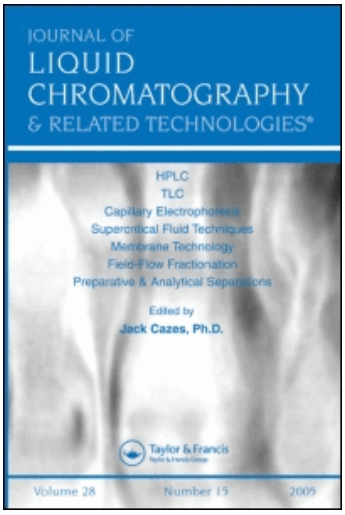

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Isolation and Identification of Process Impurities in Crude Valsartan by HPLC, Mass Spectrometry, and Nuclear Magnetic Resonance Spectroscopy

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Abstract: Three unknown recurring impurities were isolated from crude valsartan by a combination of analytical and preparative liquid chromatography. One of the impurities was identified as (*S*)-*N*-valeryl-*N*-{[2′-(1-methyl-tetrazol-5-yl) biphenyl-4-yl]-methyl}-valine by mass spectrometry and nuclear magnetic resonance spectroscopy. The tentative mechanism for the formation of the impurities is also discussed.

Keywords: Valsartan, Impurities, Liquid chromatography, Electrospray ionization mass spectrometry, Nuclear magnetic resonance spectroscopy, Degradation

INTRODUCTION

The quality and safety of pharmaceuticals can be significantly affected by the presence of impurities. Consequently, the testing and establishment of limits for impurities in active pharmaceutical ingredients have become important

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initiatives by government and the pharmaceutical industry. Guidelines of the International Conference on Harmonization (ICH) have focused on thresholds for identification, qualification, and reporting of impurities.[1] In particular, ICH requires identification of any recurring impurities at or above the 0.1% (w/w) level.

Many publications discuss the identification of pharmaceutical impurities using various technologies. Franolic et al.[2] isolated a hydrochlorothiazide—formaldehyde adduct impurity in hydrochlorothiazide drug substance by preparative chromatography, and the impurity was characterized by electrospray ionization LC-MS. Lehr et al.[3] employed the hyphenation of preparative liquid chromatography to MS and NMR for the isolation and identification of impurities in drug substances. Alsante et al.[4] reported a multidisciplinary approach to pharmaceutical impurity identification.

Valsartan, (*S*)-*N*-valeryl-*N*-{[2′-(1H-tetrazol-5-yl) biphenyl-4-yl]methyl}-valine (Figure 1), is an orally active specific angiotensin II receptor antagonist used as a hypotensive drug.[5] Angiotensin-II-receptor antagonists (ARA-IIs) are safe and effective agents for the treatment of hypertension and heart failure, either alone, or in conjunction with hydrochlorothiazide (HCT), a thiazide diuretic.[6] They have been proposed as an alternative to the more traditional angiotensin-converting enzyme inhibitors because they selectively block the angiotensin type 1 (AT1) receptor.[7]

Although various analytical methods have been developed for the quantitative determination of valsartan in tablets[8,9] and biological samples,[10–12] and furthermore, the separation of several ARA-IIs,[13–15] there has been no report in the literature on the identification and characterization of process impurities in valsartan. In addition, there are no official standards for this drug in the USP, BP, or EP.

This report describes the utilization of analytical and preparative liquid chromatography (LC) to isolate previously unknown impurities from crude valsartan. One of the impurities, impurity A, was identified by electrospray

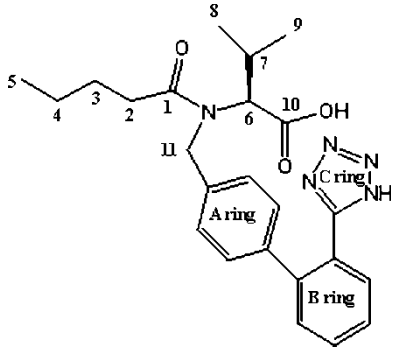


Figure 1. Structure of valsartan.

ionization (ESI)-MS and NMR. Purposeful degradation was also conducted on it. Finally, a proposed mechanism for the formation of impurity A was presented.

