilar et al., 2005). For the first time, we report here a chromatographic method for analysis of erythraline in a biological matrix - plasma. The use of UHPLC presents many advantages when compared to conventional HPLC systems such as detectability, speed, and resolution. Tandem mass spectrometry offers several advantages in terms of detectability and selectivity when compared to other detection systems. These characteristics are mainly desirable for methods applied to pharmacokinetic studies of natural products. After administered to experimental animals, the analytes might be present at very low concentration and the interference of components from biological matrices should be avoided (Xu et al., 2017).

Although not quantified due to unavailability of certified standard, the metabolite 8-oxo-erythraline was monitored in the MRM transition with *m/z* 312 > 280. The total conversion of erythraline parent ion (*m/z* 298) into its fragment (*m/z* 266) was optimized using 20 eV of collision energy. The fragmentation pathway followed by erythraline and 8-oxo-erythraline involves the loss of methoxyl group (loss of 32 Da), through a remote hydrogen rearrangement reaction (Demarque et al., 2016). This reaction is favorable once it takes place with groups in axial position and creates a fragment with three conjugated double bounds (Scheme 1). Erythraline and the IS caffeine were resolved within the run time of 4 min. The metabolite 8-oxo-erythraline did not coelute with the analytes (Fig. 1).

The analytical method for erythraline analysis in rat plasma was successfully validated considering the figures of merit selectivity, linearity, precision and accuracy. The method was selective since no co-eluting peaks were observed in the retention times of erythraline and the IS. The response was linear within the range of 5–2000 ng/ml with back calculated concentrations within ±15% of nominal values and linear coefficient regression of $r^2 = 0.99$. Precision and accuracy showed coefficients of variation and relative standard errors below 15% for concentration levels (Table 1).

The method developed here was suitable for pharmacokinetic studies of erythraline, where the detection and quantification limits allowed the analysis of erythraline up to 240 min after intravenous drug administration. Erythraline plasma concentrations versus time curves were used to calculate the pharmacokinetic parameters using non-compartmental analysis (Table 2, Fig. 2).

The AUC_{0-t}/AUC_{0-inf} ratios were used to confirm the suitability of the experimental design. Ratios higher than 80% are observed when the experimental blood sampling protocol are suitable to estimate the pharmacokinetic parameters. Here we observed a median AUC_{0-t}/AUC_{0-inf} ratio of 97.6 ± 1.8%, thus confirming that we accurately estimated the pharmacokinetic parameters (Fig. 2).

