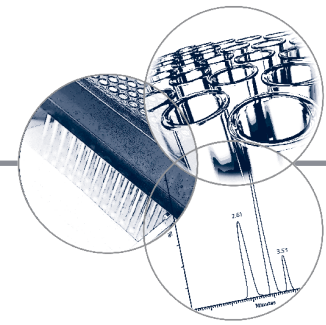
RESEARCH ARTICLE



Enantioselective analysis of ranolazine and desmethyl ranolazine in microsomal medium using dispersive liquid–liquid microextraction and LC–MS/MS

Background: An enantioselective bioanalytical method using dispersive liquid–liquid microextraction (DLLME) and LC–MS/MS was developed for the chiral analysis of ranolazine (RNZ) and one of its metabolites (desmethyl ranolazine [DRNZ]). **Results:** The analytes were extracted from microsomal medium by DLLME, using chloroform as extractor solvent and acetone as dispersive solvent. The enantiomers of RNZ and DRNZ were analyzed simultaneously for the first time using a Chiralcel OD-H®. Method validation showed recoveries in the order of 55 and 45%, and LLOQ of 25 and 10 ng ml^{-1} for the enantiomers of RNZ and DRNZ, respectively. Linearity was established in the concentration range of 10 to 1000 and 25 to 2500 ng ml^{-1} for each DRNZ and RNZ enantiomer, respectively. **Conclusion:** The unprecedented use of DLLME was demonstrated to be very useful for sample preparation of microsomal matrix. Furthermore, the *in vitro* metabolism of RNZ was enantioselective.

Ranolazine (RNZ; Figure 1) is the first approved agent of a new class of drugs against chronic stable angina in almost 30 years. The drug was originally patented in 1989, but it was only approved by the US FDA in 2006 [1,2]. Chronic stable angina describes the situation where the patients still have anginal symptoms and objective evidence of ischemia despite the best medical therapy [3]. The main pharmacological effect of RNZ is to inhibit the late sodium current, thus preventing sodium overload in the cell. In this way, RNZ prevents reverse mode sodium–calcium exchange and, thus, diastolic accumulation of calcium, possibly resulting in improved diastolic tone and improved coronary blood flow [4–6].

The RNZ is sold as a racemate, that is, a mixture of the same ratio of the enantiomers (+)-(*R*)-RNZ and (-)-(*S*)-RNZ, and is available as film-coated, sustained-release 500 mg tablets [3]. Studies addressing a possible enantioselectivity in the PK and PD properties of this drug are still underway. Only the study described by Jerling showed that the kinetic disposition of the drug in 11 healthy volunteers is not stereoselective [7]. With respect to metabolism and elimination, hydroxylation, *N*-dealkylation and *O*-demethylation are the main metabolic pathways of RNZ. Penman *et al.* described the characterization of RNZ metabolites in human plasma by LC–MS [8]. Among the metabolites observed, three Phase I and one Phase II metabolites were identified as being present at levels higher than 10% of the parent compound. These compounds were desmethyl RNZ (DRNZ; RS-88390) and the corresponding glucuronide conjugate, the *N*-dealkyl metabolite (RS-94287) and the *O*-dearylated metabolite (RS-88640). DRNZ (Figure 1), which results from the *O*-demethylation of RNZ, is the proposed target metabolite in this work. RNZ is metabolized mainly by CYP3A4 and secondarily by CYP2D6, both isoforms of cytochrome P450, and only 5–10% of the dose is excreted unchanged in urine [7].

RNZ has a low absorption response factor in the UV range, therefore, chromatographic methods using UV detectors do not have satisfactory detectability for determining this drug in biological matrices [9]. Even so, the work of Luo *et al.* has historical relevance because it demonstrates the separation of RNZ enantiomers using chiral columns based on cellulose tris (3,5-dimethylphenylcarbamate), in both polar organic and normal mode [10]. The developed method has semipreparative potential, but does not provide enough sensitivity (LLOQ of 2.9 mg ml^{-1}) for bioanalytical applications. It is also important to note that up to now there are no studies regarding the enantioselective metabolism of RNZ or the enantioselective determination of RNZ in biological matrices.

Rodrigo Almeida Simões[1,2], Thiago Barth[2] & Pierina Sueli Bonato*[2]

[1]Núcleo de Farmácia, Universidade Federal de Sergipe, Lagarto, Sergipe, Brazil
[2]Departamento de Física e Química, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, Brazil
*Author for correspondence:
Tel.: +55 163 602 4261
E-mail: psbonato@fcfrp.usp.br

FUTURE SCIENCE part of fsg

Key Terms

Liver microsomal fraction: Most popular model for *in vitro* metabolism studies. They are subcellular fractions derived from the endoplasmic reticulum of hepatic cells and are prepared by homogenization of liver followed by differential centrifugation. The microsomes are a rich source of many drug-metabolizing enzymes, especially cytochrome P450s.

Cloudy solution: Extracting solvent droplets dispersed in the sample.

Cotton effect: Amplitude variation characteristic in circular dichroism spectra in the region of an absorption band of a compound. The cotton effect is positive if the displacement from the zero of the rotation plane of the circularly polarized light is positive and is negative for the opposite displacement.

Extracting solution: Appropriate mixture of extracting and disperser solvents.

So, the aim of the present work includes the development and validation of a LC–MS/MS method employing dispersive liquid–liquid microextraction (DLLME) for the enantioselective determination of RNZ and DRNZ in rat **liver microsomal fractions**, and application of the developed method to study RNZ *in vitro* metabolism.

DLLME was introduced by Assadi *et al.* in 2006 [11]. In this sample preparation technique an appropriate mixture of extraction solvent (ES) and disperser solvent (DS) is rapidly injected into the aqueous sample using a syringe, with consequent production of a high turbulence. Thus, a **cloudy solution** is formed. After phase separation, the ES containing the analyte is recovered and analyzed. The advantages of DLLME are simplicity of operation, speed, low cost, high recoveries, enrichment factors and environmental benignity [11,12].

Experimental

Chemicals & reagents

Standard solutions: standard solutions of rac-RNZ and rac-DRNZ (purchased from Toronto Research Chemicals, Toronto, Canada) were prepared at concentrations of 1, 3, 10, 30, 75 and 100 µg ml^{-1} for rac-RNZ and 0.4, 1.2, 8, 20, 30 and 40 µg ml^{-1} for rac-DRNZ in methanol HPLC grade (Mallinckrodt, NJ, USA). All these solutions were stored at -20°C in amber glass tubes.

Mobile phase: the solvents (HPLC grade) used for mobile phase preparation were hexane containing 60% n-hexane (Mallinckrodt), ethanol and diethylamine (DEA; JT Baker, NJ, USA).

DLLME: the ES evaluated in the optimization tests of DLLME procedure were: dichloromethane and chloroform, both HPLC grade and obtained from Sigma-Aldrich (MI, USA), 1,1-dichloroethane (HPLC grade) purchased from Fluka (Buchs, Switzerland) and carbon tetrachloride (analytical grade) obtained from Merck (Darmstadt, Germany). The DS evaluated were: acetone, methanol, ethanol and acetonitrile, all of them HPLC grade and purchased from Merck. Sodium hydrogen phosphate (analytical grade) employed for buffer solution preparation was obtained from Merck (Darmstadt, Germany).

In vitro metabolism study: β-nicotinamide adenine dinucleotide phosphate ($NADP^+$), glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Sigma-Aldrich. Sodium dihydrogen phosphate, sodium hydrogen phosphate and tris(hydroxymetyl)aminomethane were obtained from Merck. Purified water was obtained from a Milli-Q-System (Millipore, DE, USA).

Instrumentation & analytical conditions

The analyses were carried out using LC–MS/MS equipment. The LC system was from Shimadzu (Kyoto, Japan) and consisted of two LC 10AD solvent pumps, a CTO 10AS column oven, a 7125 model Rheodyne injector (CA, USA) with a 20 µl loop and a SCL 10 Avp system controller. The MS system was a Quattro LC triple-stage quadrupole (Micromass, Manchester, UK) fitted with a Z-electrospray interface, operating in the positive-ion mode. A T-type valve connection (Upchurch Scientific, WA, USA) was used to split the effluent from the column and a flow rate of approximately 0.1 ml min^{-1} was directed into the stainless steel capillary probe of the MS system.