EXPERIMENTAL

Liquid Chromatographic Systems

Analytical

A Shimadzu LC-10ATvp pump, a SPD-10A UV–visible detector (Shimadzu, Japan), TL-9900 chromatography workstation (Beijing TELEH Electronics Technology Company, Beijing, China), and a Rheodyne 7125 injector with 20 μL sample loop were used. Flow rate was 1.0 mL/min; detector wavelength was 254 nm; column temperature was ambient.

Preparative

A P230 pump, a UV 228 variable wavelength UV-detector, EC2000 chromatography workstation (Dalian Elite Analytical Instruments Company, Dalian, China), and a Rheodyne 7125 injector with 1 mL sample loop were used. Flow rate was 5.0 mL/min; detector wavelength was 254 nm; injection volume was 1 mL; column temperature was ambient.

Chromatographic Columns

Analytical

ODS Hypersil column (200 mm × 4.6 mm, 5 μm) (Thermo Electron Corporation); ZORBAX SB—CN column (250 mm × 4.6 mm, 5 μm) (Hewlett-Packard Company, USA).

Preparative

Hypersil ODS2 column (250 mm × 10.0 mm, 5 μm) (Elite, Dalian, China).

Mass Spectrometer

PE-SCIEX (Applied Biosystem, Canada) Q/STAR Pulsar *i* QTOF mass spectrometer, equipped with an electrospray ionization (ESI) probe operated in the negative ion mode. The scan range was 80–600 m/z. The precursor ions were selected in the center of each cluster, for valsartan m/z 434.2 and for the impurity A m/z 448.2.

NMR Spectrometer

The Varian Mercury-Vx300-MHz spectrometer (CA, USA) was used for proton and carbon spectra collections, respectively. For comparison, the same set of experiments was carried out for valsartan in deuterated methanol. Assignments were further confirmed by running two-dimensional chemical shift correlation experiments. ChemDraw® Ultra simulation software (Cambridge soft Corporation, Cambridge, USA) was used to perform carbon and proton spectral simulations on the basis of proposed structures.

Rotary Evaporator

A RE-52CS Rotary Evaporator (Shanghai Yarong Biochemistry Instrument Factory, Shanghai, China) was utilized to evaporate solvent.

Chemicals

Acetinitrile was purchased from Beijing Fine Chemical Reagent Company (Beijing, China). Acetic acid was purchased from Shanghai Chemical Reagent Company. Other reagents were obtained from various commercial sources and were of analytical grade unless otherwise indicated. Double distilled water was used for all experiments.

Mobile Phase

Acetic acid, 0.2%, (v/v in H_2O)-acetonitrile (55 : 45, v/v) was employed for the analytical analysis. In preparative analysis, 0.2% acetic acid (v/v in H_2O)-acetonitrile (60 : 40, v/v) was used for the separation of impurity A, and 0.2% acetic acid (v/v in H_2O)-acetonitrile (62 : 38, v/v) was used for the separation of impurities B and C. All the mobile phases were filtered through a G4 fritted glass funnel and degassed prior to use.

Sample Preparation

Crude valsartan was supplied by Zhejiang Huahai Pharmaceutical Company (China). The samples injected for LC analysis were all dissolved in the solvent consisting of acetonitrile-water (8 : 2, v/v). All sample solutions were filtered through a 0.45 μm filter before injection.

The concentrations of valsartan were approximately 10 mg/mL and 30 mg/mL for analytical and preparative LC, respectively. The impurities obtained were dissolved in methanol for MS analysis and deuterated

methanol for NMR analysis. Chemical shifts were referred to residual ^{1}H in CD_3OD at 3.30 ppm and residual ^{13}C in CD_3OD at 49.07 ppm.

RESULTS AND DISCUSSION

Isolation Procedure

An LC system was established in the initial step for the analysis of these three unknown impurities. The HPLC methods reported in the literature utilized C_{18} columns, and the mobile phases consisted of acetonitrile and acidic buffer solution.[9,11,12] But, baseline separation of these three impurities was impossible under these chromatographic conditions, and the adjustment of pH couldn't improve the resolution either. So, at first, the fraction containing three impurities was collected together by preparative LC.