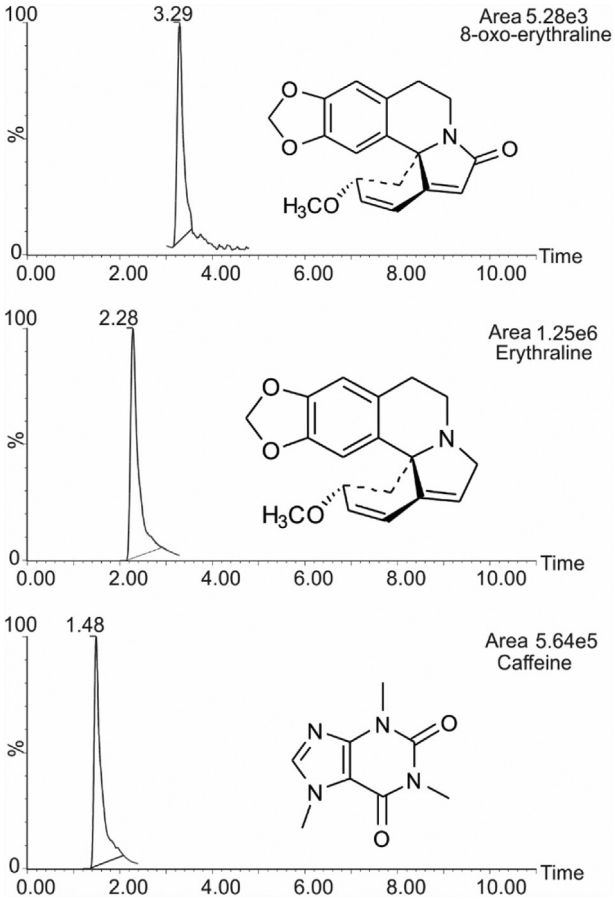


Fig. 1. Chromatograms of 8-oxo-erythraline (3.29 min), erythraline (2.28 min) and caffeine (internal standard, 1.48 min) in rat plasma samples collected after 40 min of drug administration. Column: Acquity BEH (Ethylene Bridged Hybrid) (C18, 2.1 mm × 50 mm, 1.7 μm particle size), kept at 35 °C. Mobile phase: acetonitrile (B) and water (A), both containing with 0.1% formic acid (v/v), in the gradient elution mode: 0–3 min: 5–40% B; 3–3.5 m in. 40–80% and 2.5 min of 5% B for wash and column equilibration. The flow rate was 0.3 ml/min.

After intravenous administration of 1 mg/kg in bolus, the AUC_{0-inf} of erythraline was 23.73 μg.ml/min. The plasma levels of erythraline rapidly decreased (median half-life of the 44.2 min). The fast conversion of erythraline in 8-oxo-erytraline can be the reason for the high total Cl (median: 42.1 ml/min/kg) and short MRT (median: 49.6 min). The metabolite 8-oxo-erythraline was monitored in this pharmacokinetic study and its presence was confirmed by UHPLC-MS/MS, as illustrated in Fig. 3. The 8-oxo-erythraline was verified after 20 min of erythraline administration. The *m/z* intensities of 8-oxo-erythraline increased over time while the erythraline concentrations were decreasing (Fig. 3).

Table 1
Figures of merit of the validation of the method for erythraline analysis in rat plasma samples using UHPLC-MS/MS.

Detection limit (ng/ml)	0.6
LLOQ (ng/ml)	5
Linearity	5–2000 ng/ml $r^2 = 0.99$
Intra-run precision (CV%, n = 5)	
LLOQ (5 ng/ml)	6.18
LQC (15 ng/ml)	2.30
MQC (750 ng/ml)	2.81
HQC (1750 ng/ml)	3.40
Inter-run precision (CV%, n = 5)	
LLOQ (5 ng/ml)	5.51
LQC (15 ng/ml)	6.52
MQC (750 ng/ml)	2.31
HQC (1750 ng/ml)	4.39
Intra-run accuracy (RSE, n = 5)	
LLOQ (5 ng/ml)	1.05
LQC (15 ng/ml)	0.35
MQC (750 ng/ml)	1.29
HQC (1750 ng/ml)	4.25
Inter-run accuracy (RSE, n = 5)	
LLOQ (5 ng/ml)	3.86
LQC (15 ng/ml)	5.96
MQC (750 ng/ml)	1.15
HQC (1750 ng/ml)	8.84

LLOQ: lower limit of quantification; LQC: lower concentration quality control; MQC: medium concentration quality control; HQC: high concentration quality control; CV: coefficient of variation; RSE: relative standard error.

Table 2
Non-compartmental pharmacokinetic analysis for erythraline in rats after intravenous administration of 1 mg/kg (n = 5).

PK parameters	Median (25th–75th percentiles)
AUC_{0-240} (μg/ml*min)	23.45 (16.75–32.76)
AUC_{0-inf} (μg.ml/min)	23.73 (17.12–33.42)
AUC_{0-240}/AUC^{0-inf}	0.98 (0.97–0.98)
$t_{1/2}$ (min)	44.23 (37.76–47.83)
MRT (min)	49.62 (46.30–52.11)
V_d (ml/kg)	2085.0 (1723.3–3782.1)
CL (ml/min/kg)	42.1 (33.7–74.6)

AUC_{0-240}: area under the plasma concentration-time curves calculated by the trapezoidal method; AUC_{0-inf}: area under the curve plasma concentration versus time, extrapolated to the infinity; $t_{1/2}$: half-life of elimination; MRT: Mean residence time; Vd: volume of distribution; Cl: total clearance.

