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Short communication

Microsomal metabolism of erythraline: an anxiolitic spiroalkaloid

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ABSTRACT

The genus *Erythrina*, Fabaceae, is widely distributed in tropical and subtropical regions. Their flowers, fruits, seeds and bark are frequently used in folk medicine for its effects on the central nervous system such as anticonvulsant, antidepressant, analgesic, sedative, and hypnotic effects. Erythraline has been reported as one of the active compounds from *Erythrina*, but until now there are no pharmacokinetics data about this compound and only few results showing a putative metabolism were reported. To improve the information about erythraline metabolism, this article reports and discusses, for the first time, the *in vitro* metabolism biotransformation of erythraline by cytochrome P450 enzymes.

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Introduction

Natural products have historically played a major role in the discovery and development of a diverse array of therapeutics (Kurita and Linington, 2015). They represent a rich source of chemical diversity, between 25 and 50% of approved drugs are originated from natural products. However, discovery, isolation, and development of natural products as pharmaceutical drugs are exceptionally challenging, requiring a multi-disciplinary approach to unlock their potential (Ogbourne and Parsons, 2014).

The genus *Erythrina*, Fabaceae, is widely distributed in tropical and subtropical regions (Arita et al., 2014). *Erythrina verna* Vell. is a deciduous tree found mainly in Brazilian cerrados and other tropical regions. Its flowers, fruits, seeds and bark are frequently used in folk medicine due to their effects on the central nervous system, such as: anticonvulsant, antidepressant, analgesic, sedative and hypnotic effects (Faggion et al., 2011; Flausino et al., 2007a,b). Systematic phytochemical investigations reported the occurrence of over one hundred structural *Erythrina* alkaloids derivatives (Juma and Majinda, 2004; Wanjala et al., 2002). Biological studies regarding its anxiolytic properties have supported the popular use and the mechanism of action was described as α4β2 nicotinic receptors antagonist. In the last decade, initial pharmaceutical products were commercially available, but until now there are no pharmacokinetics data of the active compounds and only few results about putative metabolism have been reported (Guaratini et al., 2014; Perdigao et al., 2013).

Metabolism of a drug candidate often decides whether it is safe and effective for clinical application. The understanding of specific drug metabolites is often a key for the success of drug discovery (Cusack et al., 2013; Chen et al., 2014) and it is crucial to inform several important structural properties as new pharmacologically inactive or toxic entities, poor bioavailability, efficacy, and safety-related events. Therefore, such an early screening in drug development is necessary (Fraga et al., 2011).

Rat and human liver microsomes (RLM and HLM) are mainly employed for metabolite profiling purposes (Spaggiari et al., 2014; Yuan et al., 2014). The system contains the main drug-metabolizing enzymes, such as the cytochrome P450 (CYP450) family and flavin monooxygenase (Chen et al., 2014), Phase I and Phase II enzymes, which can generate valuable information to predict and avoid problems during *in vivo* studies (Nowak et al., 2014).

In this way, our group has been dedicated to investigate Phase I metabolites for a series of natural products (Marques et al., 2014; Messiano et al., 2013; Moreira et al., 2013). Analysis of erythraline (**1,** ERT) metabolization by pig microbiota suggests a high stability in intestinal portion, but applying bioorganic catalysis, one putative metabolite was formed and identified as 8-oxo-erythraline (**2**) (Guaratini et al., 2014). Considering ERT (**1**) as a promising drug candidate, its metabolism still requires

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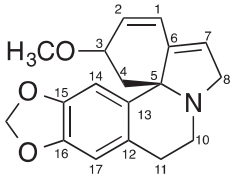
clarifications. Therefore, the aim of the present work was to investigate its *in vitro* metabolism by liver microsomes obtained from rats, as well as from human beings, in order to predict the *in vivo* metabolism, comparing with previous related results and to improve pre-clinical information of alkaloids from *Erythrina* genus.