The mobile phase for analytical LC was 0.2% acetic acid (v/v in H_2O)-acetonitrile (55 : 45, v/v). The analytical conditions were scaled up for preparative runs (column internal diameter: 10 mm) with slight adjustments of flow rate (5 mL/min vs 1 mL/min) and the ratio of acetonitrile in the mobile phase (40% vs 45%, v/v). A typical preparative HPLC chromatogram of crude valsartan is presented in Figure 2.

The total amount of three impurities was about 1.8% in crude valsartan. The impurities fraction (22 ~ 28 min) and valsartan portion (15 ~ 18 min) were collected, respectively. The valsartan portion was collected for MS

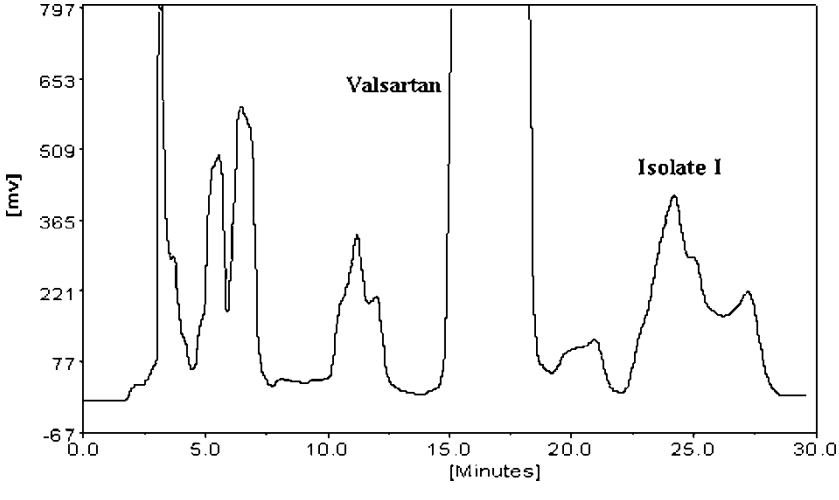
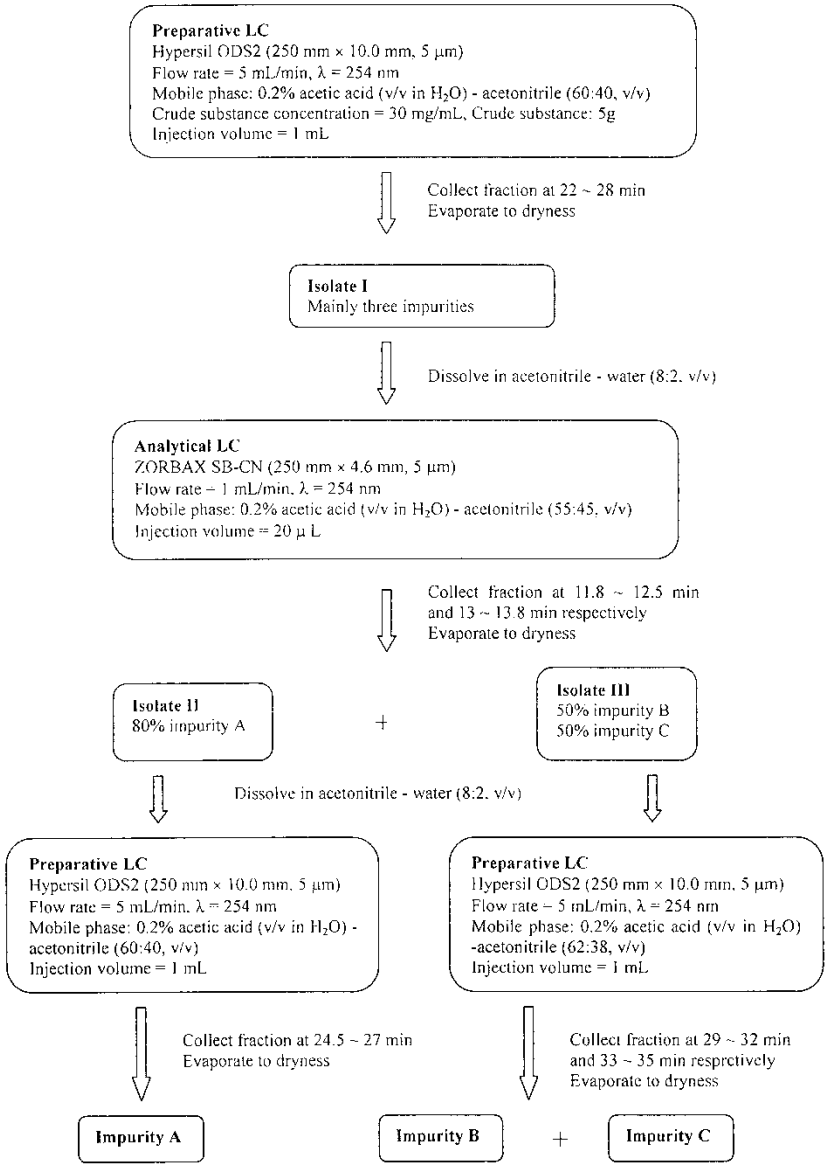