The resolution of RNZ and DRNZ enantiomers was achieved using a Chiralcel OD-H® column (150 × 4.6 mm, 5 µm particle size, Chiral Technologies, PN, USA) using a mobile phase consisting of hexane:ethanol (60/40, v/v) and 0.05% DEA, at a flow rate of 1.0 ml min^{-1}. A C18 guard column (10 × 4.6 mm, 5 µm particle size, Merck) was used to protect the analytical column.

The optimized MS/MS conditions were determined by direct infusion of standard solutions of rac-RNZ and rac-DRNZ (50 µg ml^{-1}) prepared in the mobile phase, and introduced into the detector by an infusion pump at a flow-rate of 10 µl min^{-1}. The analyses were carried out in SRM mode. The mass transition *m/z* 428 > 279 (cone voltage defined at 40 V and collision energy at 25 eV) was monitored for RNZ enantiomers. For DRNZ enantiomers, the monitored mass transition was *m/z* 414 > 266 (cone voltage defined at 39 V and collision energy at 23 eV). The temperatures of the source block and desolvation system were 100 and 250°C, respectively. Nitrogen was used as both drying and nebulizing gas and argon as the collision gas.

Elution order determination

The enantiomers of RNZ and DRNZ were obtained by semipreparative analysis of the racemates under the HPLC conditions described in the present paper (see section titled 'Instrumentation and analytical conditions'). Volumes of 20 µl of RNZ and DRNZ solutions at the

concentration of 100 µg ml^{-1} were injected several times and the column effluent was collected when the enantiomers reached the detector. Furthermore, the collected enantiomers of RNZ were analyzed on a Chiralcel OD column (25 × 4.6 mm, 10 µm particle size) using methanol 100% as mobile phase, according to the conditions described by Luo *et al.* [10]. Then, the retention times of the enantiomers from both studies were compared and the elution order established. In turn, the elution order of DRNZ enantiomers was determined by obtaining the circular dichroism (CD) spectra of the pure enantiomers of RNZ and DRNZ (200 to 400 nm) on a JASCO J-810 spectropolarimeter equipped with the temperature control apparatus JASCO PTC-423S, using a quartz cuvette with a 1.0 cm optical path at 17°C (MA, USA). Subtractions of baseline (solvent methanol) were carried out for all analyses. The standard scanning conditions were at a rate of 200 nm min^{-1} continuous mode, spectral bandwidth 1 nm, and a response of 0.5 s. According to Moen *et al.*, the enantiomer (-) is the (*S*)-RNZ, while the enantiomer (+) is the (*R*)-RNZ [13]. So, the DRNZ enantiomer that presented the same **cotton effect** of (+)-(*R*)-RNZ was considered (+)-(*R*) and the one that presented the same cotton effect of (-)-(*S*)-RNZ was considered (-)-(*S*).

■ DLLME procedure

Microsomal matrix: general composition

For the DLLME optimization, a biological matrix consisting of 0.5 ml of incubation medium (355 µl of 0.5 mol l^{-1} sodium phosphate buffer [pH 7.4], 125 µl of tris-HCl buffer at 50 mmol l^{-1}/KCl 0.154 mol l^{-1}, pH 7.4 [see section titled '*In vitro* biotransformation study'] and 20 µl rat hepatic microsomes at the concentration of 1 mg ml^{-1}) was employed. All cited reagents (analytical grade) were purchased from Merck.

DLLME procedure optimization

For the optimization of the extraction procedure, 0.5 ml of the incubation medium was placed in conical glass tubes and was spiked with rac-RNZ and rac-DRNZ (final concentration of 1 µg ml^{-1} for each enantiomer). Then, water was added to reach the final volume of 1, 2, 4 or 6 ml. After that, **extracting solution** was injected into the sample and the tubes were immediately centrifuged (HIMAC CF 15D2, Hitachi, Tóquio, Japão) at 4000 rpm for 3, 5, 10 or 20 min. In the tests in which the pH adjustment was necessary, the water was replaced by 1.5 ml of sodium phosphate buffer at 0.5 mol l^{-1} and pH 8 or 9. At the end of the centrifugation, 90 µl of the sedimented phase was transferred to another conical tube with the aid of a microsyringe, and the extract was dried under compressed air flux. The dry residues were then dissolved in 50 µl of the mobile phase prior to analysis and 20 µl was introduced into the LC system. All extractions were performed in triplicate.

■ Method validation

The recovery was evaluated by analyzing 0.5 ml of microsomal matrix samples spiked with 75, 750 and 1875 ng ml^{-1} and 30, 500 and 750 ng ml^{-1} of each RNZ and DRNZ enantiomers, respectively, in triplicate, and submitted to the DLLME procedure and chromatographic analysis. Recovery was expressed as a percentage of the amount extracted.

The calibration curves were prepared spiking 0.5 ml microsomal matrix with 25 µl standard solutions of rac-RNZ (1, 3, 10, 30, 75 and 100 µg ml^{-1}) and rac-DRNZ (0.4, 1.2, 8, 20, 30 and 40 µg ml^{-1}; n = 3 for each concentration). In this way, the final concentrations obtained were 25, 75, 250, 750, 1875 and 2500 ng ml^{-1} and 10, 30, 200, 500, 750 and 1000 ng ml^{-1} for each RNZ and DRNZ enantiomer, respectively. The results were plotted on a graph of peak areas versus analytes concentrations and the best relationship was obtained by linear least-squares regression analysis with a weighting of 1/x^2. The linearity of the method was evaluated using the correlation coefficient (r) and the deviation from the nominal value for each concentration.

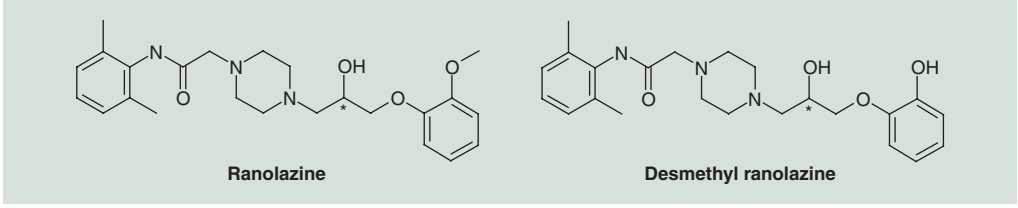


Figure 1. Ranolazine and desmethyl ranolazine.
*Chiral center.

The LLOQ of the method, defined as the lowest concentration that could be determined with accuracy and precision below 20% over five analytical runs, was determined by analyzing 0.5 ml of the microsomal matrix (n = 5) at concentrations of 25 ng ml^{-1} for each RNZ enantiomer and 10 ng ml^{-1} for each DRNZ enantiomer [14].

Within-day precision and accuracy were achieved by replicate analysis (n = 5) of control samples in three different concentrations and between-day precision and accuracy were determined during routine operation of the system over a period of 3 working days. For this purpose, 0.5 ml microsomal matrix were spiked with 75, 750 and 1875 ng ml^{-1} and 30, 500 and 750 ng ml^{-1} of each RNZ and DRNZ enantiomers, respectively, and submitted to the DLLME procedure and chromatographic analysis. The results obtained were expressed as relative standard deviation (RSD; %) and relative error (E; %).

Short-term room temperature (25°C ± 2 for 6 h) and incubation (37°C for 60 min) stabilities were evaluated for samples spiked with 75 and 1875 ng ml^{-1} and 30 and 750 ng ml^{-1} of each RNZ and DRNZ enantiomer, respectively. The quantitative response (peak areas) from the test groups were compared with the response from the samples freshly prepared and the obtained results were expressed as E. All of the experiments were carried out in sixtuplicate for each concentration.

