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Direct separation of captopril diastereoisomers including their rotational isomers by RP-LC using a teicoplanin column

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Abstract

A direct reversed-phase liquid chromatography (LC) method has been developed for the separation and analysis of captopril and its 2*R*,2*S* diastereoisomer using a teicoplanin stationary phase. The proline containing diastereoisomers, which are known to form conformers in aqueous solution, were also separated from their rotational isomers. The influence of temperature, different organic modifiers and buffer type, concentration and pH were optimised to obtain a working resolution between the two diastereoisomers and their respective rotational isomers. The diastereoisomeric purity of several commercial captopril batches was subsequently evaluated using a 0.05% triethylammonium acetate (TEAA) buffer (pH 3.8) run at 1.0 ml/min with mobile phase reservoir and column temperature controlled at 0°C. Throughout the study online UV diode array and mass spectrometry detection was carried out simultaneously to confirm that peaks eluting from the teicoplanin column were in fact captopril and not its readily converted disulphide dimer. Additionally, as a result of the greater detection sensitivity of mass spectrometry, it also facilitated a more accurate optimisation study where trace amounts of the rotational isomers were found to be present in the baseline at temperatures higher than optimum.

Keywords: Captopril; Diastereoisomer; Rotamer; Temperature; Teicoplanin stationary phase; *Cis*; *Trans*: mass spectrometry

1. Introduction

Captopril, an angiotensin converting enzyme inhibitor, has been widely used since 1980 for the treatment of hypertensive disease and congestive heart failure [1–3]. It is a relatively unstable compound that does not have an appreciable UV absorbance for facile detection making assay development for clinical studies difficult. A number of different analytical methods have therefore been described based on either radioimmunoassay [4], enzyme immunoassay [5], thin-layer chromatography [6], gas chromatography (GC) [7], gas chromatography–mass spectrometry [8–10] or liquid chromatography (LC) utilising ultraviolet (UV) [11,12], fluorescence [13], chemilumines-

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cence [14] and electrochemical detection [15,16]. A number of reports have also appeared describing derivatisation procedures prior to separation by LC [14,17–20].

Captopril has the 2*S*,2*S* configuration and of the other three diastereoisomers, only 2*R*,2*S* is considered as a possible impurity. Methods for the separation of these two are therefore needed. In the European Pharmacopoeia (EP) there is at present no test. A GC method which is based on derivatisation of the acid and amino functions with methanol and pentafluoropropionic anhydride (PFPAA), respectively was published for comment in 1996 [21]. This method has been judged to be complicated and no published data seem to be available to support it. Moreover, the PFPAA has to be of high purity as traces of trifluoroacetic acid anhydride (TFAA) will lead to a TFA-derivative of 2*S*,2*S* captopril that coelutes with the PFP-derivative of 2*R*,2*S*. This fact has resulted in complaints from some users who have not been able to reproduce the method. Therefore the primary aim of this study was to develop a simple and direct LC separation method, so that facile and reliable quantitative data relating to the diastereoisomeric purity of captopril could be attained. This was and is still complicated, however, by *cis*/*trans* rotational isomerisation (rotamers), a phenomenon previously noted by several groups when peptides [22] or peptide related drugs containing an N-protected proline residue [16,23–25] were analysed by LC.

In this paper we report our findings and a method for the direct separation of captopril diastereoisomers in reversed-phase LC using a teicoplanin bonded stationary phase. The problems relating to diastereoisomeric and *cis*/*trans* isomer (rotamer) separation are discussed before the presence or absence of the 2*R*,2*S* diastereoisomer in spiked or real captopril substance batches (2*R*,2*S* diastereoisomer) was evaluated.

2. Experimental

Acetonitrile (MeCN), methanol (MeOH), tetrahydrofuran (THF) (all HPLC grade) and glacial acetic acid (HOAc) were purchased from E. Merck AG (Darmstadt, Germany). Triethylamine (TEA), was obtained from Fluka Chemie AG (Buchs, Switzerland). Captopril CRS reference substance (2*S*,2*S* diastereoisomer) and other captopril batches were obtained from The European Pharmacopoeia Secretariat at the European Directorate for the Quality of Medicines, Strasbourg. Samples of the 2*R*,2*S* diastereoisomer were kindly delivered in small amounts by Dr A. Islam, British Pharmacopoeia Commission Laboratory, London. Deionised water (18.2 MΩ) used throughout the study was taken from a Maxima water purification system (Elga, High Wycombe, UK).

The Agilent Technologies benchtop HP1100 series gradient (LC)/mass spectrometry (MS) system (Waldbronn, Germany) using atmospheric pressure electrospray ionisation (AP-ESI) in the negative ion mode was used throughout the study. Agilent Technologies ChemStation software (Waldbronn, Germany) was used to collect and provide on-line DAD and mass-spectral data acquisition. The column used was a 250 × 4.6 mm 5 μm teicoplanin bonded phase (Chirobiotic T™, Astec, New Jersey, USA) which was immersed together with the mobile phase reservoir (where stated) in a Lauda RM 20 water bath (Lauda Dr R. Wobser GMBH & Co. KG, Lauda–Königshofen, Germany) filled with a 50% polyethylene glycol:water mixture.