Figure 2. Preparative chromatogram for the isolation of impurities and valsartan. The mobile phase consisted of 0.2% acetic acid (v/v in H_2O)—acetonitrile (60 : 40, v/v). For other chromatographic conditions, see the Experimental section.

and NMR to offer background information to characterize the structures of the impurities. The solvents were eliminated by rotary evaporation at 50°C, under vacuum, to obtain isolate I and refined valsartan. The isolation of three impurities by a combination of preparative and analytical LC is illustrated in Scheme 1.



Scheme 1. Preparative separation schematic of crude valsartan.

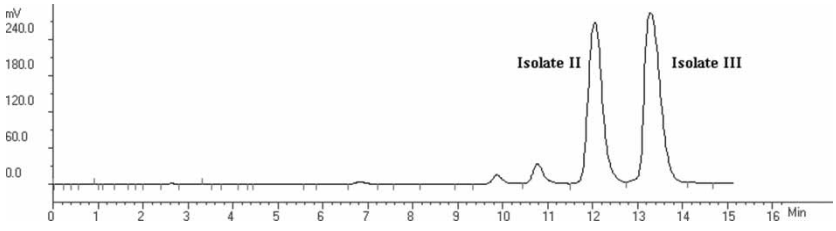


Figure 3. A typical chromatographic profile for the isolation of Isolate I by the CN column. For chromatographic conditions, see the Experimental section.

The isolate I was further separated by a CN column, as shown in Figure 3, and the baseline separation of two peaks was achieved with the CN column. The CN column exhibited quite different selectivity from that of the C_{18} columns, which was probably due to the special dipole-dipole interaction between CN stationary phase and analytes.[16] Two fractions, isolate II and III, were collected, respectively, for the following experiments.

Isolate II was further purified through preparative runs, and impurity A was finally obtained. The preparative chromatogram for the purification of impurity A is presented in Figure 4.

Two peaks were detected when isolate III was further separated with an ODS column. Two impurities, B and C, were obtained, finally. The preparative chromatogram for the isolation of impurities B and C is presented in Figure 5.

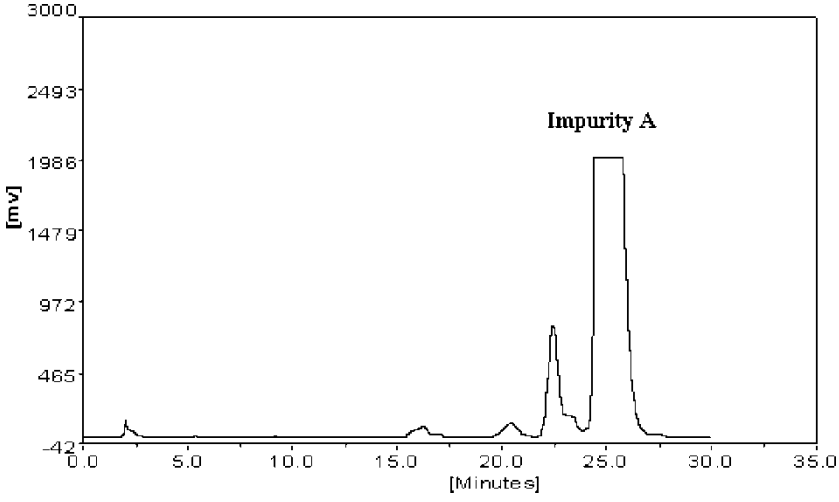


Figure 4. Preparative chromatogram for the purification of impurity A. For chromatographic conditions, see the Experimental section.