Finally, matrix effects were evaluated by comparing the peak areas obtained from the analysis of pure rac-RNZ and rac-DRNZ standard solutions prepared in the mobile phase with the peak areas obtained from extracted blank microsomal matrix spiked with 75 and 30 ng ml^{-1} of each RNZ and DRNZ enantiomer, respectively. The experiment was performed in sixtuplicate in the concentration corresponding to the LOQ.

■ *In vitro* metabolism study

The microsomal fraction was obtained from male Wistar rat (180–200 g) liver homogenates by ultracentrifugation procedures, and it was stored frozen at -70°C [15]. The use of animals in this study was approved by the ethical committee from University of São Paulo (ethical approval n. 09.1.1249.53.3).

The RNZ enantioselective *in vitro* metabolism profile was assessed by measuring the rate of disappearance of each RNZ enantiomer and monitoring DRNZ as the target metabolite in different microsomal protein concentrations and incubation times. The incubation system consisted of 125 µl NADPH-regenerating system (1.7 mg ml^{-1} NADP+, 7.8 mg ml^{-1} glucose-6-phosphate and 1.5 units ml^{-1} glucose-6-phosphate dehydrogenase in Tris–HCl buffer [50 mmol l^{-1}/KCl 0.154 mol l^{-1}, pH 7.4]), 10 to 80 µl of rat liver microsomal fraction (initial protein concentration of 25 mg ml^{-1}) in order to reach final protein concentrations of 0, 0.5, 1.0, 2.0, 3.0 and 4.0 mg ml^{-1} in the incubation medium. To complete 0.5 ml of final volume, enough amount of 0.5 mol l^{-1} sodium phosphate buffer (pH 7.4) was added. Incubations were performed (n = 3) in the same glass tubes used for DLLME procedure, using a shaking water bath at 37°C during 0, 5, 10, 20, 30, 45 and 60 min. The incubation was stopped by the addition of DLLME extraction solution. Control incubations were performed in the absence of both an NADPH-regenerating system and rat microsomes to ensure that any change in analyte concentrations was due to metabolism mediated by liver microsomes.

Results & discussion

■ Chromatographic method development

In order to separate RNZ and DRNZ enantiomers simultaneously, several chiral columns based on macrocyclic glycopeptide antibiotic (vancomycin), cellulose (3,5-dimethylphenylcarbamate, 4-methylphenylcarbamate and 4-methylbenzoate) and amylose (3,5-dimethylphenylcarbamate) derivatives were evaluated. These columns were used under normal and polar organic elution mode. The normal phase compositions were based on different ratios of hexane, 2-propanol, methanol and ethanol. On the other hand, polar organic solvents were employed to evaluate the ability of the columns to separate the RNZ and DRNZ enantiomers under polar organic mode; these solvents were acetonitrile, methanol, ethanol or 2-propanol used isolated or in mixtures.

The suitable separation in 17 min was achieved using the Chiralcel OD-H column (cellulose tris [3,5-dimetylphenylcarbamate] as chiral selector) with a mobile phase consisting of hexane:ethanol (60/40, v/v) and 0.05% DEA and a flow rate of 1.0 ml min^{-1}. Under these conditions, the resolutions between the enantiomers peaks were 4.8 and 1.25 for RNZ and DRNZ, respectively (FIGURE 2).

■ Elution order determination

The elution order for RNZ enantiomers was the same as that established by Luo *et al.* [10].

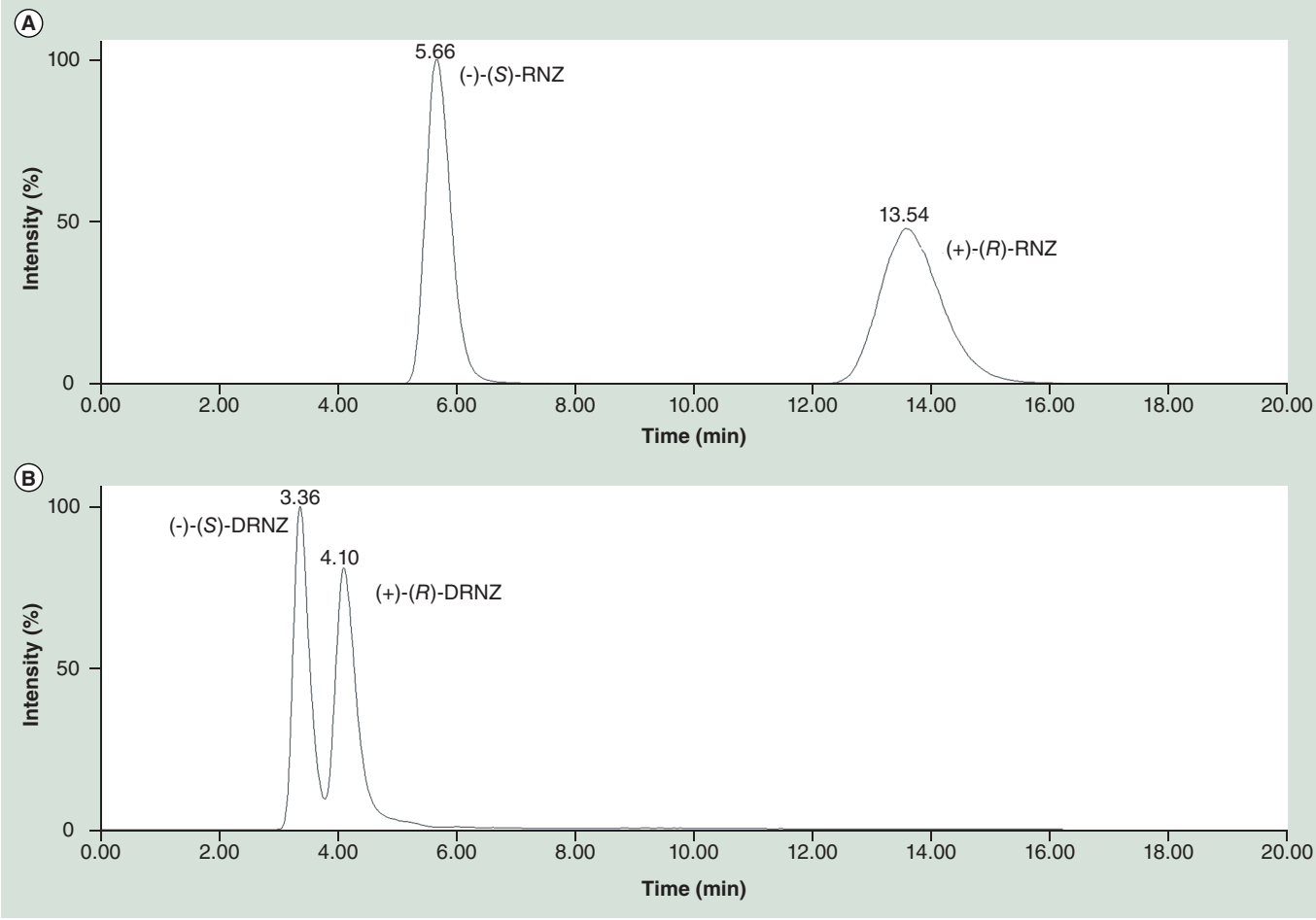


Figure 2. Representative chromatograms referring to the analysis of (A) ranolazine and (B) desmethyl ranolazine enantiomers. Chromatographic conditions: Chiralcel OD-H® column and mobile phase consisting of hexane:ethanol (60/40, v/v) with 0.05% diethylamine at a flow rate of 1.0 ml min^{-1}.
DRNZ: Desmethyl ranolazine; RNZ: Ranolazine.

The first peak in the chromatogram presented in Figure 2A corresponds to (-)-(*S*)-RNZ and the second peak to (+)-(*R*)-RNZ. The same elution order was expected due to the use of the same kind of stationary phase in both studies. Although the use of different mobile phases can result in inversion in the elution order, this was not observed in this study [16,17].