Triethylammonium acetate (TEAA) buffer mobile phases were prepared in situ by combining the desired volume of TEA to water and pH controlling with HOAc before the addition of organic modifier (where specified). Analyte stock solutions were prepared for each captopril substance in water and stored at 4°C. Analytical samples for injection were subsequently prepared by dilution of the stock solution with water and placed in the autosampler which was also cooled at 4°C.

3. Results and discussion

Initial attempts using a range of chiral stationary phases (CSPs) or achiral stationary phases while employing chiral additives and/or ion-pair-

ing agents to resolve the captopril diastereoisomers were unsuccessful. The diastereoisomers were often eluted with the solvent front despite little or no organic modifier present which reflected their polarity (Log P $\approx$ 1.5) and indicating that little interaction with the stationary phase and/or mobile phase additive under investigation was taking place. The approach of using the porous graphitic carbon stationary phase with chiral complexing additives to separate diastereoisomers [26,27] was evaluated with reasonable progress. (*S*)-metoprolol was used as a complexing agent but was not developed or pursued further, since excessively long equilibration times were required and selectivity was often lost.

Later investigations using a teicoplanin bonded CSP in reversed-phase conditions were found to be more fortuitous, and a direct separation of the captopril diastereoisomers was obtained (Fig. 1) using a TEAA buffer without any organic modifier. Direct injection of the pure diastereoisomers, used to determine their elution order, indicated that: (a) two peaks were arising from each diastereoisomer; and (b) these were separated by a 'raised baseline'. The four isomeric peaks obtained and shown in Fig. 1 were attributed to the *cis* and *trans* rotamers of each of the two diastereoisomers as outlined in Fig. 2. This profile of a raised baseline between split peaks has been observed previously in LC when peptides [22] and peptide related drugs containing a N-protected proline residue are analysed by LC [16,23–25].

All peptides can be in either the *cis* or *trans* conformation with respect to the amide bond, although almost all exist in the *trans* form [22,28]. Peptides containing a proline moiety linked via the imino nitrogen, however, have been shown to be present in both *cis* and *trans* forms under certain conditions [29,30]. This has been discussed and demonstrated earlier by Horváth and coworkers [22] and references therein who conclude that the conversion (the *cis*/*trans* composition) can be in the order of minutes and if the retention factors are not identical, peak splitting can be expected as a result of the slow kinetics of the conformational change.

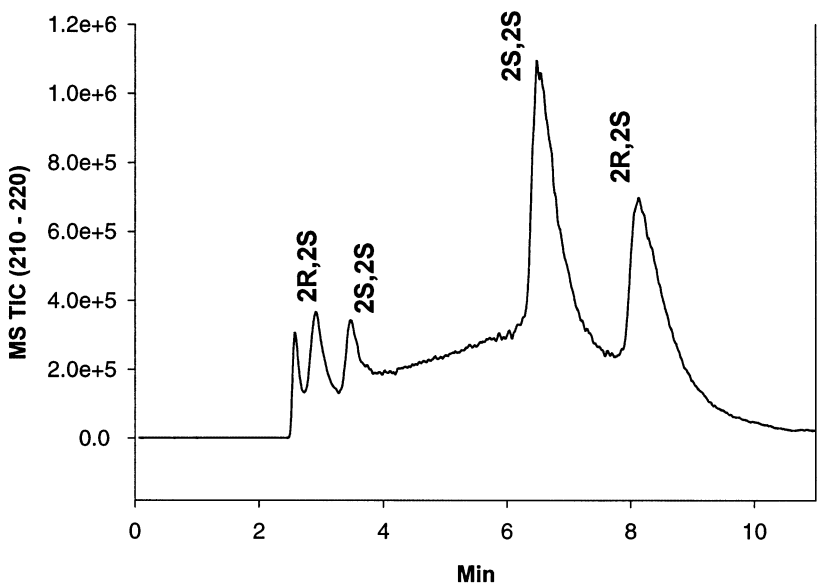

Fig. 1. Direct separation of captopril diastereoisomers, 2*S*,2*S* and 2*R*,2*S* in reversed-phase LC using a teicoplanin bonded phase. The presence of two sets of separations and the 'raised-baseline' between them indicate the slow kinetic *cis*/*trans* conversion observed when molecules containing an N-protected amide bond are analysed by LC. *Conditions*: 0.1% triethylammonium acetate (pH 3.4) delivered at 1.5 ml/min, 40 μl injection of a 0.3 mg/ml mixture of each diastereoisomer and mass spectral detection using negative ion mode scanning between 210 and 220 mass units.