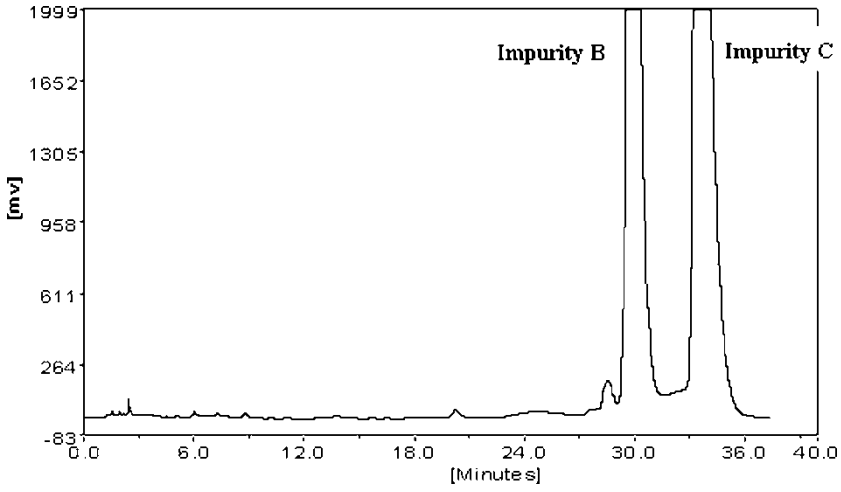


Figure 5. Preparative chromatogram for the isolation of impurities B and C. For chromatographic conditions, see the Experimental section.

Basic Degradation Study of Impurity A

When impurity A was dissolved in methanol in a basic condition (pH 11) and placed at room temperature over 24 hr, the compound of valsartan appeared at a level of approximately 80% by LC analysis. This information suggested that the impurity and valsartan may be structurally similar.

Mass Spectrometry

Portions of valsartan and impurities A, B, and C were analyzed by electrospray ionization (ESI) MS, respectively. The negative ion ESI spectra of valsartan and impurity A are presented in Figure 6. The negative ion spectrum of impurity A (Figure 6B) exhibits a [M-1] molecular ion peak at m/z 448 which is 14 mass units more than that of valsartan (Figure 6A). Considering the similar structures of impurity A and valsartan, the increase of 14 mass units suggested a methyl had replaced the hydrogen on the carboxyl or the tetrazole ring.

To obtain additional structural information, MS/MS analysis was further performed. The MS/MS spectrum of valsartan (Figure 7A) exhibited a series of product ions, m/z 116, 179, 192, 235, 304, 350, 391, while the spectrum of impurity A exhibited a series of product ions, m/z $116 + 14$, 179, 192, $192 + 14$, 235, $235 + 14$, 304, $304 + 14$, $350 + 14$ (Figure 7B). These indicated the two compounds were very similar structurally.

A key step in elucidating the impurity structure is to understand the fragmentation patterns of valsartan and the impurity A. The fragmentation