The chiral resolution and elution order of DRNZ enantiomers is not available in the literature. Therefore, to determine the elution order of DRNZ, a combination of chromatographic and CD methods was used [18,19]. Hence, the CD spectra of DRNZ enantiomers were compared with the CD spectra of RNZ enantiomers. For DRNZ, the first eluted enantiomer showed cotton effect similar to the (-)-(*S*)-RNZ and it was considered as (-)-(*S*)-DRNZ (first peak), and the second eluted enantiomer showed cotton effect similar to the (+)-(*R*)-RNZ and it was considered as (+)-(*R*)-DRNZ (second peak) (Figure 2B).

■ Development of the DLLME procedure in microsomal medium

In DLLME, the main factors affecting the extraction efficiency are allied to the ES and DS physicochemical characteristics and the volume employed. Considering that the choice of ES and DS is a crucial point in DLLME method development, the evaluation of this parameter should invariably be the first step in the extraction procedure optimization [20]. The organic solvents used as ES should be insoluble in water and denser than it. In addition, they must also have high capacity to solubilize the target analytes and, if possible, be compatible with the chromatographic system employed. Halogenated solvents such as chlorobenzene, chloroform, tetrachlorethylene and carbon tetrachloride,

among others, are extensively used as ES, especially because of their high densities. Regarding the DS, the main requirement is the solubility in both ES and aqueous sample. The most commonly used solvents as DS are methanol, ethanol, acetone and acetonitrile [21–30].

The extraction of RNZ and DRNZ from microsomal matrix was carried out using dichloromethane, chloroform, 1,1-dichloroethane and carbon tetrachloride as ES, whereas acetone, methanol, ethanol and acetonitrile were evaluated as DS. In these experiments, 600 µl of the extracting solution (100 µl of ES and 500 µl of DS), composed of all possible combinations between ES and DS, were injected into 4 ml of the samples, then the samples were centrifuged and the sedimented phase was separated and evaporated. The residues were dissolved in the mobile phase and chromatographed. All experiments were done in triplicate.

The extraction solutions that had alcohols as DS did not produce good results. By using chloroform, dichloromethane and carbon tetrachloride as ES and methanol as DS, it was not possible to obtain a limpid sedimented phase, therefore, incompatible with the chromatographic system. When ethanol was used, it was not possible to obtain the sedimented phase, probably due to high ethanol capacity of solubilizing the ES used. In contrast, extraction solutions that had acetone or acetonitrile as DS showed good results (Figure 3). Chloroform and acetone showed the best results for RNZ and DRNZ enantiomers recovery. Therefore, they were selected as ES and DS, respectively, for the next steps in DLLME optimization.

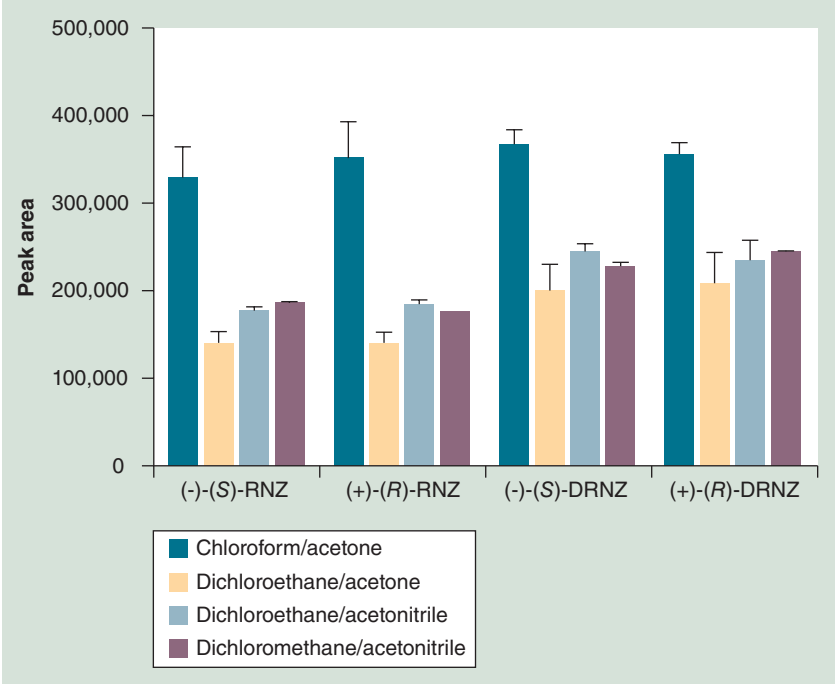


Figure 3. Evaluation of the extracting and disperser solvent type for extraction of ranolazine and desmethyl ranolazine from microsomal medium. Extraction conditions: 600 µl of extracting solution, sample volume of 4 ml, pH 7.4 and 5 min of centrifugation at 4000 rpm.
DRNZ: Desmethyl ranolazine; RNZ: Ranolazine.

The following step in method optimization was the evaluation of the influence of ES and DS volumes on the extraction efficiency. In order to characterize a microextraction technique, ES and DS volumes larger than 100 and 800 µl, respectively, were avoided. Moreover, preliminary experiments showed that ES volumes lower than 25 µl resulted in unsuitable sedimented phase. Therefore, extracting phases composed of combinations of 25, 50 and 100 µl of ES and 450 and 800 µl of DS were evaluated. The extracting solution volume was always the sum of the ES and DS volumes. Figure 4 shows that, apparently, the formation of the cloudy solution is adversely affected when a low volume of DS is used. Thus, the recovery efficiency is low [11]. The best combination of DS and ES in the extraction of the analytes was 100 µl of chloroform and 800 µl of acetone. Under these conditions it is possible to obtain good efficiency of cloudy solution formation, with approximately 100 µl of sedimented phase.

The volume ratio between the aqueous sample and the extracting solution can improve or decrease the dispersion formation and, consequently, the sedimented phase formation. Therefore, the effect of sample volume was evaluated by extracting different final volumes of the sample spiked with the same concentration of the analytes. Final volumes of 0.5, 1, 2, 4 and 6 ml were achieved by adding 0, 0.5, 1.5, 3.5 and 5.5 ml of water to 0.5 ml of liver microsome incubation samples. The extraction was performed adding 900 µl of the extracting solution (chloroform/acetonitrile). Samples with 0.5 and 1 ml of final volume did not allow the efficient formation of the cloudy solution, therefore, no proper sedimented phase. Figure 5 shows that the increase of the aqueous volume decreases the amount of the analytes recovered. This happens because the amount of RNZ and DRNZ solubilized in the sample increases when the volume ratio aqueous phase/organic phase increases, too. Hence, the final volume of 2 ml was selected for the following optimization steps.