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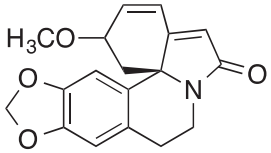

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Materials and methods

The ERT (**1**) was extracted from *Erythrina verna* Vell., Fabaceae, according to published procedures (Guaratini et al., 2014) and some control analogs were obtained as previously described (Callejon et al., 2014). Sodium chloride and sodium dihydrogen phosphate were obtained from Merck (Darmstadt, Germany). Sodium hydroxide and potassium chloride were obtained from Nuclear (São Paulo, Brazil). Glycerol and tris-(hydroxymethyl)-aminomethane were obtained from J.T. Baker (Phillipsburg, NJ, USA), ethylenediaminetetraacetic acid (EDTA) from Carlo Erba (Milan, Italy). $NADP^+$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Sigma–Aldrich (St. Louis, MO, USA).

Male Wistar rats weighing 180–220 g were obtained from the School of Pharmaceutical Sciences of Ribeirão Preto, University of Sao Paulo. The Ethical Committee from University of São Paulo approved the studies. Microsomal preparation was performed according published procedures (Marques et al., 2014). Rat liver microsome incubations (shaking water bath at 37 °C) were performed in 10 ml amber tubes in a total incubation volume of 1000 μl. Incubations contained 250 μl cofactor solution, 35 μl rat liver microsomes (29.6 mg ml^{-1} microsomal protein), 715 μl phosphate buffer (pH 7.4; 0.25 mol l^{-1}) and 25 μl ERT (**1**; 500 μg ml^{-1}). The cofactor solution consisted of $NADP^+$ (0.25 mmol l^{-1}), glucose-6-phosphate (5 mmol l^{-1}) and glucose-6-phosphate dehydrogenase (0.5 units) in Tris–HCl buffer (Tris–HCl 0.05 mol l^{-1} – KCl 0.15 mol l^{-1}, pH 7.4).

Human liver microsome incubations (BD Gentest™, Woburn, MA, EUA) were performed according Clements and Li (2011): to avoid multiple freeze–thaw cycles and maintain enzyme activity the microsomes and NADPH regenerating system were divided into single use fractions and stored at −94 °C until needed. Amber tubes in a total incubation volume of 200 μl were used. Incubations contained 100 μl human liver microsome (4 mg ml^{-1} microsomal protein), 100 μl NADPH regenerating system (NRS) (40 μl 0.5 M phosphate buffer pH 7.4, 10 μl Solution A, 2 μl Solution B, both from BD Gentest™), 5 μl ERT (**1**; 1.38 mg ml^{-1}).

After prewarming for 5 min at 37 °C, the metabolic reactions were initiated by the addition of the rat and human liver microsomes, separately. To stop metabolic reaction after 90 min, it was added 4 ml and 1 ml of chloroform, respectively, to incubations with rat and human liver microsomes. Then, the sample preparation was performed. Control incubations were performed in the absence of the cofactor solution and in the absence of the microsomal preparation. The difference between 'with' and 'without' NADPH was considered CYP450-mediated metabolism. To perform the experiments, the samples were pooled from ten single incubations.

A liquid–liquid extraction (LLE) procedure was applied to extract the ERT (**1**) from the rat and human liver microsomes. After the extraction procedure, the samples were shaken for 15 min (Vibrax VXR agitator, IKA, Staufen, Germany) and centrifuged for 5 min at 2860 × *g* (Hitachi CF16RXII, Himac, Tokyo, Japan). The supernatant was collected (3 ml and 750 μl, respectively, from rat and human microsomal preparation samples) and allowed to evaporate to dryness under a gentle stream of nitrogen. Then, the residue was reconstituted in 200 μl of methanol, and 1 μl was injected into the chromatography system.

