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Journal of Pharmaceutical and Biomedical Analysis
23 (2000) 637–651

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Development and validation of a liquid chromatographic method for the determination of the related substances of ramipril in Altace capsules

Barry L. Hogan *, Mark Williams, Anna Idiculla, Tarik Veysoglu, Ernest Parente

Quality Control Department, Aventis Pharmaceuticals, Inc., 10236 Marion Park Drive, Kansas City, MO 64137-1405, USA

Accepted 12 April 2000

Abstract

The development and validation of a reversed-phase liquid chromatographic method for the determination of the related substances of 2-[*N*-[(*S*)-1-Ethoxycarbonyl-3-phenylpropyl]-L-alanyl]-(1*S*, 3*S*, 5*S*)-2-azabicyclo[3.3.0]octane-3-carboxylic acid (ramipril) in Altace capsules is described. The method utilizes an ion-pairing agent and a simple two-step gradient for the separation of ramipril and ten related substances from each other in a 40-min run time. Four of the related substances are ramipril diastereomers. To the best of our knowledge, no method described previously in the literature has demonstrated resolution of ramipril from this set of related substances. No method for the determination of the related substances of ramipril is currently described in the United States Pharmacopoeia or the European Pharmacopoeia. The proposed method was validated with respect to accuracy, precision, linearity, and specificity. Also, the method was determined to be robust with regards to the following parameters: mobile phase apparent pH; mobile phase organic content; mobile phase perchlorate concentration; detection wavelength and time dependence of sample and standard stability.

Keywords: Ramipril; Related substances; Liquid chromatography; Altace; Method development

1. Introduction

2-[*N*-[(*S*)-1-Ethoxycarbonyl-3-phenylpropyl]-L-alanyl]-(1*S*, 3*S*, 5*S*)-2-azabicyclo[3.3.0]octane-3-carboxylic acid (ramipril, CAS no. 87333-19-5) and other angiotensin-converting enzyme (ACE) inhibitors constitute an important class of therapeutic agents for the regulation of hypertension. The chemical structures of ramipril and its potential related substances are shown in Figs. 1 and 2. It can be seen that ramipril's structure is similar to that of a proline-containing natural peptide. Other ACE inhibitors, such as enalapril, lisinopril, benazepril and quinapril also bear structural similarities to peptides [1]. Ramipril is one of several ACE inhibitors which are prodrugs. The active

* Corresponding author.

0731-7085/00/$ - see front matter
PII: S0731-7085(00)00342-3

diacid metabolite, ramiprilat, is formed through hepatic cleavage of the ramipril ester group [2].

Separation of ramipril from its potential related substances is required for accurate and precise quantitation of degradation products potentially present in formulated pharmaceuticals. Several methods for the determination of ramipril or selected precursors have been described in the literature [1–7]. The related substances presented in Figs. 1 and 2 include these precursors as well as eight additional potential related substances. This set of potential related substances was originally derived from synthetic considerations. The set of related substances includes four diastereomers of ramipril. The potential presence of these diastereomers as related substances significantly complicates the required chromatography due to the diastereomers' structural similarity to ramipril. In addition to the challenges involved in separation of these related substances, adequate detection must also be considered. Due to the low doses of ramipril utilized (typically 2.5–20 mg/day) and the small molar absorptivity of ramipril, detection of low levels of related substances of ramipril can be difficult.

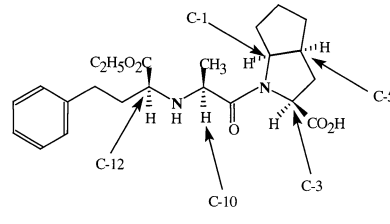
No references describing the separation of the related substances of ramipril bulk drug substance or drug product were found in a recent literature search. As of November 1999, no methods describing the determination of the related substances of ramipril are listed in the United States Pharmacopoeia (USP 23-NF 18) or in the European Pharmacopoeia (1997 edn.).

This paper describes the development and validation of a chromatographic method capable of resolving ramipril from a set of ten potential related substances. To the best of our knowledge, this method is the only one reported in the literature which is capable of resolving these potential related substances of ramipril from themselves and ramipril. A simple two-step gradient is employed and total elution times of 40 min are required. No unstable gradient baselines are generated and the method is robust.

2. Experimental

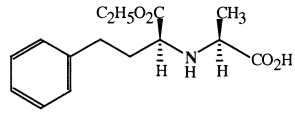
2.1. Reagents

Potassium phosphate monobasic and phosphoric acid, 85% (w/w) were obtained from Mallinckrodt. Sodium perchlorate monohydrate was obtained from Sigma. HPLC-grade acetonitrile was obtained from B&J and HPLC-grade water was obtained from Fisher. All reagents were of reagent grade unless otherwise specified.

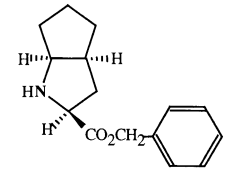


Diastereomer Abbreviation	Configuration at Listed Carbon Center				
	1	3	5	10	12
SSSSS (Ramipril)	S	S	S	S	S
SRSSS	S	R	S	S	S
SSSRS	S	S	S	R	S
RRRSS	R	R	R	S	S
SSSSR	S	S	S	S	R

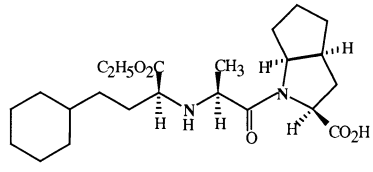
Fig. 1. Chemical structures of ramipril and four potential related substances (ramipril diastereomers).



