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Enantiomeric determination of the plasma levels of omeprazole by direct plasma injection using high-performance liquid chromatography with achiral–chiral column-switching

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Abstract

A direct injection high-performance liquid chromatographic (HPLC) method, with column-switching, for the determination of omeprazole enantiomers in human plasma is described. A restricted access media (RAM) of bovine serum albumin (BSA) octyl column has been used in the first dimension for separation of the analyte from the biological matrix. The omeprazole enantiomers were eluted from the RAM column onto an amylose tris(3,5-dimethylphenylcarbamate) chiral column by the use of a column-switching valve and the enantioseparation was performed using acetonitrile–water (60:40 v/v) as eluent. The analytes were detected by their UV absorbance at 302 nm. The validated method was applied to the analysis of the plasma samples obtained from 10 Brazilian volunteers who received a 40 mg oral dose of racemic omeprazole and was able to quantify the enantiomers of omeprazole in the clinical samples analyzed. The assay was able to determine the cytochrome P450 2C19 phenotype of the subjects participating in this study.

Keywords: Enantiomer separation; Direct plasma injection; Omeprazole

1. Introduction

The chiral compound omeprazole (Fig. 1), (5-methoxy-2-[[4-methoxy-3,5-dymethyl-2-pyridinyl)methyl]sulfinyl]-1H-benzimidazole), is used as a racemic mixture for the short and long term treatment of various acid-related gastrointestinal disorders and it is a gastric H^+,K^+-ATPase inhibitor. (*R*)-(+)-Omeprazole is stereoselectively metabolized in the liver, mainly by hydroxylation, by the polymorphic cytochrome P450 enzyme CYP2C19, while, the (*S*)-(−)-omeprazole is metabolized to the sulfone by the CYP3A4 [1–3]. Due to this metabolic difference, esomeprazole, the (*S*)-enantiomer of omeprazole, has been marketed by AstraZeneca under the trade name of Nexium [4,5].

The work of Tybring and collaborators [1,2] demonstrated that the AUC of the (+)-omeprazole was larger in poor metabolizers than in extensive metabolizers whereas the opposite happened for the 5-hydroxy metabolite indicating that the (+)-omeprazole is hydroxylated to a major extent by CYP2C19. Thus, it has been suggested that omeprazole could be used as a probe drug to CYP2C19 [1–3,5].

Although a number of enantiomeric separations for measuring the enantioselective metabolism of omeprazole are described in the literature [1,3,6–8], the main restriction of those methods are the time spent on sample preparation.

Cairns and collaborators made use of a C_2 solid phase extraction cartridge followed by filtration on a nylon filter for the enantioselective measurement of omeprazole using a Chiral AGP column [7]. While, the work of Tybring et al. [1] reports that the enantioselective separation of omeprazole was preceded by isolation of the enantiomers using a C_{18} column. A Chiralpak AD column and detection using a triple quadrupole mass spectrometer with deuterated analogues as internal standards and liquid–liquid extraction for sample preparation is also reported as an enantioselective assay of omeprazole in plasma samples [8].

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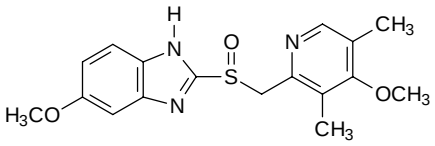

Fig. 1. Chemical structure of omeprazole.

This work reports the analysis of plasma levels of omeprazole's enantiomers by direct sample injection. This was achieved by the use of a two dimensional chromatography system using a RAM BSA C_8 column in the first dimension for extraction and clean-up and a polysaccharide column, under reversed-phase mode of elution, in the second dimension for the enantioseparation.

2. Experimental

2.1. General

Acetonitrile, HPLC grade, was purchased from Mallinckrodt Baker (St. Louis, MO, USA) and water was purified using a Milli-Q system (Millipore, Ribeirão Preto, SP, Brazil).