Evidence herein demonstrates the importance of pharmacokinetic evaluation for future studies focused on metabolites of erythrinian alkaloids, such as 8-oxo-erythraline. Evaluating the metabolism of erythrinian alkaloids is essential to understand the long-term effects on the CNS, plasma concentration, and to predict rational dosing regimens for patients who will use medicines containing the erythrinian alkaloids as active constituent.

Despite some limitations, several studies have been using metalloporphyrins as biomimetic reactions to show that the metabolites formed in these reactions also occur *in vivo* (Schaab et al., 2010; Niehues et al., 2012). This fact is especially important for natural products, since it allows for an assessment of a likely metabolite profile with a reduced number of isolated components. Previous reports from our research group have used this strategy to demonstrate the correlation of metabolites obtained by biomimetic reactions with the metabolites produced *in vivo* assays. Among these studies we highlight that the main metabolites generated *in vitro* assays of chlorogenic acid, di-caffeoylquinic acid, piperine, lapachol, and grandisin were also observed in *in vivo* tests (Santos et al., 2008; Schaab et al., 2010; Ferreira et al., 2012; Niehues et al., 2012). In agreement with other studies, our data confirm the correlation between metalloporphyrins and *in vivo* experiments.

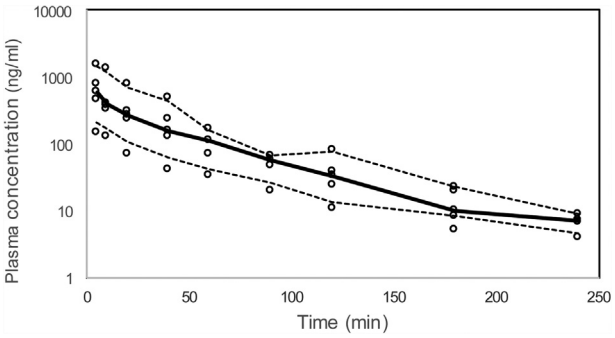


Fig. 2. Plasma concentration of erythraline in rats after intravenous administration of 1 mg/kg (n = 5). Data presented as median (solid line), 5^{th}-95^{th} percentiles (dashed lines). Observed data presented as solid circles.

In summary, the validated UHPLC-MS/MS for analysis of erythraline in rat plasma was successfully applied to estimate its pharmacokinetic parameters in rats after intravenous administration. Previous biomimetic models for in vitro metabolism of erythraline showed to effectively correlate with the *in vivo* kinetic disposition in rats. Further studies involving erythrian extract or alkaloids must consider short half-life of the alkaloid erythraline and 8-oxoerythraline as a metabolite action, as, on the CNS.

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Authors' contributions

DPD, DRC, LGP, DRG and TC designed the study and performed the laboratory work. DPD contributed to chromatographic analysis. DPD, DRC and DRG contributed to statistical analysis. NVM, JLCL, JNCL and NPL contributed to critical reading of the manuscript and

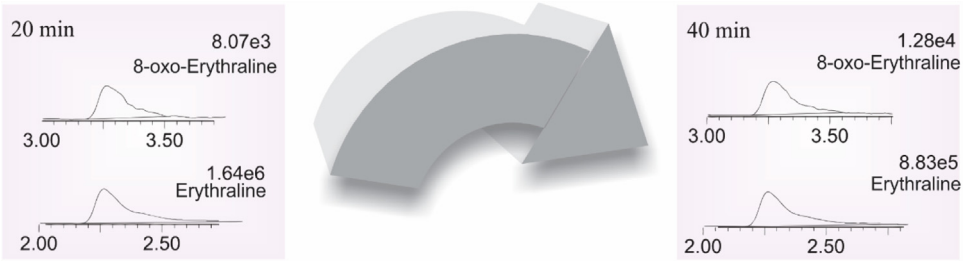

Fig. 3. Comparison between the intensity erythraline signal and its metabolite 8-oxo-erythraline before (20 min) and after (40 min) *i.v.* injection.

analyzing and interpreting the results. All the authors have read the final manuscript and approved the submission.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflicts of interest

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.bjp.2019.07.002.

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