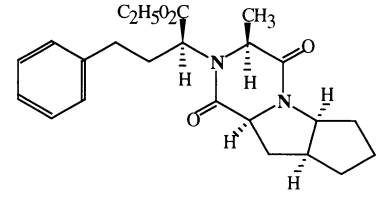
Precursor I



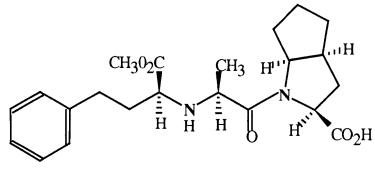
Precursor II-8



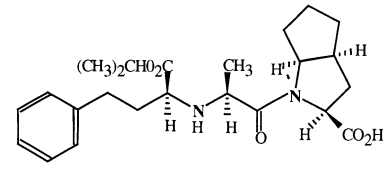
Hexahydro-ramipril



Ramipril-Diketopiperazine



Ramipril Methyl Ester



Ramipril Isopropyl Ester

Fig. 2. Chemical structures of six additional relevant related substances of ramipril.

2.2. Standards

The following standards were obtained from Hoechst AG (Frankfurt): ramipril, ramipril–diketopiperazine, ramipril isopropyl ester, ramipril precursor I, ramipril precursor II-8, ramipril methyl ester and hexahydro–ramipril.

The following ramipril stereoisomer reference standards were also obtained from Hoechst AG (Frankfurt): (1*S*, 3*R*, 5*S*, 10*S*, 12*S* or *SRSSS* stereoisomer); 1*S*, 3*S*, 5*S*, 10*R*, 12*S* or *SSSRS* stereoisomer); (1*S*, 3*S*, 5*S*, 10*S*, 12*R* or *SSSSR* stereoisomer) and the (1*R*, 3*R*, 5*R*, 10*S*, 12*S* or *RRRSS* stereoisomer).

2.3. Solutions and sample preparation

Mobile phase A consisted of a mixture of acetonitrile and an aqueous buffer (27:73, v/v). Mobile phase A contained 0.025 M potassium phosphate and 0.0569 M sodium perchlorate. Mobile phase B consisted of a mixture of acetonitrile and an aqueous buffer (38:62, v/v). Mobile phase B contained 0.025 M potassium phosphate and

0.014 M sodium perchlorate. The apparent pH of both mobile phase solutions was adjusted to 2.0 ($\pm$0.1).

A sample solution containing ramipril at a concentration of 0.5 mg/ml allowed accurate and precise quantitation of ramipril–diketopiperazine. This sample solution was prepared from a 20-capsule composite of Altace capsules. A 4 (or 5) capsule equivalent mass of the capsule contents was weighed into the appropriate volumetric flask as indicated in Table 1. After careful removal of the powdered contents by gentle tapping, the hard gelation shells were discarded. Mobile phase A was added to $\sim$ 30–40% of the flask's capacity. Each flask was shaken vigorously until all product clumps were dissolved. Each flask was then sonicated at 40°C for 10 min. The flasks were then cooled to room temperature and the contents of the flasks shaken. The flasks were diluted to volume with mobile phase A and mixed well. Approximately 10 ml of the contents of the flask were transferred to a 15-ml centrifuge tube and centrifuged at 3000 rpm for 15 min. A portion of the supernatant liquid was transferred into an HPLC vial. The concentration of ramipril in these sample solutions was $\sim$0.5 mg/ml. The concentration of excipient in each sample solution was slightly different as the four Altace capsules dosage forms were of a constant mass and possessed formulations which varied only in the ratio of mass of active ingredient to the mass of the placebo.

A related substances working standard/system sensitivity test solution containing ramipril–diketopiperazine at 0.04 mg/ml (8% w/w relative to ramipril) was utilized for quantitation. This solution also contained ramipril at the 0.0005 mg/ml level (0.1% relative to the 0.5 mg/ml ramipril target concentration). The peak area response due to the ramipril peak served as a sensitivity check for the HPLC detector. The percent relative standard deviation of the ramipril peak area after three replicate injections of this working standard/system sensitivity test solution was consistently below ten percent. A resolution test solution was utilized to verify satisfactory chromatographic separation of ramipril and related substances. The concentrations of rampril, each of the diastereoisomeric impurities and the non-stereoisomeric impurities in the resolution test solution were 0.5, 0.00625 and 0.01 mg/ml, respectively. The components of the resolution test solution and the working standard/system sensitivity test solution were dissolved in mobile phase A.

Table 1
Sample dilution scheme for Altace capsules

Strength (mg)	No. of capsule equivalents	Volumetric flask (ml)
1.25	4	10
2.50	5	25
5.0	5	50
10.0	5	100

2.4. Apparatus

Sample analyses were performed on an HPLC system consisting of a Perkin–Elmer (P–E) Series 200 pump, a P–E ISS 200 sample processor and a P–E LC 295 UV–vis detector. The chromatographic column utilized in these studies was a Zorbax SB-C18, 150 mm $\times$ 4.6 mm (i.d.) column packed with 3.5-μm diameter particles. A 12.5 mm $\times$ 4.6 mm (i.d.) guard column packed with 5-μm diameter Zorbax SB-C18 packing was also utilized. The detection wavelength was 210 nm. The mobile phase flow rate was 1.5 ml/min. Ten microliters of sample were injected into the HPLC for each analysis. A Waters column heater module was used to maintain a constant column temperature of 65°C.