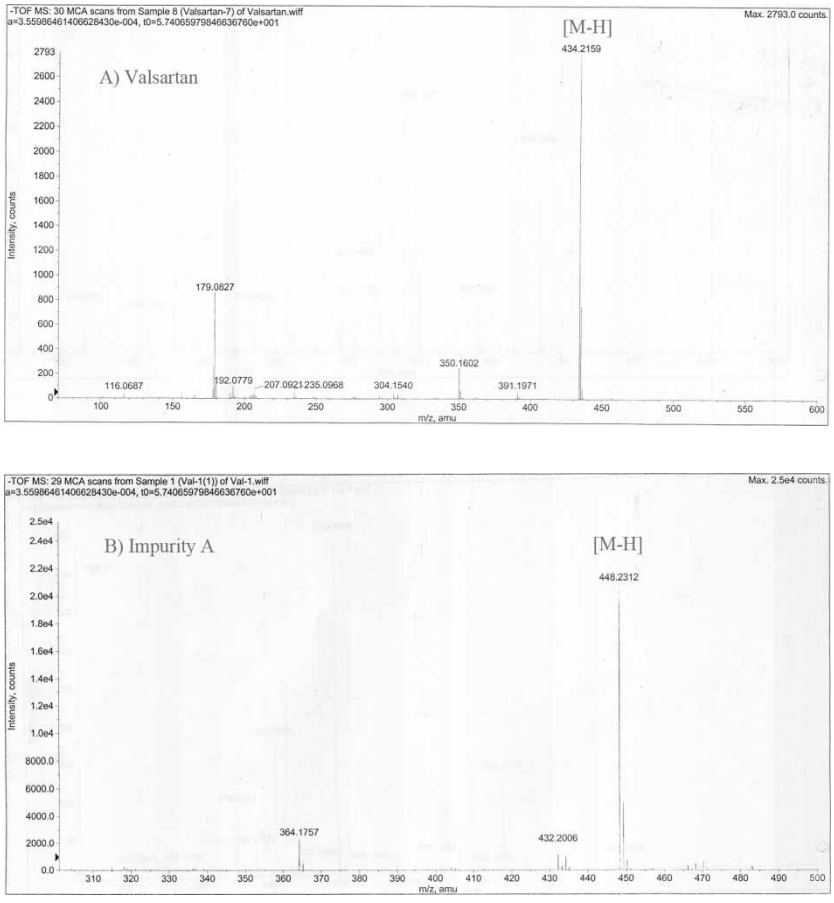


Figure 6. ESI mass spectra of (A) valsartan and (B) impurity A.

pattern of valsartan was similar to that of losartan.[17] The proposed fragmentation of valsartan and impurity A are presented in Figure 8. Cleavage on the bond of the acyl nitrogen and the bridging methylene group of valsartan resulted in the product ion at m/z 235, [CH_2 ($C_{12}H_8$) (CN_4H)]. The product ions of impurity A at m/z 235 and 249 suggested a methyl had replaced the hydrogen on the tetrazole ring rather than the one on the carboxyl, which was confirmed by the simultaneous existence of product ions at m/z 192 and $192 + 14$.

NMR Spectroscopy

The ^{1}H-NMR and ^{13}C-NMR spectra of valsartan and impurity A are presented in Figures 9A, 10A, 9B, and 10B, respectively. Full NMR characterization of