Racemic omeprazole (batch number 7119.7/0) was donated from Libbs Farmacêutica Ltda (São Paulo, SP, Brazil). Losec® Mups® capsules (from AstraZeneca) containing 20 mg of omeprazole were purchased at a local drugstore. The elution order was determined by injection of (*R*)-(+) and (*S*)-(−)-omeprazole at the established separation conditions. The enriched enantiomers were obtained by chiral separation using a amylose tris(3,5-dimethylphenylcarbamate) coated (20% w/w) onto APS-Nucleosil (7 μm particle size and 500 Å pore size) as stationary phase using hexane:ethanol (70:30 v/v) as mobile phase. The optical activity of the separated enantiomers was defined using a Perkin-Elmer Model 241 polarimeter.

The collection of blood samples from the volunteers was made at São Francisco University Hospital, Bragança Paulista, SP, Brazil. Pooled control human plasma was also supplied by the University Hospital and stored at −20 °C until use. For analysis, the plasma samples were thawed at room temperature and centrifuged for 10 min at 5000 × *g* at 20 °C, using a JOUAN B4*i*/BR4*i* centrifuge.

Written consent was obtained from each volunteer prior to the study. The protocol was approved by the São Francisco University Medical School Ethics Committee and is in accordance with the Declaration of Helsinki.

2.2. Equipment and columns

The HPLC system consisted of two Shimadzu LC-10ADVP pumps (Kyoto, Japan), with one of the pumps having a valve FCV-10AL for selecting solvent, an auto injector model SIL 10AVP, a SPD−10AV UV-Vis detector, a SPD-M10AVP diode array detector, and a SCL 10AVP interface. A sample valve HPLC 7000 Nitronic EA (Sulpelco, St. Louis, USA) was used for the automated column switching. Data acquisition was done on a Shimadzu CLASS-VP software.

The chiral column of amylose tris(3,5-dimethylphenylcarbamate) coated (20% w/w) onto APS-Nucleosil (7 μm particle size and 500 Å pore size) (150 mm × 4.6 mm i.d.) and the RAM BSA-octyl column (Luna C8 (2) Phenomenex®, 10 μm particle size and 100 Å pore size) (100 mm × 4.6 mm i.d.) were prepared as described elsewhere [9,10].

2.3. Standard solutions and sample preparation

Stock solutions of (±)-omeprazole were prepared by dissolving the drug in methanol to a final concentration of 200 μg/ml. From these stock solutions eight calibration standard solutions containing 1.0, 1.5, 3.0, 6.0, 12.0, 24.0, 48.0 and 96.0 μg/ml and three quality controls solutions at concentrations of 1.8, 38.0 and 72.0 μg/ml were prepared in methanol. The solutions were stable for at least 3 months when stored at −20 °C, and no evidence of degradation of the analytes was observed on the chromatograms during this period.

To prepare the samples, aliquots (20 μl) of the appropriated solution were placed in a culture tube and the solvent was evaporated under a stream of nitrogen. The dried analytes were reconstituted using 200 μl of plasma or mobile phase as the solvent (for the extraction and transfer evaluation) and the solutions were vortex-mixed for 15 s. A 180 μl aliquot was transferred to autosampler vials and 100 μl were injected onto the HPLC system.

2.4. Method validation

Linearity was determined using calibration standards prepared in triplicate as described in Section 2.3. Plasma calibration curves were constructed by plotting the peak area against the concentration of each enantiomer of omeprazole.

The precision and accuracy of the method were evaluated by quintuplicate analyses at the three quality control samples. Calibration standards and quality controls were analyzed on three different days in order to determine intra- and inter-day precision and accuracy. Precision was estimated from the coefficients of variation (C.V. %) and the accuracy was evaluated by back-calculation and expressed as the percent deviation between amount found and amount added at the three concentrations examined.

The extraction and transfer efficiency of each enantiomer of omeprazole from human plasma was determined by analyzing the quality controls samples. The efficiencies were calculated by dividing mean peak areas obtained from the spiked plasma samples by mean peak areas obtained from samples of the omeprazole prepared in mobile phase as the solvent (Section 2.3).