In order to investigate the formation of metabolites, the samples were analyzed by a gas chromatograph (GC-MS-QP-2010, Shimadzu) coupled to a quadruple mass spectrometer with electron impact (EI) ionization at 70 eV. The gas chromatograph was equipped with an auto sampler AOC-20i, 1 μl split injections 1/10 were performed at 250 °C. Separations were carried out on a 5% phenyl methyl siloxane (DB-5ms) column (30 m × 0.25 mm × 0.25 μm film thickness). The oven temperature program for separation conditions were 100 °C for 0.8 min followed by temperature increases to 220 °C at 6 °C min^{-1}, 220 °C for 10 min, temperature increases to 290 °C at 6 °C min^{-1}, and 290 °C for 11 min. The carrier gas was ultrapure helium *helium* (grade 5.5) at constant flow rates of 1.10 ml min^{-1}. A mass range of *m*/*z* 50–500 was recorded in the full-scan mode. Finally, the metabolites were compared with authentically samples previous isolated.

To accumulate enough metabolite used for ^1H NMR analysis, rat liver microsome incubation was performed as previous described to 150 individual tubes (ERT, **1**, 2000 μg ml^{-1}). Then, this pool was submitted to semipreparative LC using the following conditions: C18 column (Shim-pack Prep-ODS, 5 μm, 20 mm × 25 cm, Shimadzu), flow rate 9 ml min^{-1} and acetonitrile (B) and H_2O (A) both with TFA 0.02% (v/v) as solvents. The elution profile was 0–25 min (15–65%, B), 25–30 min (25–80%, B), 30–32 min (80–100%, B) and 32–35 min (100%, B). The isolated compound was characterized by NMR (Bruker Avance® DRX-500) in a Shigemi symmetrical susceptibility-matched microtube.

Results and discussion

Pure ERT (**1**) elutes in GC–MS analysis at R_t 30.6 min (Fig. 1, charts A and B) and no other signal was observed. The chromatogram of the test reaction employing rat and human liver microsomes presented a new peak at the same R_t 29.81 min showing that there was a correlation between both microsomal models (Fig. 1, charts A and B). The absence of signals in the blank and control samples confirm the enzymatic conversion probably thought an oxidative reaction. The electron ionization mass spectra of the signal 1 at R_t 29.81 show an increment of 14 mass units (Fig. 1, chart D), related to ERT (Fig. 1, chart C). This result suggests an oxidation through a carbonyl group formation as previous observed for other natural products (Niehues et al., 2012; Santos et al., 2005, 2008). The fragmentation pattern of both compounds showed similar neutral eliminations confirming the homology between the two structures.

To confirm the metabolite structure large-scale rat liver microsome incubation was performed as previous described (applied 150 individual tubes of ERT at 2000 μg ml^{-1}). Then, this pool was submitted to semipreparative LC affording 900 μg. After the solvent elimination, the sample was characterized by NMR (Bruker Avance® DRX-500) in a Shigemi symmetrical susceptibility-matched microtube. The ^1H NMR ($CDCl_3$, 500 MHz) spectra of the isolated metabolite exhibited the signals (see supplementary data associated with this article): δ_H 6.33 (m, H-1), 6.72 (s, H-14), 6.72 (s, H-17), 6.89 (m, H-2), 6.15 (s, H-7), 5.94 (d, O-CH_2-O), 5.91 (d, O-CH_2-O), 3.90 (m, H-3), 3.78 (m, H-10), 3.68 *m* (m, H-10), 3.35 (s, —OCH_3), 3.15 (m, H-11), 2.99 (m, H-11), 2.82 (m, H-4), 2.10 (m, H-4). The data are in agreement with 8-oxo-erythraline (**2**), which was previous described by Mantle et al. (1984). Finally ESI-HRMS (pos.