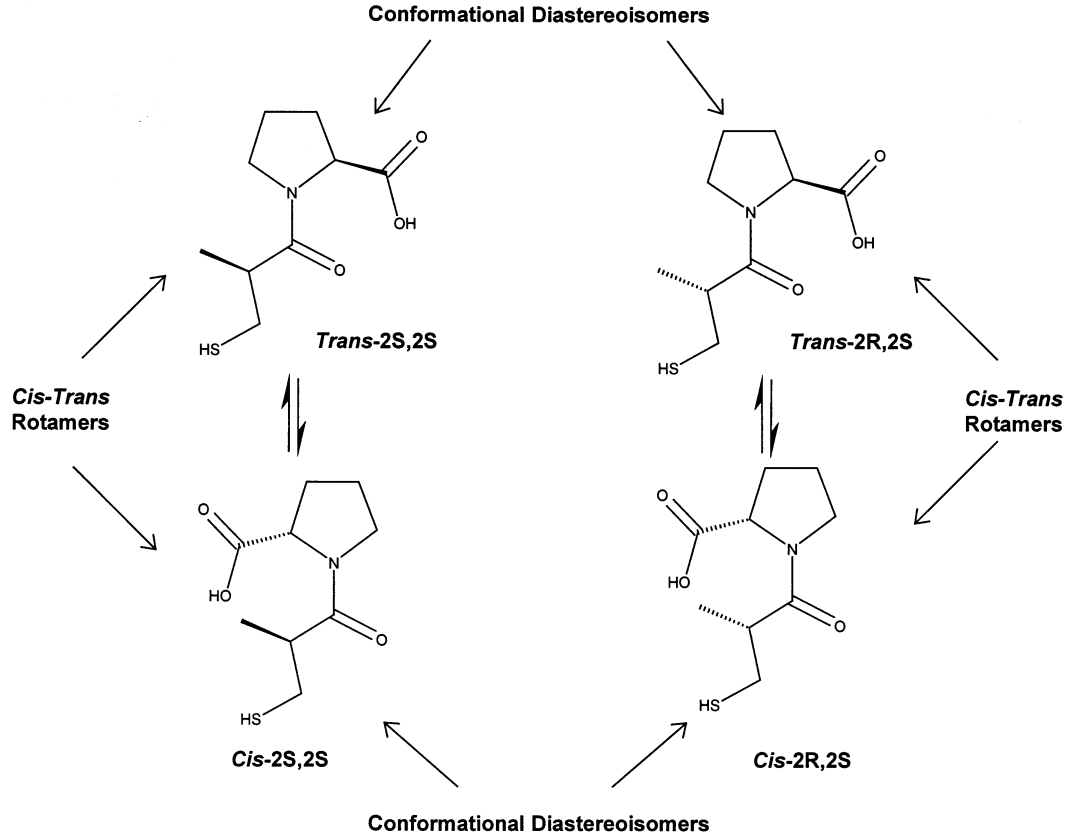

Fig. 2. The chemical structures of captopril arising from diastereoisomeric and rotational isomerisation.

It is not possible to ascribe definitively the isomeric peak elution order shown in Fig. 1 with the structures outlined in Fig. 2, since each diastereoisomer will inherently adopt a *cis*/*trans* equilibrium for which no standards could be available. One may postulate, but must do with great caution however, that the second set of diastereoisomeric peaks which are larger in area thus represent the more stable configuration can be attributed to the *trans* configuration, since others have demonstrated that *trans* is more stable for similar molecules [22]. This might be further supported with the postulation that *trans* would yield a more stable configuration due to potential intra-molecular hydrogen bonding between the carboxylic acid hydroxyl group and the carbonyl moiety in the side chain. To therefore explain why the subsequent larger *trans* peaks elute later that the smaller *cis* peaks, one must consider the interaction with the teicoplanin bonded phase which will be predominantly driven through ionic and/or hydrogen bonding, the isomers having greater potential for interaction will be retained to the greatest extent. Since the *trans* configuration with its intra-molecular hydrogen bonding effectively 'locks' the acid and carbonyl moiety together they will present greater possibility for interaction with the teicoplanin phase than the *cis* configuration and will thus elute with greater retention. Since this is only postulation, therefore for the purpose of simplicity the first set of peaks (the smaller peaks as shown in Fig. 1) will be referred to as the minor set of diastereoisomers while the second set of peaks (the larger peaks shown in Fig. 1) will be referred to as the major set of diastereoisomers.

It was necessary to optimise the separation depicted in Fig. 1 further since reliable quantitative data could not be expected from this separation. In earlier LC studies examining this peak splitting phenomenon only one diastereoisomer was examined. In this study, however, since the primary focus of the paper was to develop a direct LC method for the separation of the diastereoisomers and not to study the peak splitting due to the *cis*/*trans* conversion, its effect had still to be studied and minimised so that it would not impact on the reliability of any future separations. In previous studies, organic modifier type and content in the mobile phase, buffer pH and concentration and temperature have all been shown to influence the *cis*/*trans* conversion and thus separation of this type of molecule in LC [16,22–25]. These parameters were thus subsequently evaluated in order to attain a quantitative method for the determination of the 2*R*,2*S* diastereoisomer in captopril. Additionally, given the stability record of captopril (it is readily converted into its disulphide dimer [17]) and its low UV absorbance, mass spectral detection was used during this optimisation process. This detection technique also proved advantageous over UV during the temperature studies and will be discussed accordingly below.