The acceptance criteria for the limit of quantitation was that the precision and accuracy for three-extracted sample

be under 20% variability, while the limit of detection was determined taking a signal-to-noise ratio of three.

At all analytical runs, samples of blank pooled plasma were analyzed to evaluate the selectivity of the method. Plasma samples of the volunteers were assayed using a photodiode array UV-Vis detector and the peak purity of each enantiomer was evaluated. Quality control samples were run daily to ensure day-to-day repeatability.

The stability of omeprazole in spiked plasma samples has already been reported [8] and no degradation was observed over a 24 h period, at room temperature, for quality control plasma samples analyzed in triplicate.

2.5. Human study

A single 40 mg (2 capsules containing 20 mg) dose of racemic omeprazole was administered orally to 10 healthy volunteers after an overnight fast. Venous blood samples were collected in heparinized Vacutainer tubes at 0 (predose) and 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0 and 8.0 h after dosing. The tubes were centrifuged at 2000 × *g* for 10 min, the plasma collected and stored at −70 °C until analysis.

3. Results and discussion

3.1. Method development

Sample preparation is the most vital step in HPLC analysis of drugs and their metabolites in biological fluids. Proteins in the biological fluids can precipitate or denature and adsorb into the packing material, leading to back-pressure build-up, changes in retention time and decreased column efficiency. Some of the most commonly used sample preparation techniques include liquid–liquid extraction, protein precipitation and solid-phase extraction. However, these methods increase the total analysis time and reduce the total recovery of the analyte of interest [11,12]. To eliminate problems such co precipitation of analytes with proteins, loss of analytes during extraction and avoid the adsorption of protein onto the analytical column direct injection of the sample using a system switching is becoming the method of choice [11–15].

In the last decade, supports possessing restricted-access properties have been developed to allow the direct injection of biological matrices into on-line HPLC systems. Restricted-access supports combine size-exclusion of proteins and others high-molecular mass matrix components with the simultaneous enrichment of low-molecular mass analytes at the often hydrophobic inner pore surface. A large number of different restricted access supports have been designed and commercialized in recent years [16–18].

Cass and collaborators have reported the preparation and application of a RAM BSA column coupled to an amylose tris(3,5-dimethoxyphenylcarbamate) chiral stationary phase for the analysis of pantoprazole enantiomers [10] and for the determination of metyrapol enantiomers and metyrapone [19] in human plasma by achiral–chiral chromatography. The RAM BSA column can be characterized by a hydrophilic outer phase and a hydrophobic inner phase. Thus, large molecules such as proteins are excluded in the void volume while the small hydrophobic analytes are selectively retained. In order to evaluate the efficiency of exclusion of the plasma proteins by the RAM column, the recovery of proteins from the column was first evaluated [19]. The method used was the Bradford's method [20].