Photodiode array spectra were obtained from a Waters 2690 separations module equipped with a model 996 photodiode array detector. Peak purity analysis was carried out over a wavelength range of 208–350 nm through the use of the Waters millenium chromatography manager software (version 2.15.01). The photostability chamber utilized during forced degradation studies was a Heraeus Suntest CPS controlled by a Heraeus DSET temperature controller.

Table 2
Gradient profile used for the separation of the related substances of ramipril in Altace capsules

Gradient time (min)	%A mobile phase	%B mobile phase	Profile
0	100	0	Isocratic
17	100	0	Isocratic to 17 min
22	0	100	Linear ramp to 100% B
34	0	100	Isocratic to 34 min
35	100	0	Linear ramp to 100% A
40	100	0	Isocratic to 40 min

3. Results and discussion

3.1. Method development

Optimal separation of related substances from each other and from ramipril was achieved with an acetonitrile/perchlorate mobile phase gradient. The gradient profile consisted of two isocratic mobile phase runs connected by a short linear transition between mobile phase A and B. The mobile phase gradient employed is described in Table 2.

Perchlorate was utilized as the ion-pairing agent in the proposed method. An ion-pairing agent was important for the separation of precursor I and precursor II-8 at pH 2.0 as well as the separation of ramipril–diketopiperazine and hexahydro–ramipril. A mobile phase temperature of 65°C was employed for the separation. Separations conducted at this elevated temperature exhibited significantly improved efficiency relative to those conducted at ambient temperatures. No significant degradation of ramipril was observed at 65°C during ramipril's 15-min elution time. Typical retention times and elution orders observed for ramipril and the ten related substances are presented in Table 3.

Quantitation of all related substances other than ramipril–diketopiperazine was conducted on an area/area basis relative to the ramipril peak area in each injection. An external standard was utilized for the quantitation of ramipril–diketopiperazine as this species is the only significant degradation product of ramipril.

3.2. Leaching study

An investigation was conducted to determine the sonication time required for complete leaching of the ramipril from the sample matrix. Eight 0.5-g portions of a composite of Altace capsules (1.25 mg) composite and eight 0.625-g portions of Altace capsules (10 mg) composite were prepared.

Table 3
Typical retention times observed for ramipril and the related substances of ramipril using the conditions of the proposed method

Component	Retention time range (min)	Relative retention time
Precursor I	3.35–3.67	0.23–0.25
Precursor II-8	7.09–7.46	0.48–0.51
Ramipril methyl ester[a]	8.52–9.80	0.58–0.67
RRRSS stereoisomer	10.18–11.82	0.69–0.81
SSSRS stereoisomer	11.89–13.83	0.81–0.94
Ramipril	13.70–15.70	1.00
S/*R* stereoisomer	16.20–18.82	1.10–1.28
Ramipril isopropyl ester[a]	22.06–23.42	1.50–1.59
Hexahydro –ramipril[a]	26.27–27.11	1.79–1.85
Ramipril diketopiperazine	28.26–29.23	1.92–1.99
SSSSR stereoisomer	30.67–31.88	2.09–2.17

[a] Process impurity — do not quantitate.

Table 4
Effect of sample sonication time on leaching of ramipril for a composite sample of Altace capsules (1.25 mg)

Sample number	Sonication time (min)	Ramipril label claim (%)
1[a]	10	99.63
2[a]	10	101.35
3	10	101.58
4	10	101.02
5	30	100.28
6	30	101.15
7	60	104.36
8	60	101.59

[a] Control procedure known to fully leach ramipril from Altace capsules.

Table 5
Effect of sample sonication time on leaching of ramipril for a composite sample of Altace capsules (10 mg)

Sample number	Sonication time (min)	Ramipril label claim (%)
1[a]	10	96.80
2[a]	10	98.02
3	10	96.80
4	10	96.45
5	30	95.81
6	30	99.67
7	60	97.63
8	60	99.38

[a] Control procedure known to fully leach ramipril from Altace capsules.

For each strength studied, two samples were also prepared using a control procedure known to fully leach ramipril. The remaining samples were prepared via the proposed method with total sonication times of 10, 30 and 60 min.

Tables 4 and 5 present the assay results for sample solutions for 1.25 and 10 mg Altace capsules, respectively, prepared using different sonication times during sample leaching. Based on the results obtained, a 10-min sonication time at 40°C was specified in the proposed method.

3.3. Specificity

As indicated by ICH, the specificity of the proposed method was challenged by forced degradation of the drug product. Samples of a composite of Altace capsules (1.25 mg) were subjected to stress conditions of light, heat, acid, base and oxidation in order to evaluate the ability of the proposed method to separate ramipril from both known and unknown degradation products.

All stressed samples were compared to an unstressed time zero reference solution. The time zero reference solution was a composite sample that was prepared according to the proposed method immediately prior to the analysis of the degradation and control samples. The time zero solution provided a reference assay value for the unstressed product. The extent of degradation in the stressed and control samples was calculated relative to this assay value. Samples were degraded to levels where the assay for ramipril showed the sample to have a ramipril content less than 90.0 percent of the level present in the unstressed time zero reference solution.

Chromatograms of the sample solutions of sufficiently degraded samples were analyzed for the presence of degradation products detectable in the range of 208–350 nm. The chromatographic conditions were considered validated for specificity under each degradation condition if, for that condition, degradation products were sufficiently resolved from the ramipril peak in the sample solution such that peak purity analysis of the ramipril peak showed the peak to be spectrally homogeneous over the wavelength range of 208–350 nm.

