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Journal of Chromatography B, 766 (2001) 153–160

JOURNAL OF CHROMATOGRAPHY B

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Enantiomeric determination of pantoprazole in human plasma by multidimensional high-performance liquid chromatography

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Received 3 May 2001; received in revised form 19 September 2001; accepted 3 October 2001

Abstract

Multidimensional HPLC is a powerful tool for the analysis of samples of a high degree of complexity. This work reports the use of multidimensional HPLC by coupling a RAM column with a chiral polysaccharide column to the analysis of Pantoprazole in human plasma by direct injection. The enantiomers from the plasma samples were separated with high resolution on a tris(3,5-dimethoxyphenylcarbamate) of amylose phase after clean-up by a RAM BSA octyl column. Water was used as solvent for the first 5 min in a flow-rate of 1.0 ml/min for the elution of the plasmatic proteins and then acetonitrile–water (35:65 v/v) for the transfer and analysis of pantoprazole enantiomers, which were detected by UV at 285 nm. Analysis time was 28 min with no time spent on sample preparation. A good linear relationship was obtained in the concentration range of 0.20 to 1.5 μg/ml for each enantiomer. Inter and intra-day precision and accuracy were determined by one low (0.24 μg/ml), one medium (0.70 μg/ml) and one high (1.3 μg/ml) plasma concentration and gave a C.V. varying from 1.80 to 8.43% and accuracy from 86 to 92%. Recoveries of pantoprazole enantiomers were in the range of 93.7–101.2%. The validated method was applied to the analysis of the plasma samples obtained from ten Brazilian volunteers who received an 80 mg oral dose of racemic pantoprazole and was able to quantify the enantiomers of pantoprazole in all clinical samples analyzed.

Keywords: Enantiomer separation; Pantoprazole

1. Introduction

Pantoprazole (Fig. 1), 5-(difluoromethoxy)-2-[(3,4- dimethoxy -2- pyridyl)methylsulfinyl]1H-benzimidazole, is a selective and long-acting proton pump inhibitor. It is chiral due to the asymmetrical substituted sulfoxide center and is used clinically as a racemic mixture [1].

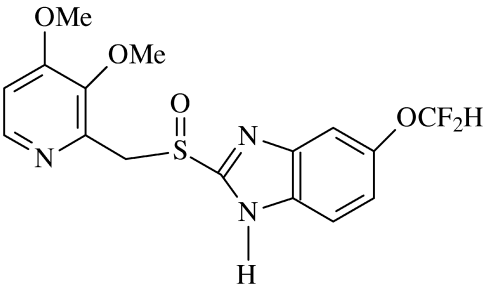

Fig. 1. Chemical structure of pantoprazole.

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1570-0232/02/$ – see front matter
PII: S0378-4347(01)00472-8

Pantoprazole, such as omeprazole and lanzoprazole, is mainly metabolized by the polymorphically expressed cytochrome P450 (CYP) isoform *S*-mephenytoin hydroxylase (CYP2C19). Because of this, few individuals in a population metabolize these benzimidazoles slowly compared with the majority of the population [1,2].

Based on findings that poor metabolizers of pantoprazole showed significant difference in the disposition of the two enantiomers [3], that omeprazole is stereoselectively metabolized by CYP2C19 [4], and also knowing that the prevalence of poor metabolizers in Caucasian population is in the order of 2–5%, while among the oriental population it is very much higher, 15–20% [2], we decided to develop a method to investigate the enantioselectivity disposition of these benzimidazoles in Brazilian health volunteers.

This investigation is important due to the great racial miscegenation observed in the Brazilian population, given that these compounds are currently used in this country and no such study has yet been related.

To perform this investigation a multidimensional HPLC method was developed for the analysis of the enantiomers of pantoprazole in plasma. The method is based upon a restricted access media (RAM) bovine serum albumin (BSA) column coupled with a chiral polysaccharide column.

This paper reports the development and validation of this novel method. The assay has been successfully used to analyze the plasma of ten volunteers who ingested 80 mg capsules of racemic pantoprazole.

2. Experimental

2.1. General

Solvents were either HPLC grade from Merck (Darmstadt, Germany) or Chromar HPLC grade from Mallinckrodt Baker (St. Louis, MO, USA).