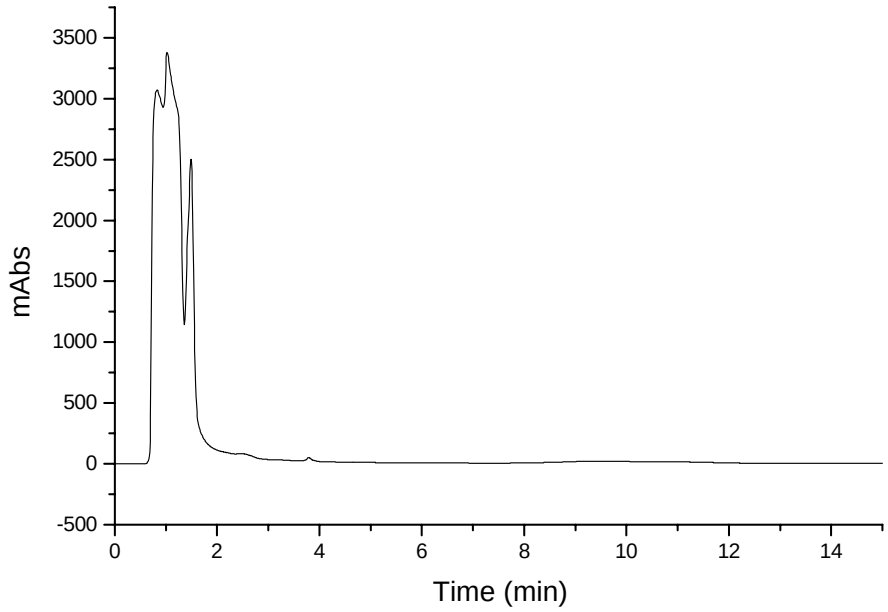

Fig. 2. Typical elution profile for a human plasma free drug sample (200 μl) from a RAM BSA C_8 column, using water as a mobile phase at a flow-rate of 1 ml/min with detection at 280 nm.

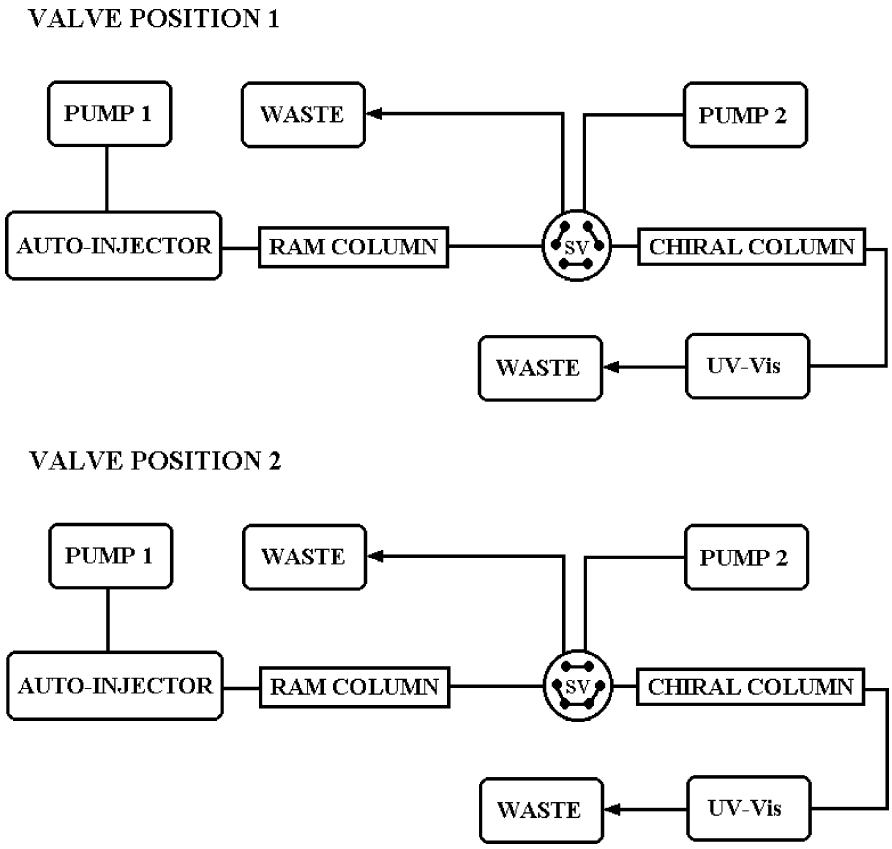

Fig. 3. Schematic diagram of the column-switching system.

Fig. 2 shows a typical elution profile by the RAM column of a plasma sample.

The column-switching system used for the coupling of the RAM and the chiral columns are showed schematically on Fig. 3.

The role of the RAM column in this column-switching system is to remove the proteins and fractionate a zone containing the analyte. The sample was applied when the valve was in position 1 (Fig. 3). Water, delivered by pump 1 at a flow rate of 1.0 ml/min eluted mainly the proteins of the sample from the RAM column to waste while the analytes were retained on the hydrophobic phase of the sorbent. Five minutes after the sample injection, a step gradient of acetonitrile:water (30:70 v/v) was applied for compression of the omeprazole chromatographic band and the switching valve was rotated to position 2, coupling the RAM column to the chiral column. The switching time was set from 9.7 to 12.0 min to transfer the omeprazole to the analytical column. The transfer time was established by connecting the UV detector directly to the end of the RAM column. The application of back flush technique was discharged by the risk of the transfer of more retained compounds, absorbed on the top of the column, to the chiral column.