The proposed chromatographic conditions were found to be specific under all applied stress conditions. In the cases of stress by light, acid and oxidation, it was observed that rigorous stress of the composite samples did not cause significant degradation. For these studies, stress was ended at the 2, 7, and 5-day time points, respectively. Stress by base and heat, however, did cause significant degradation and these stress studies were ended at the 1- and 3-h time points, respectively. Stress by heat produced ramipril–diketopiperazine as the principal degradation product. While ramipril did degrade in basic solution, the absorption of moisture into the dosage form is not expected to significantly influence the degradation of ramipril as the excipient matrix is not strongly basic.

3.4. Method validation

3.4.1. Targeting of related substances for validation

A review of the chemical structures of the potential related substances and the results of the forced degradation studies conducted for this technical report reinforce the fact that ramipril–diketopiperazine is the only major degradation product actually formed in Altace capsules.

In order to confirm the primary significance of ramipril–diketopiperazine as a degradation product of ramipril, an extensive study of nine expired lots of Altace capsules was conducted. The samples included in the study were three 1.25-mg lots, two 2.5-mg lots, two 5-mg lots and two 10-mg lots. All nine of the lots were past their respective expiration dates and several were past expiration by as much as 1 year. These samples represent a broad cross-section of worst-case degradation samples.

The results of the study indicated that ramipril–diketopiperazine was the only significant degradation product of ramipril in Altace capsules. It should be noted that one lot of Altace capsules (10 mg) was found to contain hexahydro–ramipril at 0.18%. This peak was not present to this extent in any of the other eight analyses of expired Altace capsules lots. From synthetic considerations, hexahydro–ramipril is not a degradation product of ramipril. Any hexahydro–ramipril detected is present as a process impurity. From similar considerations, ramipril–isopropyl ester and ramipril–methyl ester are also only present as process impurities. Figs. 3 and 4 show typical chromatograms of the resolution test solution for the proposed method and a 10-mg Altace capsule sample solution, respectively.

Ramipril–diketopiperazine levels found in the analysis of the expired lots ranged from 4.9 to 0.6% (area/area) for the 1.25 and 10 mg Altace capsules, respectively. Other than ramipril–diketopiperazine, none of the remaining six potential degradation products to be quantified were found to be present at levels exceeding 0.1% (area/area) relative to ramipril. In fact, of the fifty-four determinations made on these six related substances (nine lots analyzed by six related substances potentially present in each lot), no detectable degradation product was found in 37 cases. The mean percent area/area response of the remaining seventeen determinations of individual degradation products was 0.036%.

The analysis of these expired lots of Altace capsules represent strong evidence that ramipril–diketopiperazine is the only known degradation product of quantifiable significance in Altace capsules.

Because of the considerations described above, only ramipril–diketopiperazine was targeted for quantitation in this method validation. An external standard was utilized for the quantitation of ramipril–diketopiperazine. Quantitation of other known impurities was conducted via an area/area approach. Response factors for unknown impurities were set at unity. Relative response factors for precursor I and precursor II-8 were 1.4 and 1.3, respectively. Relative response factors for the other eight separated related substances were unity.

3.4.2. Linearity

3.4.2.1. Ramipril. The linearity of the relationship of detector response measured as peak area versus concentration of ramipril in sample solutions was investigated over a range extending below the reporting level of the impurities (0.1% w/w) to 250% of the 0.5 mg/ml target concentration of ramipril. These levels correspond to ramipril concentrations of 0.0005 and 1.25 mg/ml, respectively.

Solutions were prepared such that the concentrations analyzed for ramipril were at the 250, 150, 100, 60, 20, 12, 6, 2, 1.2, 0.6, 0.2 and 0.06% (w/w) levels relative to the 0.5 mg/ml target concentration for ramipril.

For ramipril, the relationship of detector response measured as peak area versus concentration was linear over the range of 0.1–250% of the target concentration of 0.5 mg/ml of ramipril in the sample solution. The product-moment correlation coefficient, R, was 0.999995 ($n = 24$). The slope was 15586.7 mV s/mg per ml. The value for the y-intercept was 4.061 mV s. The lower and

upper 95% confidence interval limits were −11.522 and 19.645 mV s, respectively. At the 95% confidence level, the y-intercept was not statistically different from zero. The use of a one-point standard was therefore justified.

3.4.2.2. Ramipril–diketopiperazine. The linearity of the relationship between detector response measured as peak area and concentration of ramipril–diketopiperazine was investigated over a range of 0.1–9.6% (w/w) relative to the concentration of ramipril in the sample solution, 0.5 mg/ml. The actual concentrations of ramipril–diketopiperazine in solution ranged from 0.0004351 to 0.054388 mg/ml. This range extends from the reporting level of 0.1% (w/w) to 120% of the degradation product's specification limit of 8% (w/w).

Solutions were prepared for concentrations such that the concentrations analyzed for ramipril–diketopiperazine were the 10, 7, 4, 2, 1, 0.2, 0.14 and 0.08% (w/w) levels relative to the 0.5 mg/ml target concentration for ramipril.