The columns were packed using a Shandon packer.

The racemic pantoprazole standard was generously supplied by BYK Química e Farmacêutica Ltda (São Paulo, SP, Brazil).

The elution order was determined by injection of (+) and (−)-pantoprazole at the established separation conditions. The enriched-enantiomers were obtained by enantiomeric separation using an amylose tris[(*S*)-1-phenylethylcarbamate] as stationary phase using hexane:ethanol (70:30 v/v) as mobile phase. The optical activity of the separated enantiomers were defined using a Perkin-Elmer Model 241 polarimeter.

Pantozol® enteric coated capsules containing 40 mg of pantoprazole were purchased at a local drugstore.

The collection of blood samples from the volunteers was made at Clinical Pharmacology Unit, São Francisco University Medical School, Bragança Paulista, SP, Brazil. Pooled control human plasma was also supplied by the University Hospital.

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

2.2. Equipment

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-6AV UV–Vis detector, a photodiode array model SPD-10AVP was also used, 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 CLASS-VP software.

2.3. Columns

The chiral columns were prepared as described elsewhere [5,6] and consisted of amylose tris(3,5-dimethoxyphenylcarbamate) coated onto APS-Nucleosil (7 μm particle size and 500 Å pore size) (20% w/w, 150×4.6 mm I.D.).

The BSA restricted access phase column (100× 4.6 mm I.D.) was prepared as follows: an octyl-silica column (Hypersil, 10 μm particle size and 120 Å

pore size) was packed by the ascending slurry method using methanol for the preparation of the slurry (50 ml) and also for the packing. The packing was carried out at a pressure of 7500 p.s.i. and after the column was conditioned for about 4 h with methanol at a flow-rate of 1.0 ml/min the immobilization of BSA was done in situ based on Menezes and Felix protocol [7]. The column was first eluted at a flow-rate of 1.0 ml/min with 0.05 *M* phosphate buffer (pH=6.0) (50 ml) before passing a 1.0 mg/ml solution of bovine serum albumin (Fraction V powder minimum 98%; Sigma, St. Louis, USA) prepared in 0.05 *M* phosphate buffer (pH=6.0) (25 ml), and then a 25% (v/v) solution of glutaraldehyde (5 ml; Merck, Darmstadt, Germany). After 5 h, the column was eluted with an 1.0 mg/ml solution of sodium borohydride (10 ml; Aldrich, St. Louis, USA) and then with water (60 ml).

2.4. Standard solutions

A stock solution of (200 μg/ml) was prepared by dissolving (±)-pantoprazole (2.0 mg) in methanol (10 ml). From this stock solution seven standard solutions in the following concentrations were prepared: 30.0; 25.0; 20.0; 16.0; 12.0; 8.0 and 4.0 μg/ml. Stock solutions were stable for 4 months when stored at −20°C and no evidence of degradation of the analyte was observed on the chromatograms during this period.

2.5. Sample preparation

To prepare the spiked samples, aliquots (20 μl) of the appropriated standard solution were placed in a culture tube. The solutions were evaporated under a stream of nitrogen and then 200 μl of plasma was added to each tube containing the dried analytes. They were vortexed for 15 s and then 180 μl was transferred to autosampler vials. A 100 μl aliquot of the spiked plasma was injected to a column-switching HPLC system.

2.6. Column-switching procedure

The flow-rate used was of 1.0 ml/min and the enantiomers were detected at 285 nm.

Step 1 (0–5.00 min): Valve is in position 1.

Pump 1 (solvent A, H_2O): Plasma samples are injected onto the RAM column and the plasma proteins are excluded.

Pump 2 (CH_3CN–H_2O 35:65 v/v): Conditioning of the chiral column.

Step 2 (5.01–16.00 min)

Pump 1 (solvent B, CH_3CN–H_2O 35:65 v/v): Elution of retained components from the RAM column to the analytical column.

Step 3 (8.00–13.00 min): Valve is switched to position 2 and the (±)-pantoprazole is transferred to the chiral column.

Step 4: Valve is in position 1.

(16.00–22.00 min) – Pump 1 (solvent C, CH_3CN): Washing of RAM column.