3.1. Effect of organic modifier

It is well known that mobile phase composition with respect to the type and content of organic modifier present can strongly influence both achiral and chiral interaction on a number of chiral and achiral stationary phases. Moreover, Gustafsson et al. when investigating the *cis*/*trans* kinetics of the N-protected proline drug, ramipril and its metabolite ramiprilate in LC, showed that the type of modifier used strongly influenced the results obtained [25]. Since the separation for the two captopril diastereoisomers in Fig. 1 was attained using 0.1% TEAA buffer at pH 3.4, the effect of additional MeCN, MeOH, THF, 1-propanol or 2-propanol to the aqueous phase were subsequently examined. In all cases resolution between each diastereoisomer and between the *cis*/*trans* isomers was significantly reduced or totally lost. With modifier concentrations as low as 1% in the mobile phase one peak corresponding to the diastereoisomeric mixture eluted just after the solvent front indicating that all ionic interaction between the diastereoisomers and the teicoplanin phase was lost. Furthermore, when other 100% aqueous phases were examined, water, acetic acid acidified water, TEA buffer pH controlled with trifluoroacetic acid or ammonium nitrate for example, little or no separation was attained leaving interpretation of the separation mechanism difficult but still likely to be strongly influenced by ionic interactions. These data exemplify earlier work indicating the selectivity that can be obtained using the TEAA buffer system with macrocyclic antibiotic stationary phases [31].

3.2. Effect of buffer concentration and pH

The effect of TEAA buffer concentration on peak retention and resolution was studied in a range of 0.05–1% TEA at pH 3.8. Three resolution values which corresponded to: (a) the separation between the earlier eluting minor set of diastereoisomers; (b) the later eluting major set of diastereoisomers; and (c) the separation between the 2*S*,2*S* *cis*/*trans* isomers were evaluated throughout these studies (Fig. 1). It was found that on increasing the TEAA concentration, an approximate 30% reduction in resolution was observed for all three values outlined above with optimum results obtained using 0.05% TEAA. This value was subsequently used to study the effect of buffer pH between 3.4 and 6.7, which exceeded the manufacturers recommended both the maximum and minimum operating limit for the column [32]. The pH was shown to have a significant effect on both the diastereoisomeric and *cis*/*trans* resolution values and on the capacity factors (k) obtained on this teicoplanin phase (Fig. 3). These data are in contrast to that shown earlier in separate studies for L-alanyl-L-proline [22] and ramiprilate [25] but are similar to those obtained for ramipril [25] and idrapril [24] where optimum conditions for *cis*/*trans* resolution were indicated at lower pH values.