For ramipril–diketopiperazine, the relationship of detector response measured as peak area vs. concentration was linear over the range of 0.1–9.6% (w/w) of the target concentration of ramipril in the sample solution (0.5 mg/ml). The product-moment correlation coefficient, R, was 0.99977 ($n = 16$). The slope was 15408.6 mV s/mg per ml. The value for the y-intercept was −3.646 mV s; the lower and upper 95% confidence limit inter-

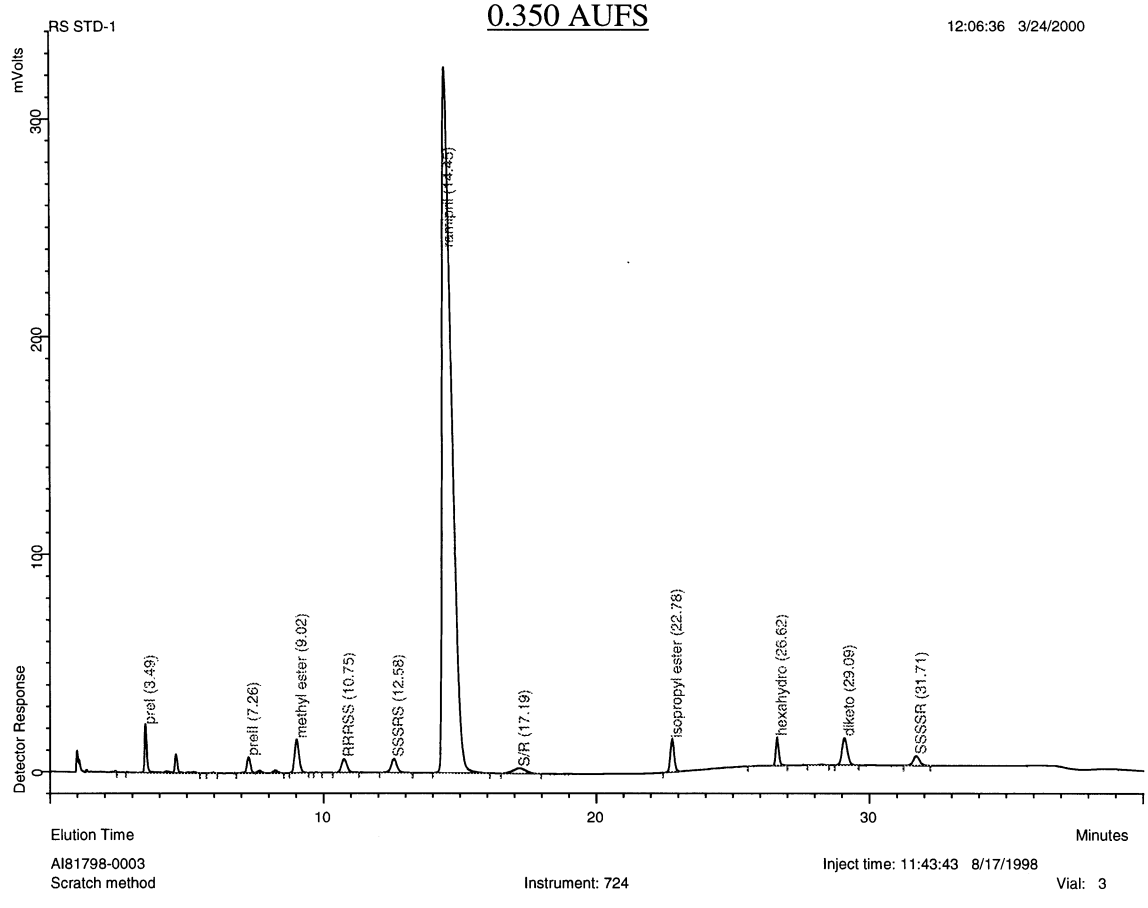


Fig. 3. Chromatogram of the resolution test solution for the proposed mixture. See Table 3 for identities and retention times of related substances.