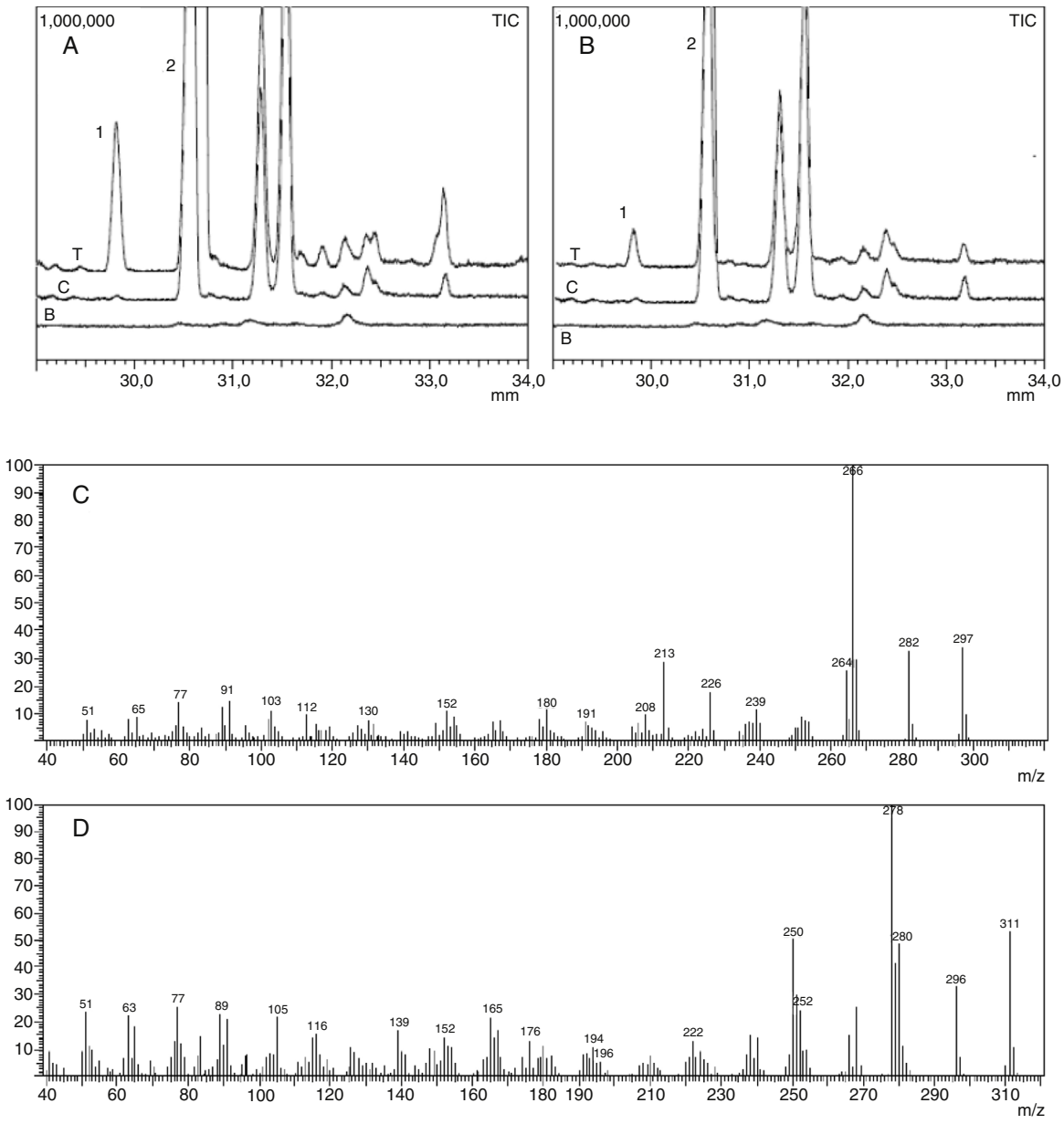

Fig. 1. Representative GC chromatograms of rat (chart **A**) and human microsomes extracts (chart **B**), signal 1: 8-oxo-erythraline (R_t = 29.81 min) and signal 2: ERT (R_t = 30.6 min). Lines T (test samples) of ERT metabolism after incubation; lines C (control) in both microsomal preparation without NADPH cofactor; lines B (blank): microsomal preparation without ERT. Left: rat liver microsomes; right: human liver microsomes. Chart **C** shows the EI-MS data of ERT (R_t = 30.6 min) and chart **D** shows the EI-MS data of 8-oxo-erythraline (R_t = 29.81 min).

ion mode) confirmed the molecular structure (observ. *m/z* 312.1228 $[M+H]^+$; calcd. for $C_{18}H_{18}NO_4^+$ 312.1230, error = 0.6 ppm).