The enantioselective analyses were performed using acetonitrile:water (60:40 v/v), which was delivered by pump 2, at a flow rate of 0.5 ml/min (Position 1; Fig. 3). The RAM column was first cleaned with 100% acetonitrile and then, it was conditioned with water by pump 1 while the separation was carried out on the chiral column. The sequence time used is listed in Table 1.

Table 1
Time events for the switching of columns and of mobile phases

Time (min)	Pump	Event	Valve position
0.00–5.00	1 (eluent A)	Plasma proteins are excluded by RAM column	1
0.00–5.00	2	Conditioning of the chiral column	
5.01–17.00	1 (eluent B)	Elution of retained components on the RAM	1
9.70–12.00	1 (eluent B)	Analytes are transferred to the chiral column	2
12.01–30.00	2	Analysis of the omeprazol enantiomers	1
17.01–22.00	1 (eluent C)	Washing of RAM column	1
22.01–30.00	1 (eluent A)	Conditioning of RAM column	1

Eluents used in pump 1: (A) H_2O; (B) CH_3CN:H_2O (30:70 v/v); (C) CH_3CN, flow rate: 1.0 ml/min. Eluents used in pump 2: CH_3CN:H_2O (60:40 v/v), flow rate: 0.5 ml/min. λ: 302 nm.