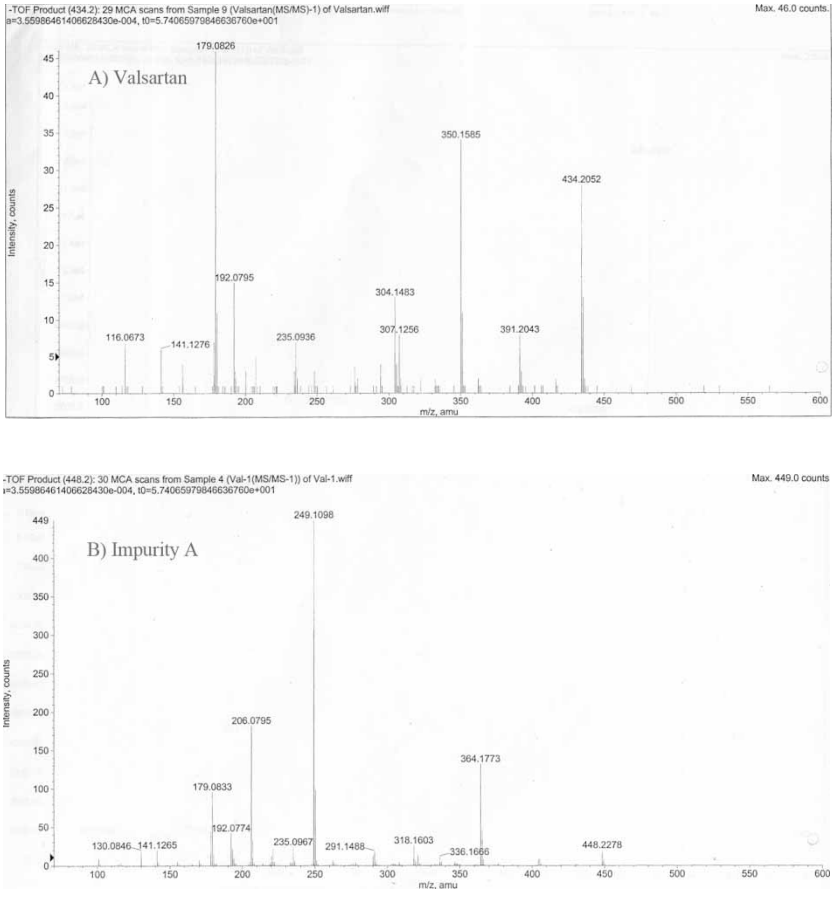


Figure 7. ESI MS/MS of (A) valsartan and (B) impurity A.

valsartan was used as background information. The ^{1}H spectra of impurity A and valsartan were found to be almost identical, except for the presence of a methyl singlet at 3.36 ppm in the spectrum of impurity A.

As for the carbon resonance assignments, the only difference was an obvious increase in the signal intensity at 34.3 ppm in the spectrum of impurity A, implying the methyl carbon and 2-C had same resonance at 34.3 ppm. According to the proton and carbon spectral simulations, chemical shifts of the protons on the ester methyl and tetrazole methyl were similar, but the chemical shifts of carbons on the ester and the tetrazole methyl were 51.9 ppm and 29.7 ppm, respectively. So, the signal at 34.3 ppm was undoubtedly assigned to the tetrazole methyl.

Because of the existence of carbonyl, the carbon–nitrogen single bond behaved as double bonds to a certain degree, which led to spin hindrance.

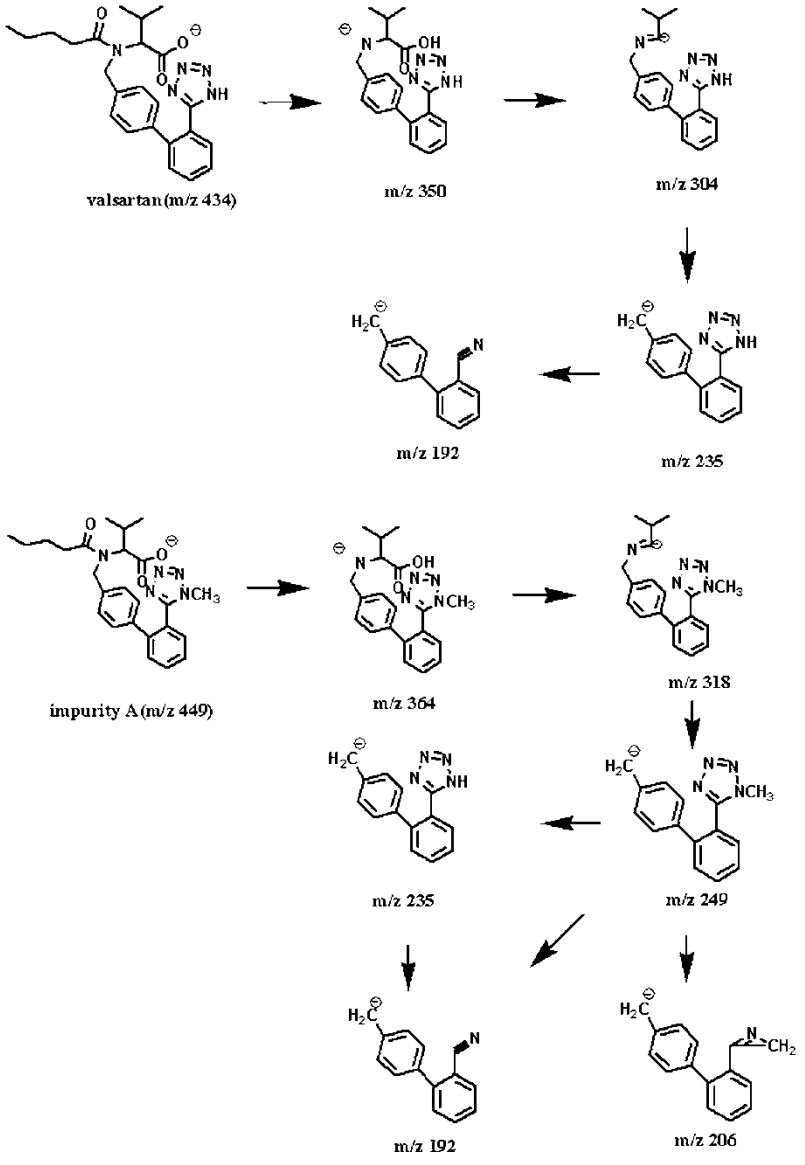


Figure 8. Proposed fragmentation of valsartan and impurity A.