Conclusion

This paper describes, for the first time, the CYP450-mediated metabolism of a promising natural product, ERT (**1**), using rat and human liver microsomes. Its metabolism showed the formation of the previously formed 8-oxo-erythraline (**2**) by bioinorganic catalysis (Guaratini et al., 2014). This compound was previously isolated as a minor compound in some cultivar of *E. verna* and biological evaluation applying macrophage and *Leishmania* cells revealed low cytotoxic activity for the ERT analog alkaloids investigated. In this manner, this compound should be considered as possible metabolite in the *in vivo* metabolism and its toxicological effect must be further investigated.

Authors' contributions

LMMM, FAA, TG, DBS, DRC conducted extraction and isolation of the eritralin and metabolites from plant and in microsomal biotransformation, respectively and the interpretation of all these data. LMMM, ARMO, NPL, JLCL and TG wrote and revised the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2015.05.011.

References

Arita, T., Miyazaki, S., Teramoto, S., Yoshitama, K., 2014. Major anthocyanin biosynthesis in the brilliant crimson petals from *Erythrina crista-galli* L. Sci. Hortic. 168, 272–280.

Callejon, D.R., Riul, T.B., Feitosa, L.G.P., Guaratini, T., Silva, D.B., Adhikari, A., Shrestha, R.L.S., Marques, L.M.M., Baruffi, M.D., Lopes, J.L.C., Lopes, N.P., 2014. Leishmanicidal evaluation of tetrahydroprotoberberine and spirocyclic erythrina-alkaloids. Molecules 19, 5692–5703.

Chen, T.L., Zhang, Y.B., Xu, W., Kang, T.G., Yang, X.W., 2014. Biotransformation of isoimperatorin by rat liver microsomes and its quantification by LC–MS/MS method. Fitoterapia 93, 88–97.

Clements, M., Li, L., 2011. Strategy of using microsome-based metabolite production to facilitate the identification of endogenous metabolites by liquid chromatography mass spectrometry. Anal. Chim. Acta 685, 36–44.

Cusack, K.P., Koolman, H.F., Lange, U.E.W., Peltier, H.M., Piel, I., Vasudevan, A., 2013. Emerging technologies for metabolite generation and structural diversification. Bioorg. Med. Chem. Lett. 23, 5471–5483.

Faggion, S.A., Cunha, A.O.S., Fachim, H.A., Gavin, A.S., Santos, W.F., Pereira, A.M.S., Beleboni, R.O., 2011. Anticonvulsant profile of the alkaloids (+)-erythravine and (+)-11-α-hydroxy-erythravine isolated from the flowers of *Erythrina mulungu* Mart ex Benth (Leguminosae–Papilionaceae). Epilepsy Behav. 20, 441–446.

Flausino Jr., O., Pereira, A.M., Bolzani, V.S., de Souza, R.L.N., 2007a. Effects of erythrinian alkaloids isolated from *Erythrina mulungu* (Papilionaceae) in mice submitted to animal models of anxiety. Biol. Pharm. Bull. 30, 375–378.

Flausino Jr., O., Santos, L.A., Verli, H., Pereira, A.M., Bolzani, V.S., de Souza, R.L.N., 2007b. Anxiolytic effects of erythrinian alkaloids from *Erythrina mulungu*. J. Nat. Prod. 70, 48–53.

Fraga, A.G.M., Silva, L.L., Fraga, C.A.M., Barreiro, E.J., 2011. CYP1A2-mediated biotransformation of cardioactive 2-thienylidene-3,4-methylenedioxybenzoylhydrazine (LASSBio-294) by rat liver microsomes and human recombinant CYP enzymes. Eur. J. Med. Chem. 46, 349–355.

Guaratini, T., Silva, D.B., Bizaro, A.C., Sartori, L.R., Humpf, H.U., Lopes, N.P., Lotufo, L.V.C., Lopes, J.L.C., 2014. In vitro metabolism studies of erythraline, the major spiroalkaloid from *Erythrina verna*. BMC Complement. Altern. Med. 14 (61), 2–5.