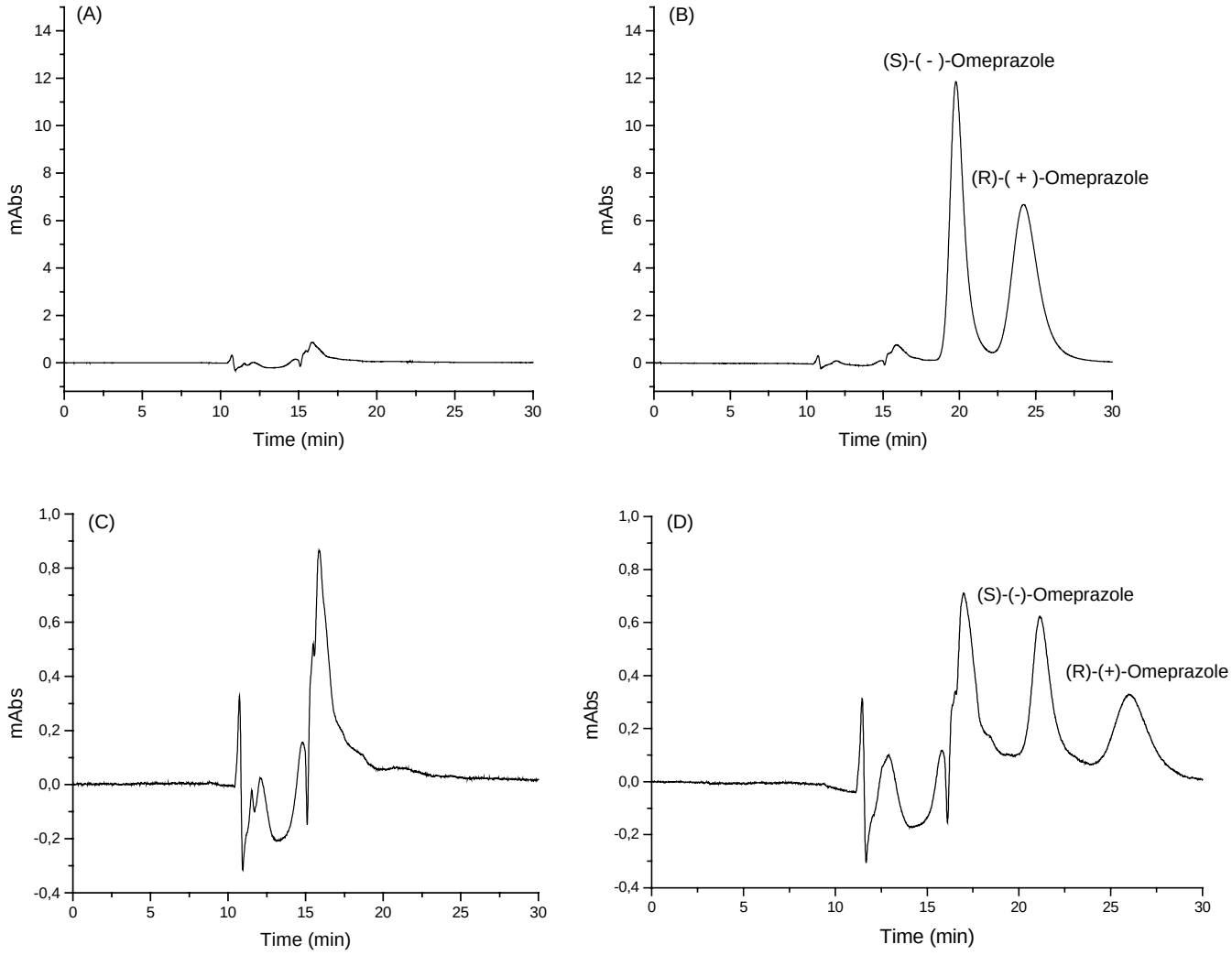

Fig. 4. Typical chromatograms of (A) and (C) plasma free drug and (B) and (D) spiked plasma with (±)-omeprazole (3.8 and 0.18 μg/ml, respectively).

3.2. Validation

Representative chromatograms of the analysis of blank plasma samples and spiked plasma with omeprazole analyzed at the established conditions are shown in Fig. 4. The chromatograms show that no endogenous compounds are interfering with the detection of the omeprazole enantiomers.

Plasma calibration curves were constructed by plotting the peak area against the concentrations of each omeprazole enantiomer from 0.05 to 4.80 μg/ml. The following regression equations and correlation coefficients were obtained: $y = 0.0241546x + 2.05352 \times 10^{-6}$ ($r = 0.998851$) for the (*S*)-(−)-enantiomer and $y = 0.0284553x + 2.12468 \times 10^{-6}$ ($r = 0.99952$) for the (*R*)-(+)-enantiomer. The C.V. of each calibration standard ($n = 3$) varied from 0.38 to 11.7% with accuracy that varied from 92.9 to 115% for the (*S*)-(−)-enantiomer and a C.V. of 0.75–7.60% with accuracy of 96.4–120% for the (*R*)-(+)-enantiomer. The lower concentration calibration standard (0.05 μg/ml) was taken as the limit of quantification in both cases (115 and 120% accuracy). The limit of detection was 0.0063 μg/ml for each enantiomer.

The extraction and transfer efficiencies were excellent for both enantiomers analyzed at the three quality control levels and are given on Table 2.

The accuracy and intra and inter-day precision of the method were determined by analyzing five replicates of the

Table 2
Extraction efficiencies of the omeprazole enantiomers from human plasma ($n = 5$)

Concentration added (μg/ml)	Extraction efficiency (%)	
	(*S*)-(−)-Omeprazole	(*R*)-(+)-Omeprazole
0.090	92.5	97.4
1.90	90.9	93.6
3.60	97.0	103

Table 3
Accuracy and intra and inter-day variability for the assay of omeprazole enantiomers in human plasma

Enantiomers	Concentration (μg/ml)	First day ($n = 5$)		Second day ($n = 5$)		Third day ($n = 5$)		Pooled ($n = 15$)	
		Accuracy (%)	C.V. (%)	Accuracy (%)	C.V. (%)	Accuracy (%)	C.V. (%)	Accuracy (%)	C.V. (%)
(*S*)-(−)-Omeprazole	0.090	104	6.60	111	3.27	112	4.62	109	6.62
	1.90	90.9	3.53	95.1	2.90	98.5	2.25	94.8	4.37
	3.60	94.5	4.32	96.4	0.78	102	2.15	97.5	4.07
(*R*)-(+)-Omeprazole	0.090	112	6.50	114	2.54	114	9.29	114	6.57
	1.90	93.2	3.42	95.9	3.92	99.3	2.84	96.2	4.19
	3.60	97.3	3.39	96.8	1.03	102	2.11	98.6	3.17

three quality controls on three non-consecutive days. Accuracy was evaluated by back-calculation and expressed as the percent deviation between the amount found and the amount added at the three concentrations examined and the precision is expressed as C.V. (%). These results are shown on Table 3.