(22.01–28.00 min) – Pump 1 (solvent A, H_2O): Conditioning of RAM column.

(16.00–28.00 min) – Pump 2 (CH_3CN–H_2O 35:65 v/v): Analysis of the enantiomers of pantoprazole.

Step 5 (0–5.00 min): A new sample is injected onto the RAM column.

2.7. Calibration curves

Using the appropriate standard solution of (±)-pantoprazole spiked plasma samples were prepared at the following concentrations: 0.40; 0.80; 1.2; 2.0; 2.5 and 3.0 μg/ml. The samples were prepared in triplicate. Calibration curves were constructed from a least-squares linear regression by plotting the peak area against the concentration of each enantiomer.

2.8. Recovery, precision, accuracy and selectivity

The absolute extraction recoveries of each enantiomer of pantoprazole from human plasma were estimated using spiked plasma at 0.48; 1.4 and 2.6 μg/ml of (±)-pantoprazole. The peak-area ratios of five-extracted samples at each concentration were compared with those of five injections of standard solutions to derive a percent recovery.

Inter and intra-day variability of the method were evaluated by replicate analysis at the same three concentrations that were used for the recovery experiments. Five samples of each concentration were prepared on three non-consecutive days.

The accuracy of the method was evaluated by

back-calculation; it was also tested using blinded unknowns, at two different concentrations, which were prepared by a different analyst.

At all analytical runs, samples of blank pooled plasma were analyzed to evaluate the selectivity of the method. Plasma samples of one volunteer were assayed using a photodiode array UV–Vis detector and the peak purity of each enantiomer was evaluated.

2.9. Limits of detection and quantification

The limit of detection was calculated taking a signal-to-noise ratio of 3 as criteria was measured by preparing spiked plasma sample with serial diluted solutions. The acceptance criteria for the LOQ were that the C.V. and accuracy for three-extracted samples were under 20% variability.

2.10. Human study

A single 80 mg dose of racemic pantoprazole was administered orally to ten healthy volunteers after an overnight fast. Venous blood samples were collected in heparinized Vacutainer tubes at 0 (predose) and 0.5; 1; 1.5; 2; 2.5; 3; 4; 6 and 8 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

Tedious and time-consuming pretreatment procedures such as protein precipitation and/or solid or liquid extraction is part of the routine work with biological fluids. Different approaches have been investigated to deal with the unwanted proteins. Among these, the use of restricted access media (RAM) for the analysis of low molecular mass compounds in complex matrices has been shown to be the method of choice [8–10].

Various types of restricted access phases have been developed [11,12], the unique features of these phases is that they prevent the access of the matrix components while selectively retaining the small hydrophobic analytes.

Menezes and Felix [7,13] reported the use of RAM BSA and HSA (human serum albumin) columns easily prepared for the analysis of pesticides in milk by direct injection.

As the goal of this work was to develop an enantioselective HPLC assay that would be simple and reliable for use in a number of clinical samples for the pharmacokinetic studies of the benzimidazoles series of proton pump inhibitors, the use of a RAM BSA column coupled to a polysaccharide-based column was taken into account. Pantoprazole was selected for initiating these studies.

The polysaccharide-based columns have been used with success for the enantioseparation of a variety of chiral sulfoxides [14,15] and also for these benzimidazoles [16–20], justifying the selection.

Previously a complete study for the enantioresolution of omeprazole, lanzoprazole and pantoprazole using cellulose and amylose tris(3,5-dimethylphenylcarbamate) phases and amylose tris[(*S*)-1-phenylethylcarbamate] and tris(3,5-dimethoxyphenylcarbamate) phases, on multimodal elution, was carried out. Great differences in enantioselectivity for each phase were observed on the different modes of elution for the series of benzimidazoles investigated. The results obtained were fundamental for this work and will be published elsewhere.

The amylose tris(3,5-dimethoxyphenylcarbamate) phase which is readily prepared from commercially available materials [5,6], showed the highest enantioselectivity for pantoprazole on the reversed-phase mode of elution with a separation factor (α) of 1.58 and resolution (R_s) of 3.85 using acetonitrile:water (1:1 v/v) as solvent. Excellent enantioselectivity was also observed for lanzoprazole ($\alpha = 1.72$ and $R_s = 3.57$). It is interesting to note that with this chiral phase any of the three benzimidazoles were enantioresolved when it was used on normal elution mode.