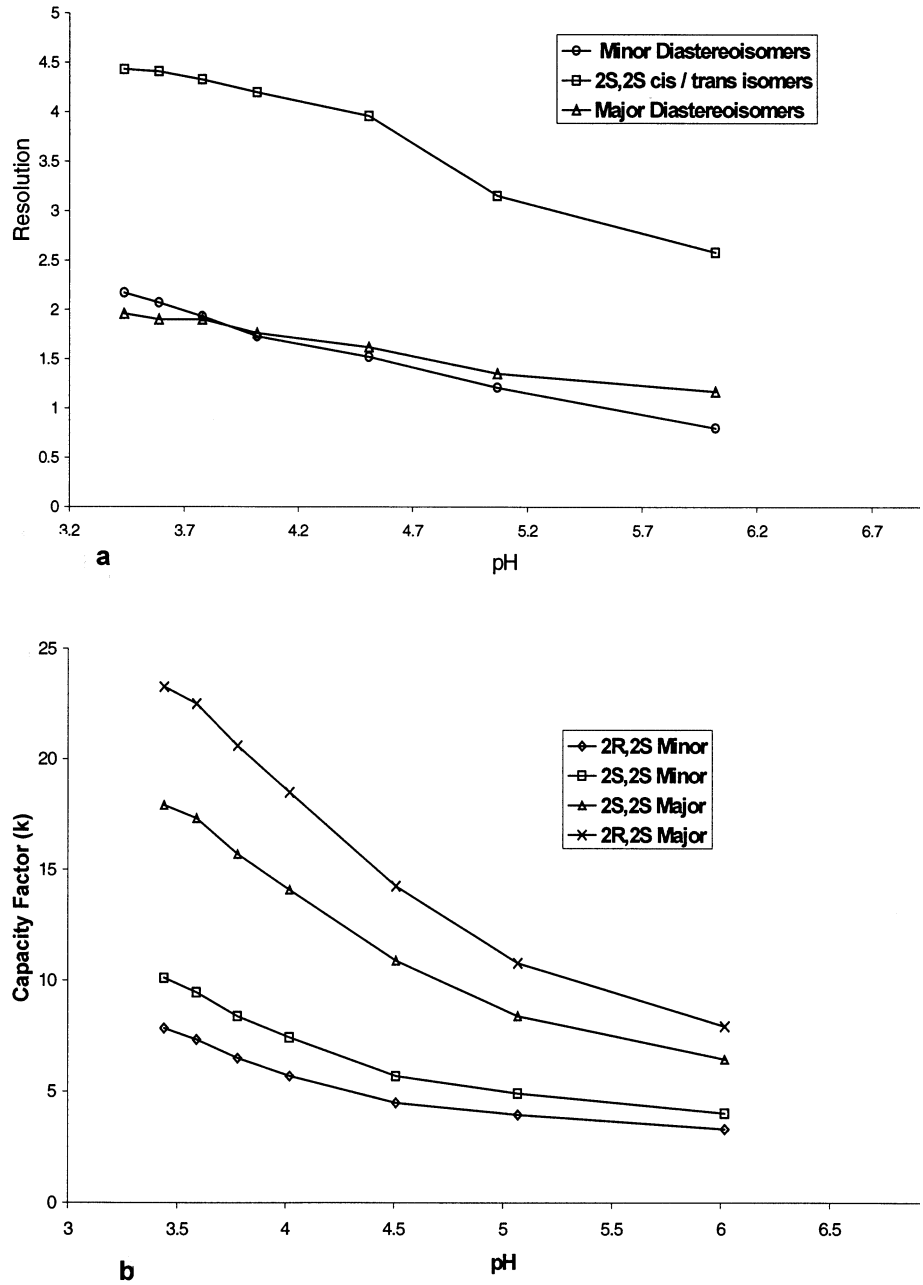

Fig. 3. Effect of TEAA buffer pH on the: (a) resolution; and (b) peak capacity factor (k) of captopril diastereoisomer peaks. Conditions as described in Fig. 1 with the exception of a 0.05% TEAA buffer, 1.0 ml/min and column together with mobile phase reservoir temperature controlled at 0°C.

3.3. Effect of temperature

In earlier studies described above investigating the parameters influencing multiple peaks obtained from peptide like molecules containing an N-blocked proline moiety, the effect of temperature was always examined and shown to have significant effect. With the separation described in Fig. 1, which was carried out at ambient temperature (22°C), the typical 'raised baseline' attributed to the slow kinetics of *cis*/*trans* isomerisation dominated the separation. The effect of temperature on this separation was therefore subsequently examined using the optimised mobile phase conditions described above (0.05% TEAA at pH 3.8 which was the recommended pH limit). The teicoplanin column and mobile phase reservoir were immersed in a temperature controlled polyethylene glycol bath and which was equilibrated for 30 min prior to investigation. The effects of temperature on the separation of the captopril diastereoisomers is shown in Fig. 4. It is important to note that two processes are occurring simultaneously in this separation: (a) the separation of the diastereoisomers; and (b) the separation of the *cis*/*trans*-isomers (which account for the second pair of diastereoisomeric peaks). At temperatures approaching 0°C the 'raised baseline' which results form the slow *cis*/*trans*-isomerisation is progressively reduced as a result of decreased rotation about the N-blocked amide bond of captopril [22,24,25]. At higher temperatures, however, resolution between both the diastereoisomers and the *cis*/*trans* isomers is lost. This has been attributed earlier to the kinetic effects of the isomerisation where the rate of rotation about the amide bond is faster than the chromatographic time scale which results in a more monodisperse peak. It is expected that at even higher temperatures (70–80°C) a single peak corresponding to both *cis* and *trans* isomers would be observed [22]. This higher temperature was not investigated in this study since: (a) the teicoplanin phase would deteriorate over time at these temperatures but more importantly; (b) the goal of this study would be defeated since resolution between the diastereoisomers is lost at higher temperatures. This loss in resolution at higher temperatures is consistent with earlier chiral studies with this column where lower binding affinities were observed at higher temperatures between the solutes of interest and the complex teicoplanin phase resulting in lower resolution values [33].

An interesting feature observed during these studies was the greater detection sensitivity of MS compared to UV detection and therefore its greater ability to fully attribute the existence or non-existence of *cis*/*trans* rotamer isomerisation. As described above and shown in Fig. 4, the isomerisation was reduced when the analysis was carried out at lower temperatures. If this process of temperature optimisation was monitored by UV detection, it is possible that one would describe the separation carried out at 8°C and shown in Fig. 5a to be acceptable. However, when the MS total ion current chromatogram for this separation is evaluated (Fig. 5b) it is clear that an appreciable amount of isomerisation is still occurring since the baseline between the two diastereoisomeric peaks is still raised to some extent. This was easily confirmed by obtaining a mass spectrum from the baseline between the diastereoisomeric peaks which indicated that the intensity of the captopril negative ion (216 mass units) was greater than an order of magnitude compared to the baseline before or after the elution of captopril. Moreover, it was not until the temperature was decreased further to 0°C before an acceptable baseline was achieved indicating little or no isomerisation was taking place.