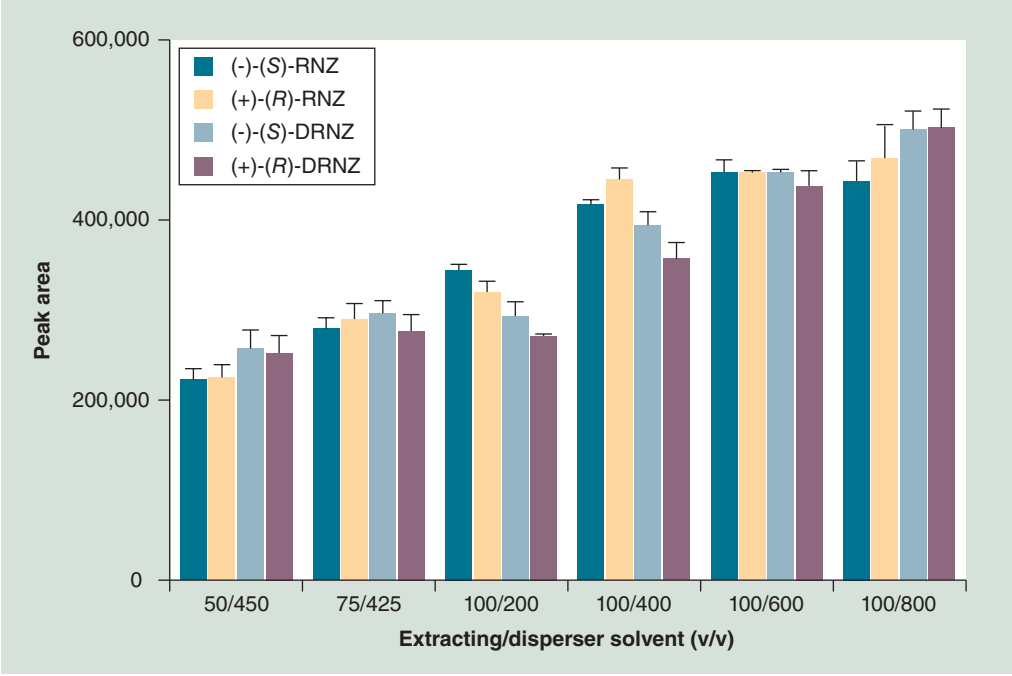


Figure 4. Evaluation of extracting and disperser solvent volume for extraction of ranolazine and desmethyl ranolazine from microsomal medium. Extraction conditions: chloroform as extraction solvent and acetone as disperser solvent, sample volume of 4 ml, pH 7.4 and 5 min of centrifugation at 4000 rpm.
DRNZ: Desmethyl ranolazine; RNZ: Ranolazine.

Both RNZ and DRZ behave as weak bases and have pKa close to 6.5 [101]. Therefore, the influence of sample pH adjustment in the extraction of these compounds was evaluated for up to two units above their pKa, since in this pH range all of the molecules are statistically in the nonionized form and, thus, have higher affinity for the chloroform (ES). pH 7.4 was also particularly evaluated, because this is the same pH of the incubation medium. Despite the pKa values of the analytes, the data presented in Figure 6 show no statistic difference in extraction efficiency between pH 7.4 (incubation medium pH), 8.0 and 9.0. This is a very interesting point, since in this way, there is no need for adjusting samples pH.

Extraction time is one of the most important factors in most of the extraction procedures, especially in microextraction techniques such as solid-phase microextraction and hollow-fiber liquid-phase microextraction, because these procedures are not exhaustive and the equilibrium of the analyte distribution between the sample and the extracting phase is strongly time-dependent [11,31]. In DLLME, extraction time is defined as the interval between the extracting solution injection into the sample and centrifugation. After the formation of the cloudy solution, the surface area between the extracting solvent and the aqueous phase is extremely large, thus the equilibrium state is reached rapidly and, consequently, the extraction time is achieved promptly. Numerous studies in the

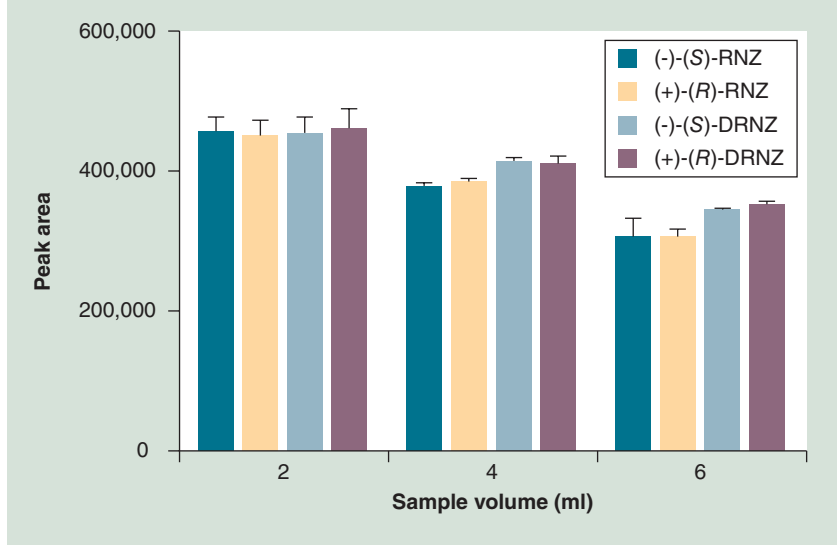


Figure 5. Evaluation of the sample volume in the extraction of ranolazine and desmethyl ranolazine from microsomal medium. Extraction conditions: 100 µl chloroform, 800 µl acetone, pH 7.4 and 5 min of centrifugation at 4000 rpm.
DRNZ: Desmethyl ranolazine; RNZ: Ranolazine.

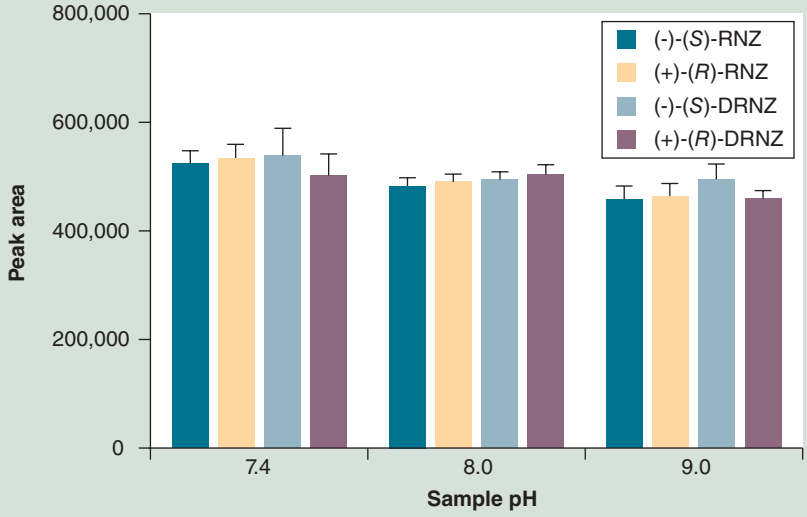


Figure 6. Evaluation of the influence of sample pH in the extraction of ranolazine and desmethyl ranolazine from microsomal medium. Extraction conditions: 100 µl chloroform, 800 µl acetone, sample volume of 2 ml and 5 min of centrifugation at 4000 rpm.
DRNZ: Desmethyl ranolazine; RNZ: Ranolazine.

literature show clearly that this variable is statistically insignificant for DLLME [21–30]. Hence the influence of time on extraction efficiency was not systematically evaluated in this present work. Only the influence of the ultrasound-assistance time was evaluated.

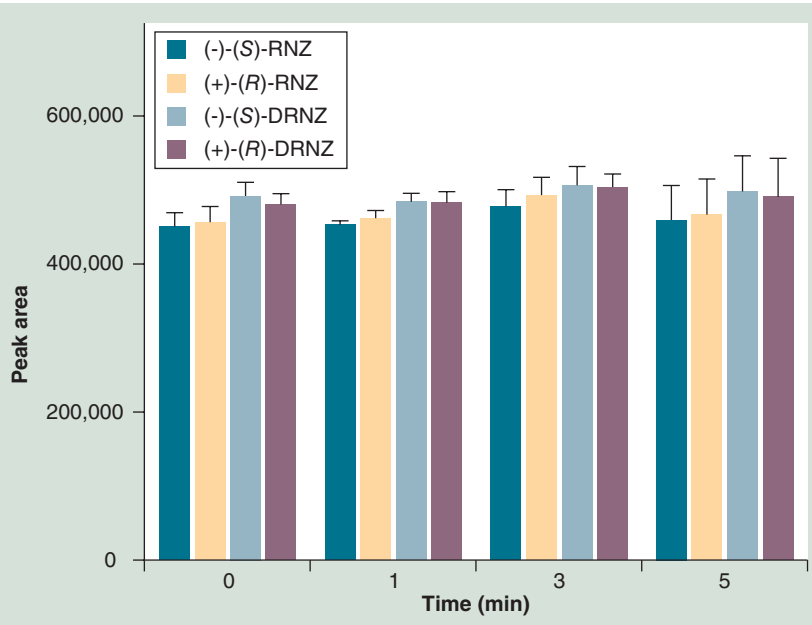


Figure 7. Evaluation of the ultrasound-assistance in the extraction of ranolazine and desmethyl ranolazine from microsomal medium. Extraction conditions: 100 µl chloroform, 800 µl acetone, sample volume of 2 ml, pH 7.4 and 5 min of centrifugation at 4000 rpm.
DRNZ: Desmethyl ranolazine; RNZ: Ranolazine.