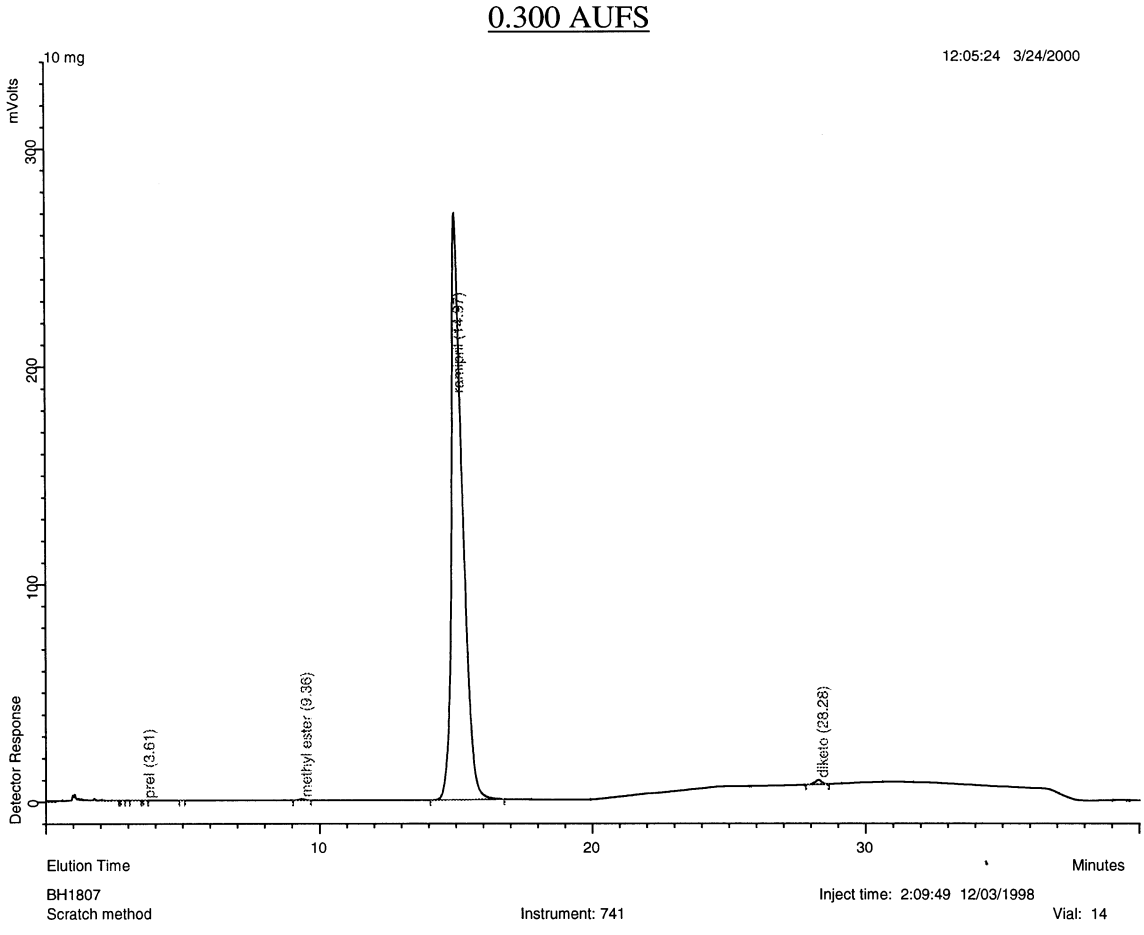


Fig. 4. Chromatogram of the sample solution for 10 mg Altace capsules prepared as per the proposed method.

vals were −11.9251 and 4.633 mV s, respectively. At the 95% confidence level, the y-intercept was not statistically different from zero. Use of a one-point standard was therefore found to be suitable for quantitation of ramipril–diketopiperazine.

3.4.3. Accuracy

The accuracy of the method for quantitation of ramipril–diketopiperazine was investigated by analysis of solutions of actual samples of Altace 1.25 and 10 mg capsules. The sample solutions were prepared with ramipril at the target concentration of 0.5 mg/ml and were spiked with ramipril–diketopiperazine at the following percent (w/w) levels:

% Ramipril–diketopiperazine: 9.6, 8, 6, 3, 1 and 0.1%

These levels extend from the area cutoff of the method (0.1%) to 120% of the specification limit (8% w/w) for ramipril–diketopiperazine.

Two approaches were used to prepare spiked solutions over this range of concentrations. All accuracy studies conducted on the 1.25 mg composite utilized wet spikes for all levels due to the inherent difficulties involved in handling microgram masses of solids. Accuracy studies conducted on the composite of the 10 mg Altace capsules utilized dry spikes for the four highest levels and wet spikes for the two lowest levels (1 and 0.1%).

Injections of three similarly prepared reference solutions containing samples of the composite were used to correct for the inherent presence of ramipril–diketopiperazine in the product composite. These solutions were not spiked with any additional quantity of ramipril–diketopiperazine. The mean ramipril–diketopiperazine content found in the unspiked composite was subtracted from the spiked composite results.

For each Altace product strength studied, four sets of accuracy analyses were conducted by each of two analysts. Each analyst ran two sets, each set on a different HPLC system using a different column. Two different HPLC systems were utilized to conduct the analyses. Each column was packed with a different lot of packing material and all columns were from the same manufacturer. Use of different systems, analysts and columns allowed the introduction of robustness testing regarding variations in analyst, HPLC system and columns.

Accuracy results are presented in Tables 6 and 7. The grand means for recovery of ramipril–diketopiperazine in 1.25 and 10 mg composites were 94.29 and 103.75%, respectively. In review of the data, it was noted that the mean recovery values at the 0.1% spiking level differs significantly from the mean recovery values at higher spiking levels. The grand means calculated with this 0.1% level excluded would be 101.53 and 101.93%, respectively. It is important to note that the 0.1% spiking level represents the LOQ for the method and that this spiking level involves the measurement of ramipril–diketopiperazine peaks with net peak areas of only ~ 5–8 mV s.

Conducting accuracy investigations by spiking a related substance into actual product inherently containing significant amounts of that related substance necessarily adds imprecision to the assessment of analyte recovery. In order to obtain the 'net' amount of related substance found, the level already present in the product is first estimated through analysis (typically $n = 3$) of the

Table 6
Results for spiked ramipril–diketopiperazine recovered from samples of Altace capsules (1.25 mg) obtained by applying the proposed method

	Sample no.	Percent (w/w) added	Recovery (%)				Mean	% RSD
			Analyst 1		Analyst 2			
			Day 1	Day 2	Day 1	Day 2		
	1	9.6	103.4	102.4	108.4	104.8	104.8	2.5
	2	8.0	103.2	101.3	109.2	106.0	104.9	3.3
	3	6.0	104.7	101.3	107.5	104.0	104.4	2.4
	4	3.0	108.4	100.5	111.8	104.6	106.3	4.6
	5	1.0	41.6	99.0	111.4	97.4	87.4	35.6
	6	0.1	25.1	24.8	42.2	140.0	58.0	95.2
Mean			81.1	88.2	98.4	109.5		
% RSD			46.10	35.24	28.04	13.95		
Grand mean			94.29					
	All levels	Levels 1–5 only						
Within-run S.D.	28.973	14.345						
Within-run % RSD	30.727	14.127						
Between-run S.D.	3.624	3.282						
Between-run % RSD	3.843	3.232						
Total S.D.	29.198	14.715						
Total % RSD	30.966	14.492						