3.4. Analysis of captopril batches

Use of the minor set of diastereoisomers (it is not known if these correspond to either the *cis* or the *trans* conformation) for any quantitation of the 2*R*,2*S* diastereoisomer in captopril batches was favoured since it eluted first allowing facile quantitation. It was not actually possible to detect the 2*R*,2*S* diastereoisomer below 2% when the major set of diastereoisomers were used since it eluted last (Fig. 1). It was decided to carry out a linearity study to investigate the possibility of this but it had to be limited due to unavailability of adequate quantities of the 2*R*,2*S* diastereoisomer. Consequently, linearity was investigated by vary-

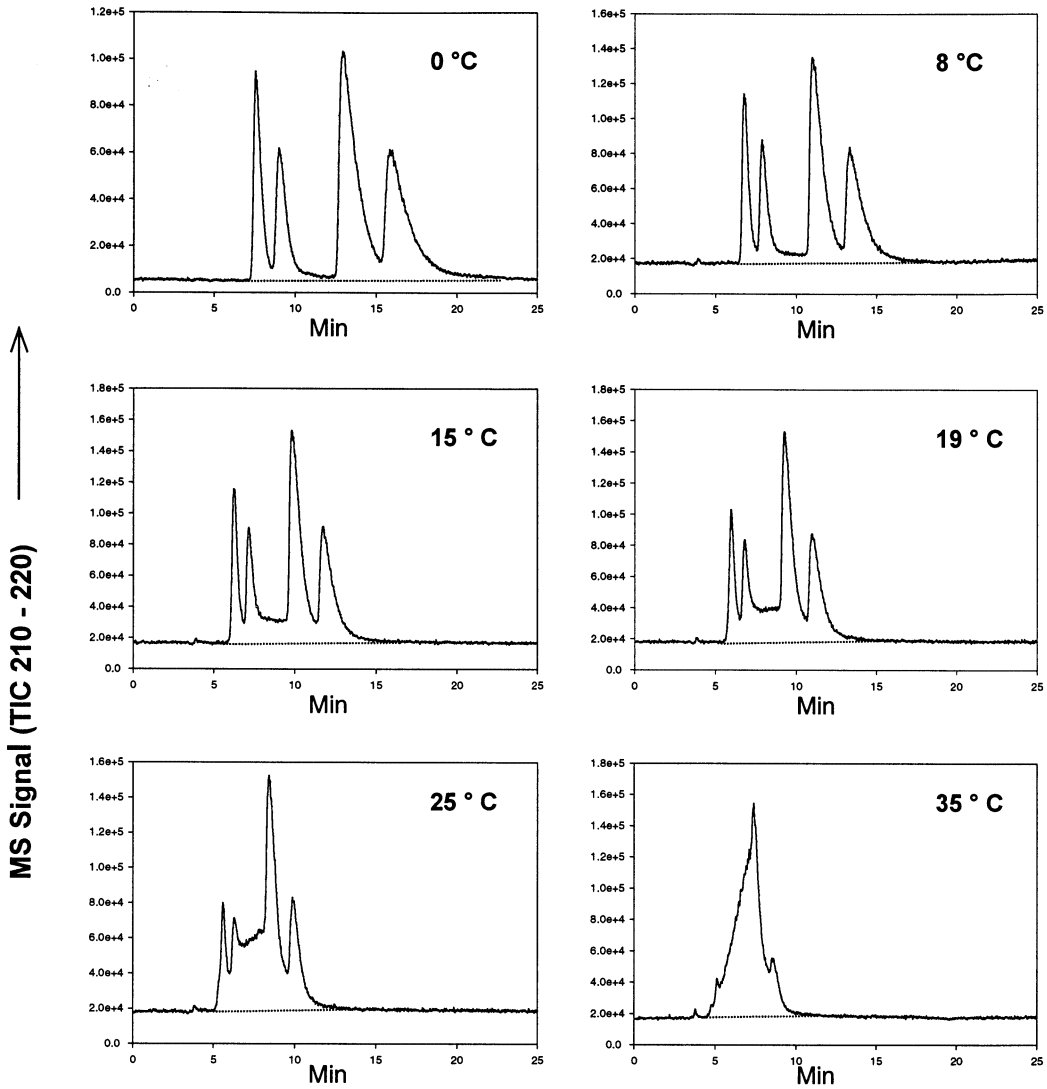

Fig. 4. Effect of temperature on the separation of captopril diastereoisomer peaks using a teicoplanin bonded phase under aqueous conditions. Conditions: 0.05% TEAA (pH 3.8), 1.0 ml/min and mass spectral detection using negative ion mode scanning between 210 and 220 mass units.

ing the amount injected to the column instead of separate solutions of differing concentrations which allowed a seven point calibration curve to be constructed. Linearity was calculated for each of the four diastereoisomeric peaks shown in Fig. 1, and found to yield R^2 (coefficient of determination) values greater than 0.998 for each. This method using the quantity injected to the column has its limitations nevertheless serves as a good indicator to show that the minor set of diastereoisomers could be used for adequate quantitation of any 2*R*,2*S* diastereoisomer in a captopril batch. The repeatability of the separation was also tested and found to yield acceptable relative standard deviation values ($n = 10$) for retention time (0.2%), peak areas (1.5%) and resolution (0.6%).