Reversed-phase is the preferred mode of elution for working with biofluids by direct injection. Pantoprazole has previously been efficiently quantified in serum and plasma by direct injection using either an achiral [21] or more recently a chiral column [3].

The column-switching system used for the coupling of the RAM and the chiral columns are the one showed schematically in Fig. 2. The time schedule for the switching events is given in the experimental section.

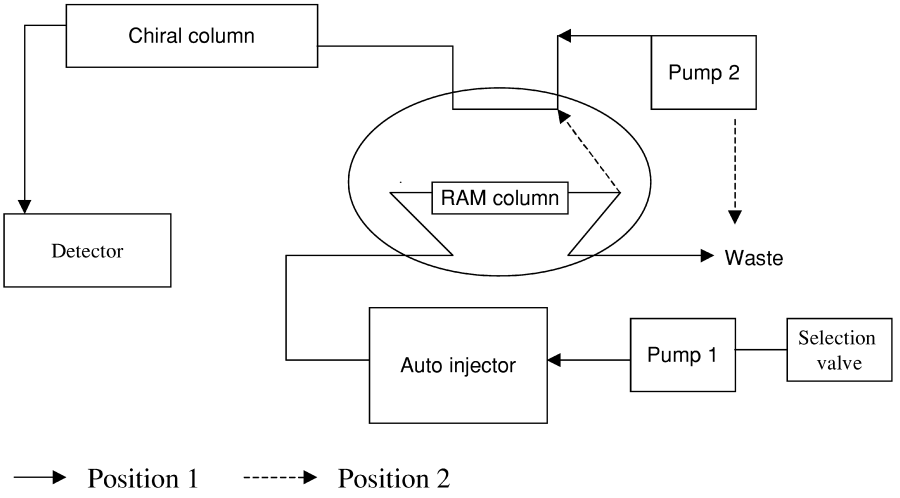

Fig. 2. Schematic diagram of the column-switching system.

To determine the elution profile of the sample matrix, the RAM column was initially connected to the UV detector. The complete elution of the proteins with 100% of water was possible in 5 min. The percentage of organic modifier in the mobile phase for the transfer of the pantoprazole from the RAM column to the chiral column was also well evaluated to give a narrow band with a symmetric peak. To get the right selectivity by the chiral column the solvent strength was well adjusted. The injection volume was 100 μl to satisfy the desired sensitivity. The RAM column was first cleaned with 100% acetonitrile and then, it was conditioned with water while the separation was carried out by the chiral column. The increase in backpressure of the RAM column during the method development could be amended by the cleaning of the sealings of the RAM column, which can get clogged by proteinaceous compounds [22].

Fig. 3 shows typical chromatograms of (a) blank plasma and (b) spiked plasma samples analyzed at the established conditions.

Least square regression calibration curves were constructed by plotting the pantoprazole enantiomers concentration versus the peak area and were linear from 0.20 to 1.5 μg/ml of plasma. The following regression equations and correlation coefficients were obtained: $y = -7844.2 + 1.6 \times 10^{-5}x$ ($r =$