Two blinded samples produced accuracies of 97.3–103.5% for the (*S*)-(−)-omeprazole and 96.4–102% to the (*R*)-(+)-omeprazole, at the concentration levels of 0.20 and 0.75 μg/ml for each enantiomer.

3.3. Application of the method

The present method was designed to examine the enantioselective fate in vivo of omeprazole after a 40 mg oral dose administration to each of 10 heath volunteers.

The same RAM column was used during the method development and validation and also for the analysis of the clinical samples collected from two volunteers. After about 17.3 ml of plasma were injected, the RAM column showed some performance deterioration and it was substituted for a freshly prepared one. The transfer time of omeprazole in this new RAM column was re-adjusted from 9.7–12.0 to 9.3–12.0 min and the method was re-validated before the analysis of all clinical samples. The chromatographic performance of the second RAM column was maintained after 28.0 ml of plasma injection. The performance of the chiral column was excellent during the complete work. The broadening or alteration in the retention time of the solutes by the RAM columns after plasma injection is often granted to change in surface characteristics [18]. The upper limit of plasma samples has not yet been determined for the RAM BSA columns [9,19–21].

The assay proved to be adequate for establishing pharmacokinetic parameters in all samples evaluated.

3.4. Disposition of omeprazole enantiomers in healthy subjects

Considering that the AUC of (+)-omeprazole is larger for poor metabolizers than for extensive [1–3], nine out of the 10 volunteers could be phenotyped as extensive metabolizers while one was considered to be a poor metabolizer. This is in agreement with previously observed results of phenotying CYP2C19 using pantoprazole with this same group of volunteers [10]. The comparison of these results will be published elsewhere. The plasma concentration-time profile of both enantiomers in the plasma samples of the single volunteer classified as poor metabolizer and in the plasma

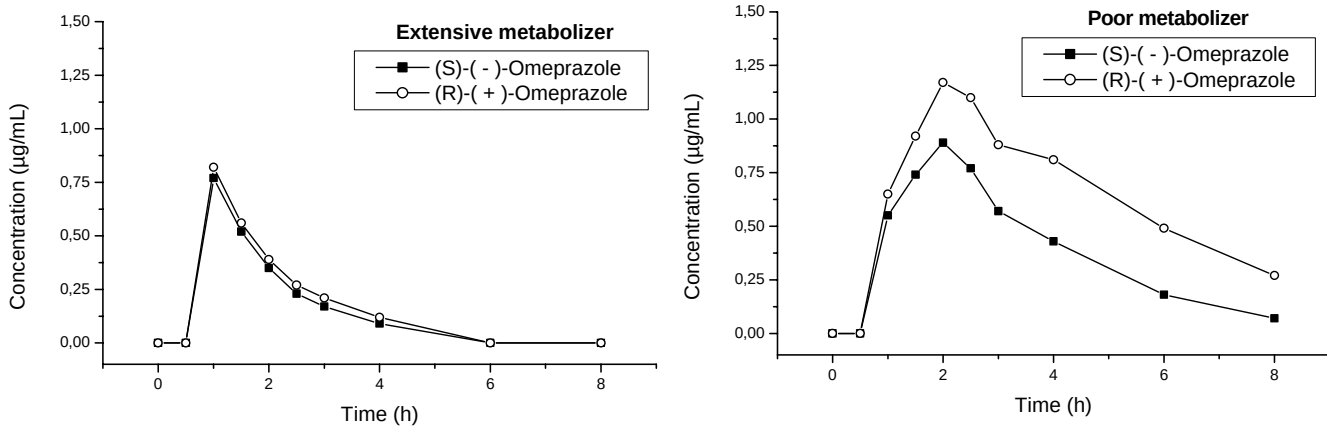

Fig. 5. Pharmacokinetic profiles for both enantiomers after a single oral dose of 40 mg of (±)-omeprazole for one volunteer classified as an extensive metabolizer and of the volunteer classified as a poor metabolizer.