The method was subsequently used to analyse a number of captopril substance (2*S*,2*S* diastereoisomer) batches for the presence of the 2*R*,2*S* diastereoisomer. A representative chromatogram of captopril CRS substance (2*S*,2*S* diastereoisomer) spiked with 0.5% of the 2*R*,2*S*

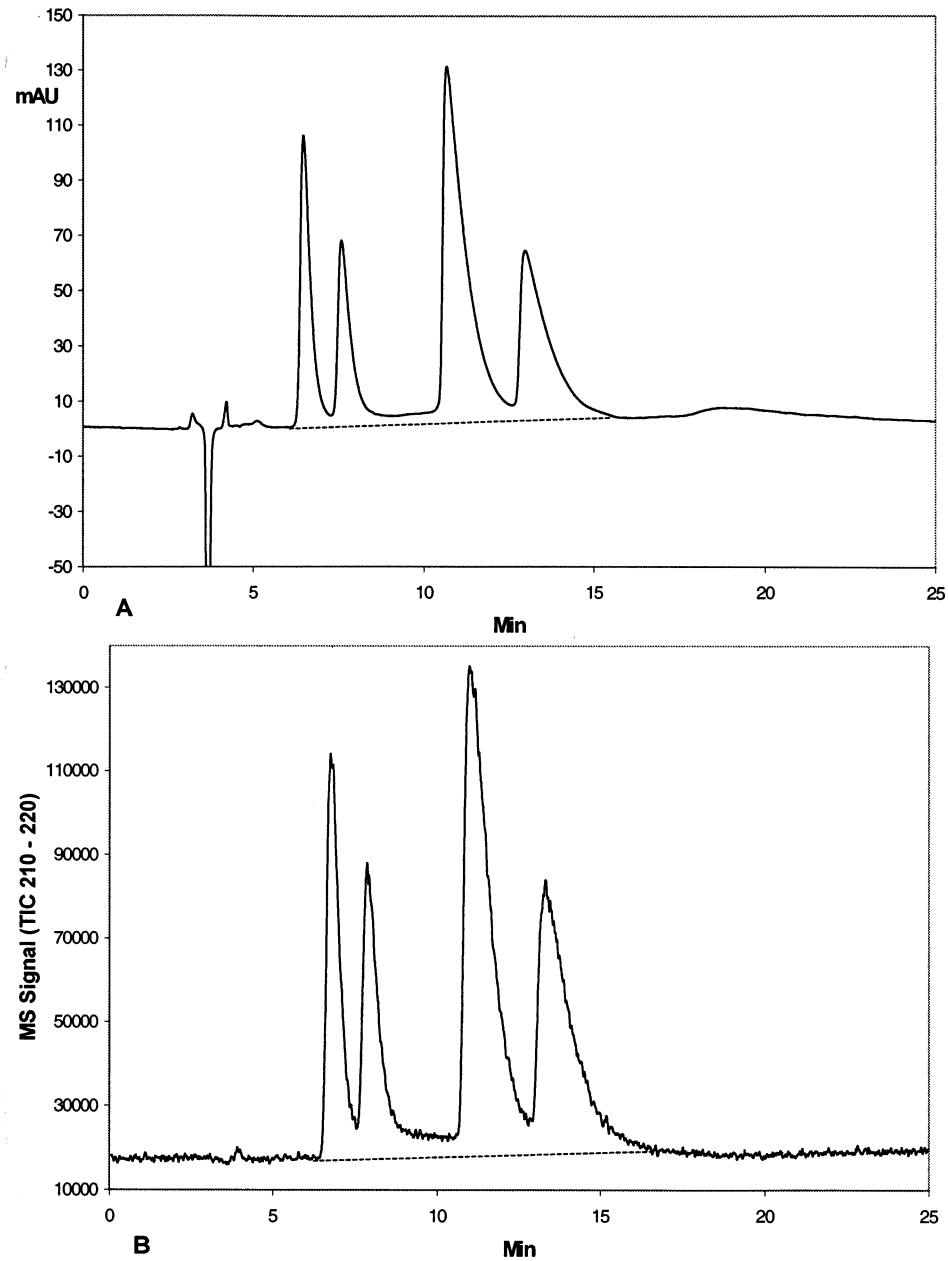

Fig. 5. Separation of captopril diastereoisomers and *cis*/*trans* isomers in reversed-phase LC at 8°C using: (a) UV detection at 220 nm; and (b) mass spectrometry detection in the negative ion mode scanning between 210 and 220 mass units. Conditions as described in Fig. 4.