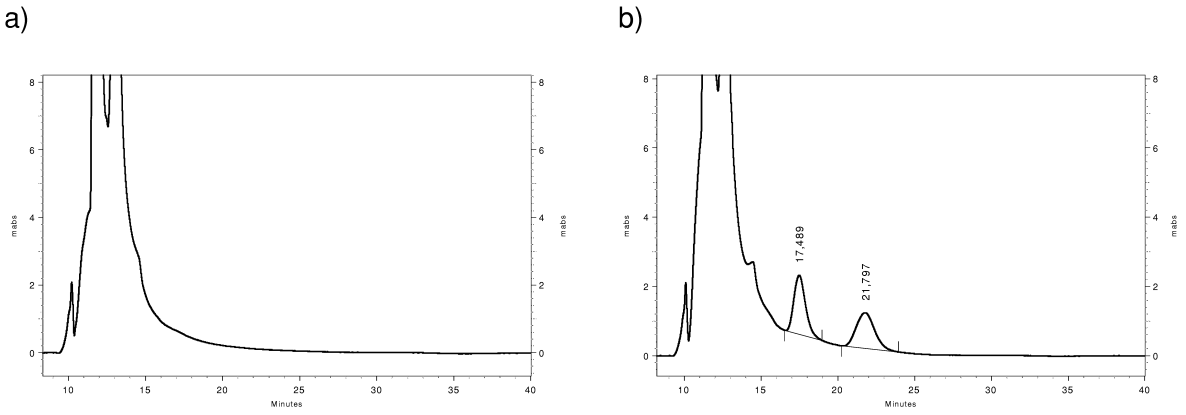

Fig. 3. Typical chromatograms of (a) plasma free drug and (b) spiked plasma with (±)-pantoprazole (1.2 μg/ml).

Table 1
Recovery of the enantiomers of pantoprazole from human plasma

Concentration μg/ml	(+)-Enantiomer %	(−)-Enantiomer %
0.24	94.3	93.7
0.70	100.7	96.6
1.30	101.2	100.8

0.998) for the first eluted enantiomer and $y = -4539.0 + 1.6 \times 10^{-5}x$ ($r = 0.997$) for the second enantiomer.

The excellent recoveries obtained at the three quality control levels analyzed is given in Table 1. A representative chromatogram obtained during the recovery assay is given in Fig. 4.

The intra and inter-day precision were evaluated using the data of three quality controls analyzed over a 3-day period. The results are expressed as coefficients of variation. The accuracy was evaluated from back-calculation and expressed as the percent deviation between amount found and amount added for each enantiomer at the three concentrations examined. These results are shown in Table 2.

Two blinded samples containing unknown concentrations to the analyst produced accuracies of 91 to 94% for the first eluted enantiomer and 92 to 101% to the second one at the concentration levels of 0.30 and 1.2 μg/ml.

The limit of quantification was 0.20 μg/ml for each enantiomer, while the limit of detection was 0.05 μg/ml.

The chromatograms shown in Fig. 3 indicate that no endogenous compounds interfered with the detection of the enantiomers of pantoprazole. To evaluate if the selectivity of the method was maintained in the presence of pantoprazole metabolites, plasma samples collected from one health volunteer at the time profile used for the pharmacokinetic study, were analyzed using a photodiode array detector and the peak purity of each enantiomer was

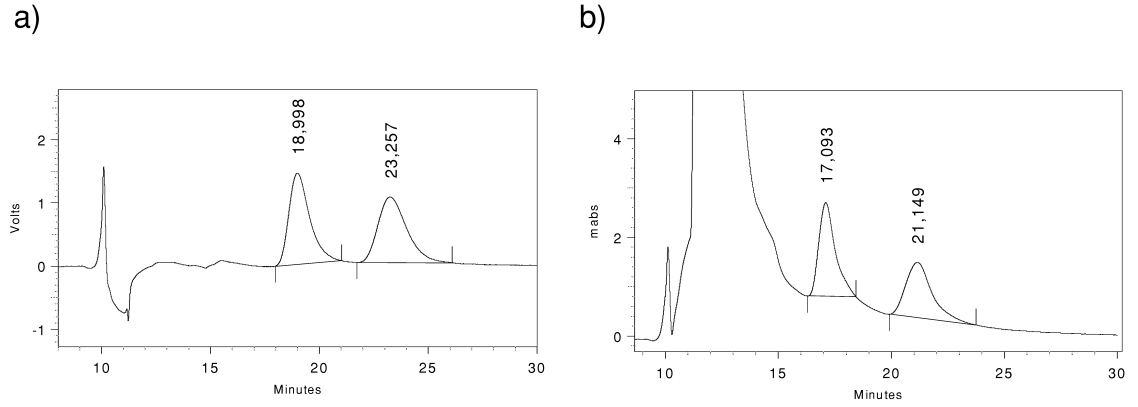

Fig. 4. Typical chromatograms obtained at the recovery studies; (a) a solution of (±)-pantoprazole (1.4 μg/ml) and (b) spiked plasma with (±)-pantoprazole (1.4 μg/ml).