Juma, B.F., Majinda, R.R.T., 2004. Erythrinaline alkaloids from the flowers and pods of *Erythrina lysistemon* and their DPPH radical scavenging properties. Phytochemistry 65, 1397–1404.

Kurita, K.L., Linington, R.G., 2015. Connecting phenotype and chemotype: high-content discovery strategies for natural products research. J. Nat. Prod. 78, 587–596.

Mantle, P.G., Laws, I., Widdowson, D.A., 1984. 8-Oxo-erythraline, a naturally-occurring principal alkaloid from *Erythrina crista-galli*. Phytochemistry 23, 1336–1338.

Marques, L.M.M., SilvaJr, E.A., Gouvea, D.R., Vessecchi, R., Pupo, M.T., Lopes, N.P., Kato, M.J., De Oliveira, A.R.M., 2014. In vitro metabolism of the alkaloid piplartine by rat liver microsomes. J. Pharm. Biomed. 95, 113–120.

Messiano, G.B., Santos, R.A.S., Ferreira, L.S., Simoes, R.A., Jabor, V.A.P., Kato, M.J., Lopes, N.P., Pupo, M.T., de Oliveira, A.R.M., 2013. In vitro metabolism study of the promising anticancer agent the lignan (-)-grandisin. J. Pharm. Biomed. 72, 240–244.

Moreira, F.L., Souza, G.H.B., Rodrigues, I.V., Lopes, N.P., De Oliveira, A.R.M., 2013. A non-michaelian behavior of the in vitro metabolism of the pentacyclic triterpene alfa and beta amyrins by employing rat liver microsomes. J. Pharm. Biomed. 84, 14–19.

Niehues, M., Barros, V.P.M., Emery, F.S., Dias-Baruffi, M., Assis, M.D., Lopes, N.P., 2012. Biomimetic *in vitro* oxidation of lapachol: a model to predict and analyse the in vivo phase I metabolism of bioactive compounds. Eur. J. Med. Chem. 54, 804–812.

Nowak, P., Wozniakiewicz, M., Koscielniak, P., 2014. Simulation of drug metabolism. Trends Anal. Chem. 59, 42–49.

Ogbourne, S.M., Parsons, P.G., 2014. The value of nature's natural product library for the discovery of new chemical entities: the discovery of ingenolmebutate. Fitoterapia 98, 36–44.

Perdigao, P.S., Serrano, M.A.R., Flausino Jr., O., Bolzani, V.S., Guimaraes, M.Z.P., Castro, N.G., 2013. *Erythrina mulungu* alkaloids are potent inhibitors of neuronal nicotinic receptor currents in mammalian. PLoS ONE 8, e82726.

Santos, M.D., Martins, P.R., Santos, P.A., Bortocan, R., Iamamoto, Y., Lopes, N.P., 2005. Oxidative metabolism of 5-*O*-caffeoylquinic acid (chlorogenic acid), a bioactive natural product, by metalloporphyrin and rat liver mitochondria. Eur. J. Pharm. Sci. 26, 62–70.

Santos, M.D., Iamamoto, Y., Lopes, N.P., 2008. HPLC–ESI-MS/MS analysis of oxidizes di-caffeoylquinic acids generated by metalloporphyrin-catalydes reactions. Quim. Nova 31, 767–770.

Spaggiari, D., Geiser, L., Serge, R., 2014. Coupling ultra-high-pressure liquid chromatography with mass spectrometry for *in-vitro* drug-metabolism studies. Trends Anal. Chem. 63, 129–139.

Wanjala, C.C.W., Juma, B.F., Bojase, G., Gache, B.A., Majinda, R.R.T., 2002. Erythrinaline alkaloids and antimicrobial flavonoids from *Erythrina latissima*. Planta Med. 68, 640–642.

Yuan, L., Jia, P., Sun, Y., Zhao, C., Zhi, X., Sheng, N., Zhang, L., 2014. Study of *in vitro* metabolism of *m*-nisoldipine in human liver microsomes and recombinant cytochrome P450 enzymes by liquid chromatography–mass spectrometry. J. Pharm. Biomed. 97, 65–71.