Dispersion is the key step for effective extraction [32]. During ultrasonic irradiation, implosion bubbles are produced due to the cavitation phenomenon, which generates severe collision waves in the surrounding liquid and high-velocity liquid jets. Such microjets can cause droplet disturbance and cause bubbles to collapse, with consequent dispersion improvement [33,34]. Therefore, the influence of sonication was evaluated from 0 to 5 min. Figure 7 shows no significant difference in the extraction efficiency with (1, 3 and 5 min) and without sonication (0 min). These data show that the injection speed of the extracting solution into the aqueous sample generated enough energy for the proper cloudy solution formation (dispersion). Thus, immediately after injection of the extracting solution the samples were centrifuged.

The centrifugation is required to destabilize the dispersion and accelerate ion-phase separation [32]. The centrifugation time was evaluated at 3, 5, 10 and 20 min. After only 3 min of centrifugation at 4000 rpm, the sample presented three distinct layers. From top to bottom: the first layer (limpid) is aqueous and rich in acetone (DS), the second one is a thin layer rich in cellular debris and macromolecules coming from the biological matrix and the third one (also limpid) is the sedimented phase (ES). The microsomal fraction components have a relatively low density as a consequence of its isolation procedure (by ultracentrifugation) [15]. This fact allows the formation of an intermediate thin layer after centrifugation. This characteristic of the sample after centrifugation is extremely interesting regarding applying DLLME to complex samples, because one of the major drawbacks of using DLLME in this type of matrix is the precipitation of some particles from the biological sample together with ES.

After method optimization, the extractions were performed as: ES: 100 µl chloroform; DS: 800 µl acetone; sample volume: 2 ml; sample pH: 7.4; centrifugation: 3 min at 4000 rpm.

■ Method validation

The method showed to be linear over the concentration range of 10 to 1000 ng ml^{-1} for each DRNZ enantiomer with r > 0.99 and of 25 to 2500 ng ml^{-1} for each RNZ enantiomer with r > 0.98. The deviations from the nominal value (E%) were <15% for all the points in the calibration curves (Table 1). The recoveries obtained by the DLLME procedure were close to 50% for all analytes (Table 2), which allowed excellent

LLOQs to be obtained: 25 ng ml^{-1} and 10 ng ml^{-1} for each RNZ and DRNZ enantiomers, respectively, with RSD and E% lower than 15% (Table 3).

The precision and accuracy of the method were tested by within- and between-day analysis. The RSD and E% were lower than 15% (Table 4).

To evaluate the short-term room temperature stability, the samples were kept at room temperature (25°C ± 2) for 6 h. Additionally, the analytes stabilities under the incubation conditions were evaluated using samples incubated at 37°C for 60 min in microsomal medium, without NADPH-regenerating system. Both short-term room temperature and incubation conditions stability studies showed no differences (E% ≤ 15%) between the results obtained from fresh samples (freshly prepared and processed) and from samples kept at room temperature and at incubation conditions (Table 5).

The results obtained for the evaluation of matrix effect on analytes ionization (Table 6) indicate that the extraction procedure was very efficient in removing matrix compounds that could suppress the ionization of RNZ and DRNZ enantiomers.

Table 1. Linearity of the method.

Analyte	Range (ng ml^{-1})	Linear equation	r†
(-)-(*S*)-ranolazine	25–2500	y = 2181x + 49,060	0.9873
(+)-(*R*)-ranolazine	25–2500	y = 2784x + 55,503	0.9891
(-)-(*S*)-desmethyl ranolazine	10–1000	y = 200x + 888	0.9953
(+)-(*R*)-desmethyl ranolazine	10–1000	y = 225x + 1179	0.9944

†*Correlation coefficient.*

Table 2. Recovery of the method.

Analyte concentration (ng ml^{-1}; n = 3)†	Recovery (%)	RSD (%)
(-)-(S)-ranolazine		
75	49.6	2.4
750	55.5	2.5
1875	56.7	2.0
Mean	53.9	7.0
(+)-(R)-ranolazine		
75	53.6	5.6
750	61.7	0.4
1875	61.6	1.1
Mean	59.0	7.8
(-)-(S)-desmethyl ranolazine		
30	38.9	4.6
500	48.5	2.8
750	49.2	1.7
Mean	45.5	12.6
(+)-(R)-desmethyl ranolazine		
30	39.8	4.8
500	49.4	1.7
750	50.6	4.1
Mean	46.6	12.6

†*Number of determinations for each concentration.*

■ Method application: RNZ *in vitro* metabolism

In order to assess the enantioselective *in vitro* metabolism profile of RNZ, the influence of two parameters was evaluated: microsomal protein concentrations and incubation times. The concentration of microsomal proteins in the medium is indicative of the cytochrome P450 enzymes concentration. Incubations were performed in triplicate in the range of 0.5 to 4.0 mg ml^{-1}of protein in the incubation medium. The nominal substrate concentration used in these experiments was 1875 ng ml^{-1} for each RNZ enantiomer. Figure 8A shows the metabolic behavior of RNZ against different microsomal protein concentrations. As the protein concentration increased, the enantiomers concentrations ratio – [(+)-(*R*)-RNZ]/[(-)-(*S*)-RNZ] – also increased, indicating that the (-)-(*S*)-RNZ enantiomer was metabolized preferentially. This increase in the enantiomer ratio showed a linear behavior over the range of protein concentration evaluated. The enantiomers of the presumed metabolite (DRNZ) were detected, but their signals were below the LLOQ of the method (10 ng ml^{-1}), indicating that less than 0.1% of the drug is transformed to this metabolite in rats. So, the decrease in RNZ enantiomers concentrations could be due to the formation of other metabolites not detected by the proposed method. Aiming to evaluate RNZ metabolic behavior over time, the substrate (1875 ng ml^{-1} for each enantiomer) was incubated for 0 to 60 min in a medium containing 1.5 mg ml^{-1} microsomal protein concentration. Figure 8B shows that the enantioselectivity of the RNZ biotransformation during the time slot evaluated followed the same linear profile observed in the evaluation of protein range.

Conclusion

The development and validation of an enantioselective method for the simultaneous analysis of RNZ and DRNZ in biological matrices is described for the first time. The optimum chromatographic conditions to separate the enantiomers of the analytes was achieved using

Table 3. LLOQ of the method.

Analyte	Nominal concentration (ng ml^{-1})	Obtained concentration (ng ml^{-1})	Accuracy: relative error (%)	Precision: RSD (%)
(-)-(*S*)-ranolazine	25	24.6	-1.4	7.1
(+)-(*R*)-ranolazine	25	24.1	-3.6	3.9
(-)-(*S*)-desmethyl ranolazine	10	9.4	-5.4	11.0
(+)-(*R*)-desmethyl ranolazine	10	9.3	-7.1	9.8

Table 4. Precision and accuracy of the method.

Analyte	Nominal concentration (ng ml^{-1})	Within-day (n = 5)†			Between-day (n = 3)‡		
		Concentration (ng ml^{-1})	RSD (%)	Relative error (%)	Concentration (ng ml^{-1})	RSD (%)	Relative error (%)
(-)-(*S*)-ranolazine	75	70.9	6.9	-5.5	76.8	6.6	2.4
	750	697.9	1.4	-6.9	728.7	7.9	-2.8
	1875	1844.1	2.0	-1.6	1811.1	4.9	-3.4
(+)-(*R*)-ranolazine	75	70.4	4.8	-6.2	74.2	4.5	-1.0
	750	727.2	3.9	-3.0	755.3	3.3	0.7
	1875	1892.7	1.3	0.9	1848.1	2.1	-1.4
(-)-(*S*)-desmethyl ranolazine	30	31.6	6.6	5.4	31.7	0.3	5.7
	500	466.8	4.4	-6.6	476.9	2.6	-4.6
	750	664.0	2.9	-11.5	694.6	4.2	-7.8
(+)-(*R*)-desmethyl ranolazine	30	31.9	3.0	6.4	32.5	1.7	8.4
	500	461.8	1.9	-7.6	469.5	2.7	-6.1
	750	665.9	2.6	-11.2	705.2	5.1	-6.0

†*Number of determinations.*
‡*Number of days.*

Table 5. Short-term room temperature and incubation stabilities.