Table 2
Accuracy and intra and inter-day variability for the assay of pantoprazole

Conc. μg/ml	(+)-Enantiomer			(−)-Enantiomer		
	(C.V.%)		Accuracy %	(C.V.%)		Accuracy %
	Intra-day $n=5$	Inter-day $n=15$		Intra-day $n=5$	Inter-day $n=15$	
0.24	7.22	8.43	88	4.80	4.89	95
0.70	1.98	3.24	88	2.03	2.97	86
1.30	1.80	2.31	92	2.06	2.38	92

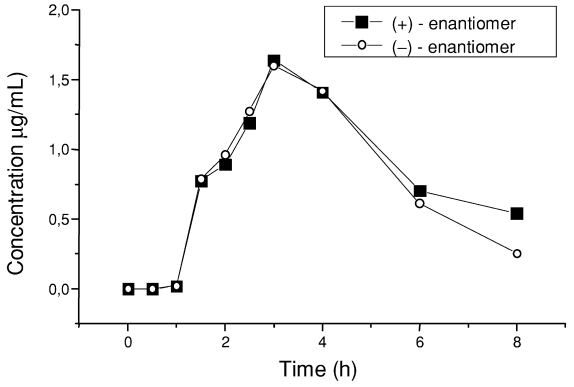

Fig. 5. Mean pharmacokinetic curves of pantoprazole enantiomers.

examined. No interfering metabolites were detected on the samples examined.

The method was applied for the determination of the enantiomers of pantoprazole in plasma samples of ten volunteers.

The mean pharmacokinetic curves of the enantiomers were similar (Fig. 5), however, differences in the pharmacokinetics of the enantiomers were observed by analyzing the data of individual volunteers, as an example, Fig. 6 shows typical chromatograms of samples of two different volunteers collected 4 h after an oral dose of 80 mg of racemic pantoprazole.

The development and application of this method was the initial part of a designed work for the enantiomeric evaluation of the series of benzimidazoles proton pump inhibitors within the Brazilian population. Work is now in progress for the enantiomeric determination of omeprazole and lanzoprazole. Pharmacokinetic results will be discussed for the three proton pump inhibitors proposed in this work.

4. Conclusion

Direct injection of biological fluids reduces the time of sample preparation and decreases the overall time of analysis. The coupling of a restricted access BSA phase to a polysaccharide chiral phase was efficiently performed and the novel method described in this work is rapid, accurate and precise and it was efficiently used for the enantioselective pharmacokinetics studies of pantoprazole. The quality of the performance of both columns was maintained with over 350 plasma injections of 100 µl each.

Acknowledgements

The authors acknowledge the financial support and grants from FAPESP. The grant from CNPq is also acknowledged.

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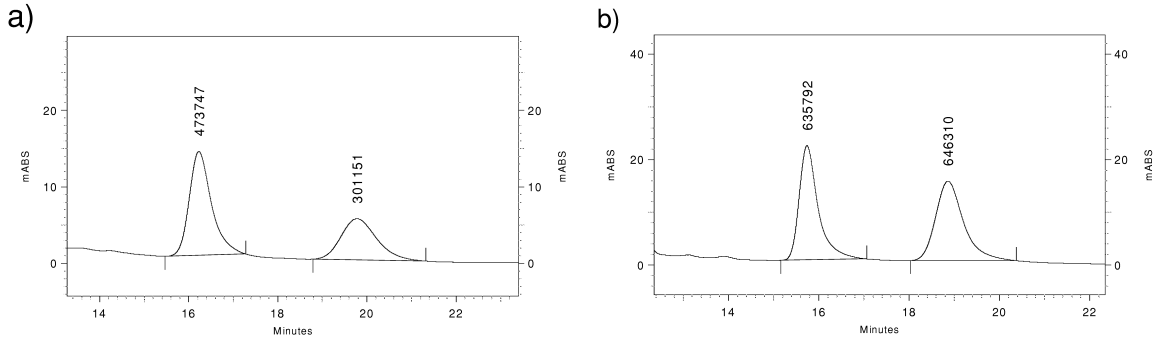

Fig. 6. Chromatograms of plasma samples from two different volunteers collected 4 h after oral dose of 80 mg of pantoprazole; chromatogram (a) shows a 22.2% of e.e. for the (+)-enantiomer while chromatogram (b) shows a 1:1 ratio for the enantiomers.

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