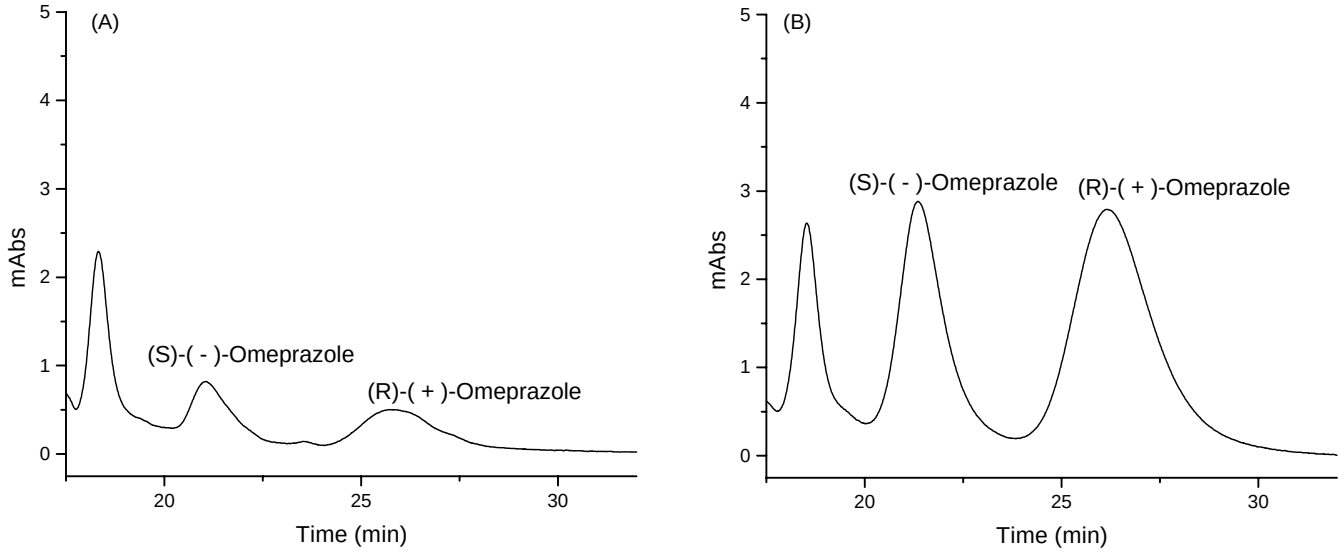

Fig. 6. Chromatograms of plasma samples from two different volunteers collected 4 h after oral dose of 40 mg of (±)-omeprazole. (A) chromatogram of plasma sample of one volunteer classified as extensive metabolizer showing a 1:1 enantiomer ratio. (B) chromatogram of plasma sample of the volunteer classified as poor metabolizer showing a 28.8% of e.e. for the (+)-enantiomer.

samples of one of the nine volunteers classified as extensive metabolizers are shown in Fig. 5. The chromatograms at Fig. 6 exemplifies the quality of the analysis of the samples collected from these two different volunteers at 4 h after an oral doses of 40 mg of racemic omeprazole.

4. Conclusions

A column-switching HPLC achiral–chiral method with UV detection, which requires only 100 μl of plasma samples, was efficiently developed and validated for assaying the enantiomers of omeprazole in human plasma. The developed method is simple and requires a total analysis time of only 30 min per sample, with no time involved for sample pretreatment. The method has proven to be useful for collecting data for the pharmacokinetics studies of omeprazole enantiomers in a series of 10 Brazilian health volunteers. The present work is useful for easy differentiation of (+)/(−)-enantiomer ratio of omeprazole and can be used for phenotyping CYP2C19.

Acknowledgements

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