Analyte (n = 6)†	Nominal concentration (ng ml^{-1})	Nominal deviation (E%)	
		Short-term room temperature	Incubation
(-)-(*S*)-ranolazine	75	8	-4
	1875	-7	-5
(+)-(*R*)-ranolazine	75	5	2
	1875	-5	-7
(-)-(*S*)-desmethyl ranolazine	30	-4	-9
	750	0	-6
(+)-(*R*)-desmethyl ranolazine	30	-11	-14
	750	-2	-3

†*Number of determinations.*

Table 6. Evaluation of matrix effects.

Analyte	Concentration (ng ml^{-1})	Matrix effect (%)	RSD (%)
(-)-(*S*)-ranolazine	25	9.5	4.3
(+)-(*R*)-ranolazine	25	13.5	6.8
(-)-(*S*)-desmethyl ranolazine	10	1.0	10.3
(+)-(*R*)-desmethyl ranolazine	10	-4.5	9.5

a Chiralcel OD-H column under normal elution mode. The unprecedented use of DLLME for sample preparation of this kind of matrix showed that this microextraction technique could be very useful for *in vitro* metabolism studies, since it allows stopping the metabolism reactions and extraction of the analytes in a single step. In addition, this technique is very simple, rapid, reproducible and provides excellent recoveries (~50%).

The developed and validated enantioselective method was applied to an *in vitro* metabolism study using rat liver microsomes. The study showed

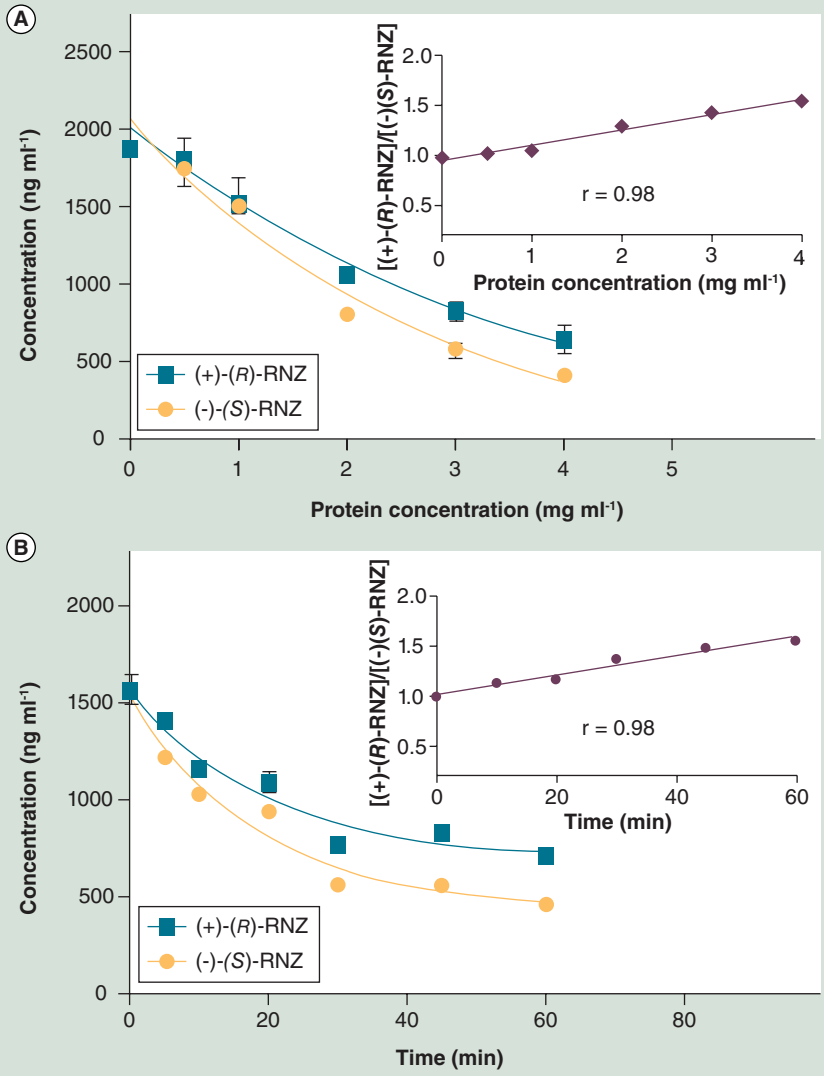


Figure 8. Ranolazine *in vitro* metabolism profile. (A) Influence of the microsomal protein concentration in the RNZ *in vitro* metabolism profile. Incubation for 15 min at 37°C. **(B)** Influence of the incubation time in the RNZ *in vitro* metabolism profile. Incubation at 37°C with 1.5 mg ml^{-1} of microsomal protein concentration. Cofactor solution: β-nicotinamide adenine dinucleotide phosphate (1.7 mg ml^{-1}), glucose-6-phosphate (7.8 mg ml^{-1}) and glucose-6-phosphate dehydrogenase (1.5 units ml^{-1}).
RNZ: Ranolazine.

that RNZ metabolism was enantioselective with preferential consumption of the (-)-(*S*)-RNZ enantiomer. However, the target metabolite was not detected in significant concentrations in the metabolism model employed.

Future perspective

DLLME is widely applied for environmental water samples, but hardly ever applied for the analysis of drugs in complex matrices such as biological fluids. However, in the present work DLLME was shown to be a promising technique for sample preparation in *in vitro* metabolism studies using liver microsomal fraction since it was possible to produce suitable sediment phase for injection into analytical instruments such as LC–MS/MS. Thus, it is necessary to evaluate the DLLME performance in other *in vitro* metabolism models, such as supersomes, liver cytosolic fractions, S9 fractions and cell cultures. If DLLME continues to show excellent performance in these kinds of samples, it will be able to be an alternative to protein precipitation by organic solvents – the most widely used

technique to stop metabolism reactions during *in vitro* metabolism studies.

Acknowledgements

The authors would like to thank L Tiemi Okano for help with the dichroism circular experiments.

Financial & competing interests disclosure

The authors are grateful to Fundação de Amparo à Pesquisa do Estado de São Paulo, Conselho Nacional de Desenvolvimento Científico e Tecnológico and to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior for financial support and for granting research fellowships. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- Dispersive liquid–liquid microextraction was used for the extraction of ranolazine and desmethyl ranolazine from liver microsomal fraction samples.
- The analytical step was carried out using LC–MS/MS with the chiral column Chiralcel OD-H® and a normal mobile phase.
- The method was used to study the *in vitro* metabolism of ranolazine.

References

Papers of special note have been highlighted as:
- ▪ of interest
- ▪▪ of considerable interest

1 Aslam S, Gray D. Ranolazine (Ranexa) in the treatment of chronic stable angina. *Adv. Ther.* 27(4), 193–201 (2010).

2 Bhaumik U, Ghosh A, Sarkar AK *et al.* Determination of ranolazine in human plasma by LC–MS/MS and its application in bioequivalence study. *J. Pharmaceut. Biomed.* 48(5), 1404–1410 (2008).

3 Nash DT, Nash SD. Ranolazine for chronic stable angina. *Lancet* 372(9646), 1335–1341 (2008).

4 Hayashida W, van Eyll C, Rousseau MF, Pouleur H. Effects of ranolazine on left ventricular regional diastolic function in patients with ischemic heart disease. *Cardiovasc. Drugs Ther.* 8(5), 741–747 (1994).