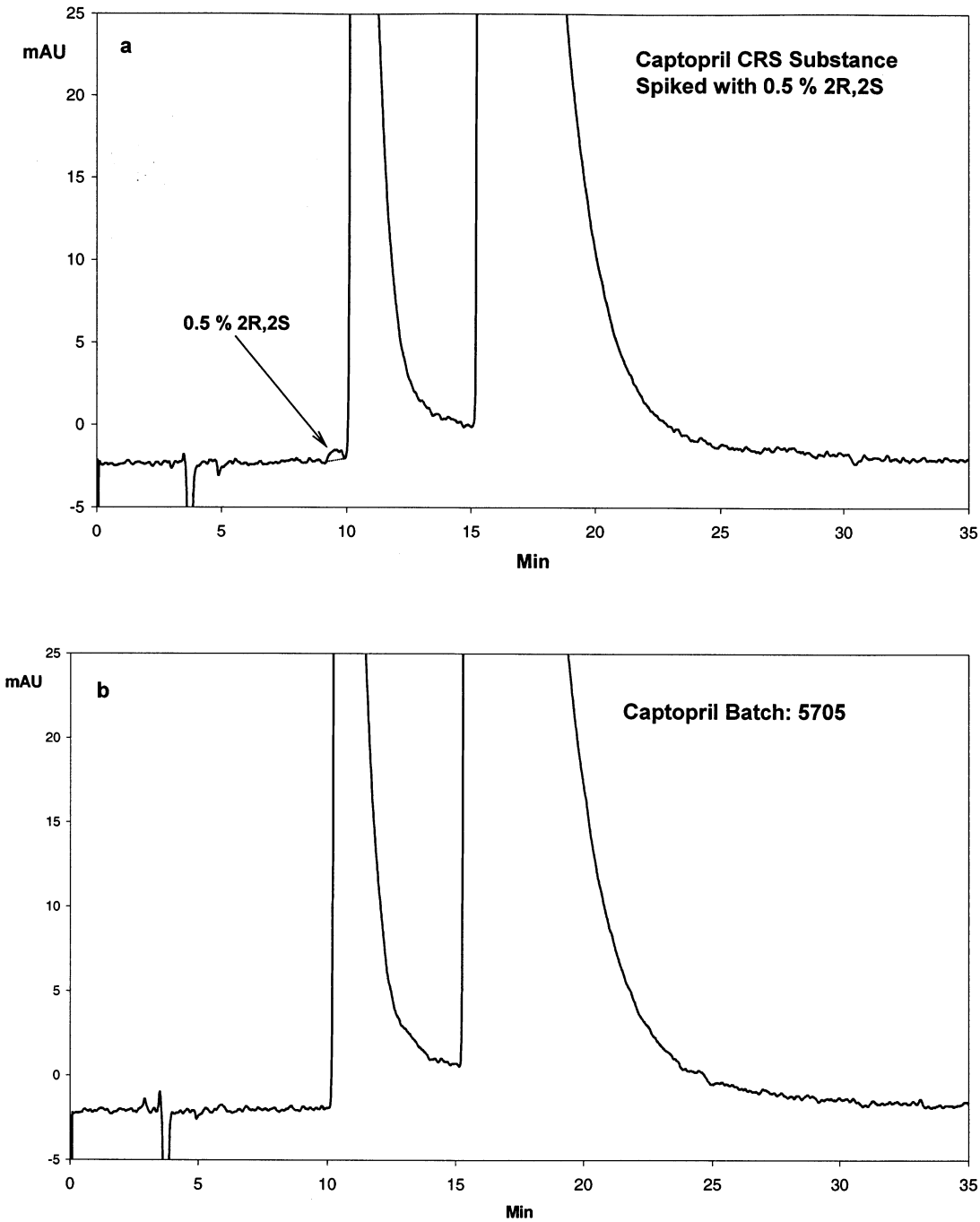

Fig. 6. analysis in reversed-phase LC using a teicoplanin column: (a) captopril CRS reference substance to which 0.5% of the 2*R*,2*S* diastereoisomer was spiked; and (b) a commercial captopril batch indicating the absence (at least the 0.5% level) of the 2*R*,2*S* diastereoisomer. Conditions as described in Fig. 4 but using UV detection at 220 nm.

diastereoisomer is shown in Fig. 6a where its calculated percentage peak area relative to the 2*S*,2*S* minor diastereoisomer as an average of duplicate injections was found to be 0.46%. A representative chromatogram of a captopril substance batch (5705) indicating the absence of the 2*R*,2*S* diastereoisomer is shown in Fig. 6b. The latter finding is in contrast to reports that even the CRS materials contained traceable amounts of the 2*R*,2*S* diastereoisomer.

The LC method and experimental details described above represent a good starting point for this captopril analysis which may need to be evaluated further to increase its robustness and applicability before validation and subsequent commercial implementation. These may include an examination of how the samples are prepared since in these studies water was used throughout and a question may arise as to the differences in rotational isomer equilibrium in water compared to that in the mobile phase employed. Additionally and although not fully evaluated in this study, a nominal limit of quantification of 0.5% diastereoisomeric purity was demonstrated. We believe, however, that this could be lowered to reach the levels required by the relevant regulatory authorities by implementing MS with its greater detection sensitivity which would allow even lower sample concentrations to be used and thus increasing the peak resolution between the 2*R*,2*S* and the 2*S*,2*S* peaks which would further aid quantification. Unfortunately, due to instrument unavailability at the time, the commercial analyses shown above were carried out using only UV diode array detection.

Even if the conditions employed in this method were considered atypical for LC, they have delivered results that are not affected by derivatisation and the inherent risks that this involves. A more elaborate study of captopril batches and samples on the market will reveal if there is a real need for this or a similar method. One additional advantage of this study has been that the 'know-how' achieved by this method can support the results of other less complicated methods and therefore the goal for this study has been achieved.

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