Table 7
Results for spiked ramipril–diketopiperazine recovered from samples of Altace capsules (10 mg) obtained by applying the proposed method

	Sample no.	Percent (w/w) added	Recovery (%)				Mean	% RSD
			Analyst 1		Analyst 2			
			Day 1	Day 2	Day 1	Day 2		
	1	9.6	99.6	99.9	103.4	100.0	100.7	1.8
	2	8.0	98.0	100.0	104.2	100.1	100.6	2.6
	3	6.0	99.7	99.6	102.1	109.0	102.6	4.3
	4	3.0	98.4	99.9	102.8	106.5	101.9	3.5
	5	1.0	103.8	100.8	103.0	108.0	103.9	2.9
	6	0.1	64.1	93.6	125.7	167.7	112.8	39.4
Mean			93.9	99.0	106.9	115.2		
% RSD			15.71	2.69	8.66	22.57		
Grand mean			103.75					

	All levels	Levels 1–5 only
Within-run S.D.	15.707	2.503
Within-run % RSD	15.139	2.456
Between-run S.D.	6.76	2.088
Between-run % RSD	6.516	2.049
Total S.D.	17.1	3.260
Total % RSD	16.482	3.198

unspiked composite. Using the 1.25 mg composite as an example, the ramipril–diketopiperazine present in the unspiked composite produces peak areas of ~ 300 mV s. The variability in these areas can be 1–4 mV s. As mentioned above, the 'net' analyte concentration spiked into the product may result in peaks with areas of 8 mV s. The process of determining the recovery of the spiked sample then involves subtracting the ~ 300 mV s (± 1–4 mV s) ramipril–diketopiperazine background from the ~ 308 mV s peak due to the ramipril–diketopiperazine background and spike. The 'analyte' recovered then would be ~ 8 mV s (± 1–4 mV s). Thus, small uncertainties in the measurement of the inherent ramipril–diketopiperazine present in the composite samples tend to disproportionately influence the percent recovery values obtained.

In considering the relevance of the repeatibility of the recovery values at the 0.1% level as shown in Tables 6 and 7, the method precision data presented in Tables 8 and 9 should also be examined. It should be noted that the precision of the method for ramipril–DKP present at the 0.8% level in unspiked product (Table 9) was 2.65%. In addition, as mentioned in Section 2.3, replicate ($n = 3$) injections of the working standard/system sensitivity test solution containing ramipril at the 0.1% level consistently provided mean detector responses with precisions of less than 10%. These facts allow us to be certain that the instrumentation is capable of reproducibly quantitating compounds at the 0.1% level and that when the method is applied to actual product containing ramipril–DKP at low levels, precise results are obtained.

Table 8
Results obtained for the determination of ramipril–diketopiperazine in Altace capsules (1.25 mg) prepared and analyzed as specified in the proposed method

	Sample no.	% Ramipril–diketopiperazine found			
		Analyst 1		Analyst 2	
		Day 1	Day 2	Day 1	Day 2
	1	5.97	6.20, 6.22	6.20	6.50
	2	5.95	6.18, 6.21	6.27	6.50
	3	5.96	6.16, 6.27	6.30	6.61
	4	5.94	6.25, 6.24	6.22	6.38
	5	6.01	6.15, 6.22	6.29	6.35
	6	6.00	6.26, 6.25	6.22	6.34
Mean		5.97	6.22	6.25	6.45
% RSD		0.47	0.63	0.67	1.66
Grand mean		6.22			
% RSD		2.62			
Within-run S.D.			0.058		
Within-run % RSD			0.928		
Between-run S.D.			0.168		
Between-run % RSD			2.694		
Total S.D.			0.177		
Total % RSD			2.850		

Table 9
Results obtained for the determination of ramipril–diketopiperazine in Altace capsules (10 mg) prepared and analyzed as specified in the proposed method

	Sample no.	% Ramipril–diketopiperazine found			
		Analyst 1		Analyst 2	
		Day 1	Day 2	Day 1	Day 2
	1	0.78	0.78, 0.80	0.82	0.83
	2	0.78	0.78, 0.81	0.81	0.84
	3	0.79	0.79, 0.81	0.82	0.85
	4	0.79	0.79, 0.84	0.82	0.84
	5	0.80	0.79, 0.81	0.82	0.83
	6	0.80	0.78, 0.84	0.82	0.82
Mean		0.79	0.80	0.82	0.84
% RSD		1.13	2.65	0.50	1.26
Grand mean		0.81			
% RSD		2.65			
Within-run S.D.			0.010		
Within-run % RSD			1.276		
Between-run S.D.			0.021		
Between-run % RSD			2.559		
Total S.D.			0.023		
Total % RSD			2.859		

3.4.4. Precision

3.4.4.1. System repeatability. System repeatability was assessed by the replicate injection of a working standard/system sensitivity test solution during the studies conducted for determination of the accuracy of the method. For the first six replicate injections performed by both analysts on both of 2 days, a mean system precision of 0.96% (range of 0.36–1.41%) for the ramipril–diketopiperazine peak was found.

3.4.4.2. Method repeatability and intermediate precision of the method. A composite sample of Altace capsules (1.25 mg) was prepared by mixing the contents of two lots of Altace capsules. One hundred capsules of each of two lots were prepared similarly for the 10 mg composite. Using the proposed method, two analysts performed the related substances determinations on six samples from the composite on 2 different days. The mean ramipril–diketopiperazine content of the 1.25 mg composite was 6.22% with an RSD of 2.62%. The mean ramipril–diketopiperazine content of the 10 mg composite was 0.81% with an RSD of 2.65%. Data for the assessment of method repeatability are presented in Tables 8 and 9.