5 Hasenfuss G, Maier LS. Mechanism of action of the new anti-ischemia drug ranolazine. *Clin. Res. Cardiol.* 97(4), 222–226 (2008).

6 Fraser H, Belardinelli L, Wang LG, Light PE, Mcveigh JJ, Clanachan AS. Ranolazine decreases diastolic calcium accumulation caused by ATX-II or ischemia in rat hearts. *J. Mol. Cell. Cardiol.* 41(6), 1031–1038 (2006).

7 Jerling M. Clinical pharmacokinetics of ranolazine. *Clin. Pharmacokinet.* 45(5), 469–491 (2006).

8 Penman AD, Eadie J, Herron WJ, Reilly MA, Rush WR, Liu Y. The characterization of the metabolites of ranolazine in man by liquid chromatography mass spectrometry. *Rapid Commun. Mass Spectrom.* 9(14), 1418–1430 (1995).

9 Liang Y, Xie L, Liu X *et al.* Simple, sensitive and rapid liquid chromatography/atmospheric pressure chemical ionization mass spectrometric method for the quantitation of Ranolazine in rat plasma. *Rapid Commun. Mass Spectrom.* 20(4), 523–528 (2006).

10 Luo XP, Zhai ZD, Wu XM, Shi YP, Chen LR, Li YM. Analytical and semipreparative resolution of ranolazine enantiomers by liquid chromatography using polysaccharide chiral stationary phases. *J. Sep. Sci.* 29(1), 164–171 (2006).

11 Rezaee M, Assadi Y, Hosseini MRM, Aghaee E, Ahmadi F, Berijani S. Determination of organic compounds inwater using dispersive liquid–liquid microextraction. *J. Chromatogr. A* 1116(1–2), 1–9 (2006).

▪▪ **First work describing the dispersive liquid–liquid microextraction techinique.**

12 Rezaee M, Yamini Y, Faraji M. Evolution of dispersive liquid–liquid microextraction method. *J. Chromatogr. A* 1217(16), 2342–2357 (2010).

▪▪ **Excellent review on the application, advantages, limitations, recent developments and challenges of dispersive liquid–liquid microextraction.**

13 Moen AR, Karstad R, Anthonsen T. Chemo-enzymatic synthesis of both enantiomers of the anti-anginal drug ranolazine. *Biocatal. Biotransformation* 23(1), 45–51 (2005).

14 US Department of Health and Human Services, US FDA, Center for Drug Evaluation and Research, Center for Veterinary Medicine. *Guidance for Industry. Bioanalytical Method Validation.* US Department of Health and Human Services, FDA, Center for Drug Evaluation and Research, Center for Veterinary Medicine, Rockville, MD, USA (2001).

15 Snell K, Mullock B. *Biochemical Toxicology: A Practical Approach.* Oxford Universtiy Press, Oxford, UK (1987).

16 Okamoto M. Reversal of elution order during the chiral separation in high performance liquid chromatography. *J. Pharm. Biomed. Anal.* 27(3–4), 401–407 (2002).

▪ **Good review reporting reversal of elution order on chiral separations.**

17 Ma S, Shen S, Lee H *et al.* Mechanistic studies on the chiral recognition of polysaccharide-based chiral stationary phases using liquid chromatography and vibrational circular dichroism: reversal of elution order of *N*-substituted alpha-methyl phenylalanine esters. *J. Chromatogr. A* 1216(18), 3784–3793 (2009).

18 Lämmerhofer M, Pell R, Mahut M *et al.* Enantiomer separation and indirect chromatographic absolute configuration prediction of chiral pirinixic acid derivatives: limitations of polysaccharide-type chiral stationary phases in comparison to chiral anion-exchangers. *J. Chromatogr. A.* 1217, 1033–1040 (2010).

19 Barth T, Simões RA, Pupo MT, Okano LT, Bonato PS. Stereoselective liquid chromatographic determination of 1′-oxobufuralol and 1′-hydroxybufuralol in rat liver microsomal fraction using hollow-fiber liquid-phase microextraction for sample preparation. *J. Sep. Sci.* 34(24), 3578–3586 (2011).

20 Wen YY, Li JH, Zhang WW, Chen LX. Dispersive liquid–liquid microextraction coupled with capillary electrophoresis for simultaneous determination of sulfonamides with the aid of experimental design. *Electrophoresis* 32(16), 2131–2138 (2011).

21 Suna JN, Shia YP, Chena J. Ultrasound-assisted ionic liquid dispersive liquid–liquid microextraction coupled with high performance liquid chromatography for sensitive determination of tracecelastrol in urine. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 879(30), 3429–3433 (2011).

22 Campillo N, Viñas P, Cacho JI, Peñalver R, Hernández-Córdoba M. Evaluation of dispersive liquid–liquid microextraction for the simultaneous determination of chlorophenols and haloanisoles in wines and cork stoppers using gas chromatography–mass spectrometry. *J. Chromatogr. A* 1217(47), 7323–7330 (2010).

23 Farajzadeh MA, Bahram M, Jönsson JA. Dispersive liquid–liquid microextraction followed by high-performance liquid chromatography–diode array detection as an efficient and sensitive technique for determination of antioxidants. *Anal. Chim. Acta* 591(1), 69–79 (2007).

24 Wei G, Li Y, Wang X. Application of dispersive liquid–liquid microextraction combined with high-performance liquid chromatography for the determination of methomyl in natural waters. *J. Sep. Sci.* 30(18), 3262–3267 (2007).

25 Farajzadeh MA, Vardast MR, Bahram M. Optimization of dispersive liquid–liquid microextraction of irganox 1010 and irgafos 168 from polyolefins before liquid chromatographic analysis. *Chromatographia* 69(5–6), 409–419 (2009).

26 Farhadi K, Matin AA, Hashemi PLC. Determination of trace amounts of phenoxyacetic acid herbicides in water after dispersive liquid–liquid microextraction. *Chromatographia* 69(1–2), 45–49 (2009).

27 Wu QH, Li YP, Wang C *et al.* Dispersive liquid–liquid microextraction combined with high performance liquid chromatography–fluorescence detection for the determination of carbendazim and thiabendazole in environmental samples. *Anal. Chim. Acta* 638(2), 139–145 (2009).

28 Zhou Q, Pang L, Xiao J. Trace determination of dichlorodiphenyltrichloroethane and its main metabolites in environmental water samples with dispersive liquid–liquid microextraction in combination with high performance liquid chromatography and ultraviolet detector. *J. Chromatogr. A* 1216(39), 6680–6684 (2009).

29 Chen H, Chen H, Ying J, Huang J, Liao L. Dispersive liquid–liquid microextraction followed by high-performance liquid chromatography as an efficient and sensitive technique for simultaneous determination of chloramphenicol and thiamphenicol in honey. *Anal. Chim. Acta* 632(1), 80–85 (2009).

30 Chen H, Ying J, Chen H, Huang J, Liao L. LC determination of chloramphenicol in honey using dispersive liquid–liquid microextraction. *Chromatographia* 68(7–8), 629–634 (2008).

31 Pawliszyn J. Sample preparation: quo vadis? *Anal. Chem.* 75(11), 2543–2558 (2003).

▪▪ **Excellent perspective about fundamental aspects of sample preparation.**

32 Liao XX, Liang B, Li ZZ, Li YF. A simple, rapid and sensitive ultraviolet-visible spectrophotometric technique for the determination of ultra-trace copper based on injection-ultrasound-assisted dispersive liquid–liquid microextraction. *Analyst* 136(21), 4580–4586 (2011).

33 Castro MDL, Priego-Capote F. Ultrasound-assisted preparation of liquid samples. *Talanta* 72(2), 321–334 (2007).

34 Song X, Li J, Liao C, Chen L. Ultrasound-assisted dispersive liquid–liquid microextraction combined with low solvent consumption for determination of polycyclic aromatic hydrocarbons in seawater by GC–MS. *Chromatographia* 74(1–2), 89–98 (2011).

■ Website

101 SciFinder. https://scifinder.cas.org