Investigation of the effect upon the method of different analysts, columns, HPLC systems and days of analysis was incorporated into the experimental design of the studies of the accuracy of the method and is also reflected in the intermediate precision presented in Tables 8 and 9. Precision of the method was determined to be satisfactory under the conditions of the proposed method.

3.4.5. Robustness and stability of sample and standard solutions

The robustness of the method relative to each operational parameter was challenged. The operational parameters investigated were:

- apparent pH of the mobile phase ($\pm$ 0.1 pH units)
- mobile phase organic content ($\pm$ 2% (absolute))
- mobile phase perchlorate concentration ($\pm$ 0.001 M)
- mobile phase temperature ($\pm$ 5°C)
- detection wavelength setting ($\pm$ 3 nm)
- time dependence of the stability of the sample and standard solutions

Robustness of the method relative to each operational parameter was assessed through analysis of the resolution test solution under variable chromatographic conditions. Resolution of critical pairs was measured at the conditions of interest in order to evaluate robustness.

The stability of working standard/system sensitivity test solution and sample solutions for Altace capsules was tested over a time frame of 42 h. Ramipril degrades to ramipril–diketopiperazine at room temperature. This stability study investigated the feasibility of extending stability time limits by utilizing storage at 4°C.

Supernatants of several replicate sample solution preparations were combined to form a composite of sufficient volume to conduct the experiment. Working standard/system sensitivity test solutions were treated similarly. A portion of each of these pooled solutions was injected onto an HPLC system as the time zero (initial value) reference solutions. The remainder of each composite was then split into two portions. One portion was stored at ambient temperature while the other portion was immediately stored at 4°C.

Twenty-four hours after the initial sample preparation, the analysis of all sample and standard solutions against a freshly prepared working standard/system sensitivity test solution was initiated. The last sample solution analyzed was injected after an 18-h delay in the autosampler. The results indicate that sample solutions may be stored for 24 h at 4°C prior to analysis. The sample solutions must be injected within a maximum of 18 h exposure to room temperature. It should be noted that the ramipril–diketopiperazine peak areas in both the 1.25 and 10 sample solutions increased by $\sim$ 4 mV s over this 42-h time frame. It should be noted that each 1 mV s increase in peak area represents approximately a 0.33 μg/ml increase in ramipril–diketopiperazine concentration found for the 10 mg sample solutions. The results for the 10 mg sample solutions therefore appear disproportionately high due to the low levels of ramipril–

Table 10
Results of a study of solution stability for working standard/system sensitivity test solutions stepwise aged (4°C for 24 h and room temperature for 18 h) and analyzed against a freshly prepared working standard/system sensitivity test solution[a]

	Sample number	Temperature	Ramipril–diketopiperazine found (%)
	1	4°C	8.08
	2	4°C	8.06
	3	4°C	8.11
Mean			8.08
Mean initial value			8.09 (S.D. = 0.028)
	4	RT	8.08
	5	RT	8.05
	6	RT	8.08
Mean			8.07
Mean initial value			8.09 (S.D. = 0.028)

[a] Standards 1–3 held at 4°C for 24 h followed by exposure to ambient temperature for 18 h. Standards 4–6 held at ambient temperature for 42 h. A ramipril–diketopiperazine content of 8% corresponds to a solution concentration of 40 μg/ml.

Table 11
Results of a study of solution stability for sample solutions stepwise aged (4°C for 24 h and room temperature for 18 h) and analyzed against a freshly prepared working standard/system sensitivity test solution[a]

	Sample number	Weight/temperature	Ramipril–diketopiperazine found (%)
	1	1.25 mg/RT	2.24
	2	1.25 mg/RT	2.24
	3	1.25 mg/RT	2.25
Mean			2.24
Mean initial value			2.20 (S.D = 0.006)
	4	1.25 mg/4°C	2.22
	5	1.25 mg/4°C	2.24
	6	1.25 mg/4°C	2.21
Mean			2.23
Mean initial value			2.20 (S.D. = 0.006)
	7	10 mg/RT	0.55
	8	10 mg/RT	0.52
	9	10 mg/RT	0.53
Mean			0.53
Mean initial value			0.46 (S.D. = 0.006)
	10	10 mg/4°C	0.49
	11	10 mg/4°C	0.50
	12	10 mg/4°C	0.51
Mean			0.50
Mean initial value			0.46 (S.D. = 0.006)

[a] Solutions 1–3 and 7–9 held at ambient temperature for 42 h prior to injection. Solutions 4–6 and 10–12 held at 4°C for 24 h followed by exposure to ambient temperature for 18 h prior to injection. A ramipril–diketopiperazine content of 2.2% corresponds to a solution concentration of 11 μg/ml. A ramipril–diketopiperazine content of 0.46% corresponds to a solution concentration of 2.3 μg/ml.

diketopiperazine initially present in the sample solutions. The results for working standard/system sensitivity test solutions are presented in Table 10. The results for 1.25 and 10 mg sample solutions are presented in Table 11.

4. Conclusions

Data are presented which demonstrate that the proposed method is accurate, precise, linear, specific and robust for the determination of related substances in Altace capsules (1.25, 2.5, 5.0 and 10.0 mg). Also, the method was determined to be robust with regards to the following parameters: mobile phase apparent pH; mobile phase organic content; mobile phase perchlorate concentration; detection wavelength and time dependence of sample and standard stability.

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