The paired resonances during the NMR examination could be explained by the influence of a conformational isomer caused by spin hindrance of acyl amide. Furthermore, the chiral carbon could influence 2-H and 11-H on the methylenes, and cause their magnetic inequivalence. Proton and carbon resonance assignments are given in Tables 1 and 2, respectively.

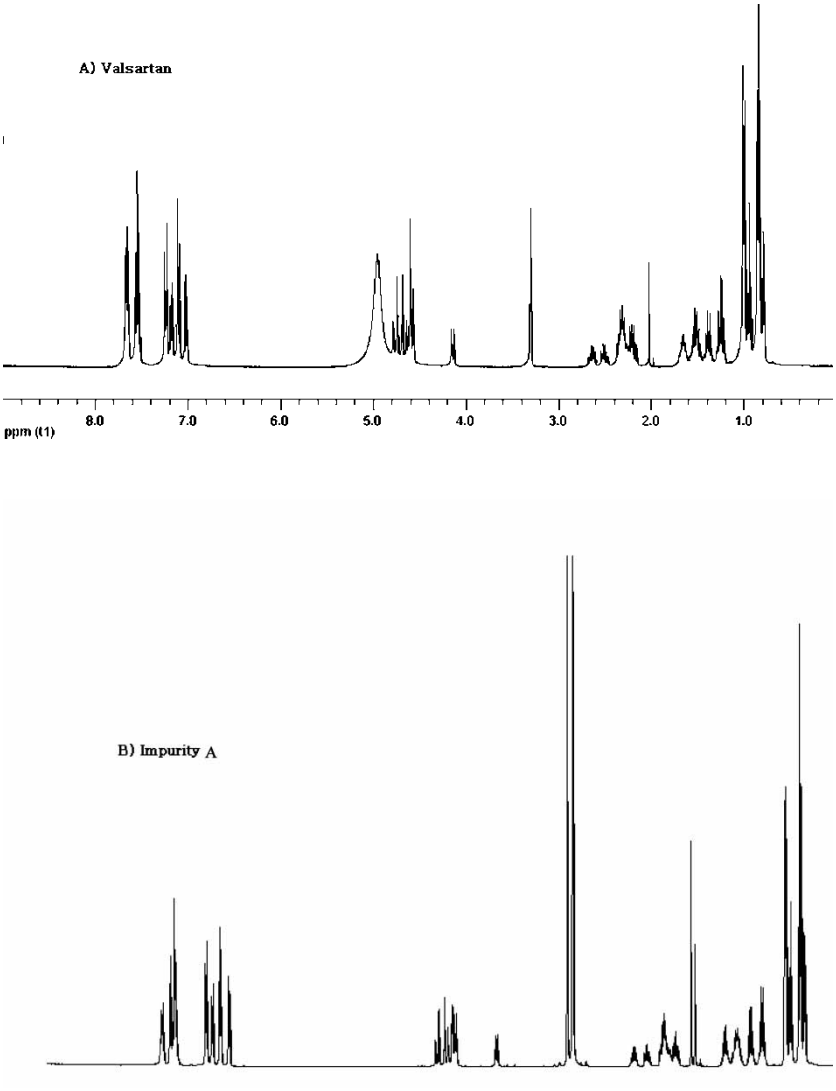


Figure 9. ^{1}H NMR spectra of (A) valsartan and (B) impurity A.

Based on the above data, impurity A was identified as (*S*)-*N*-valeryl-*N*-{[2′-(1-methyl-tetrazol-5-yl) biphenyl-4-yl]-methyl}-valine. The impurity A was deduced to form during the hydrolysis of the methylester in the last step of crude synthesis. The methyl may attack the nitrogen on the tetrazole acid isostere to form impurity A.

The structures of the other two impurities are in the process of identification.

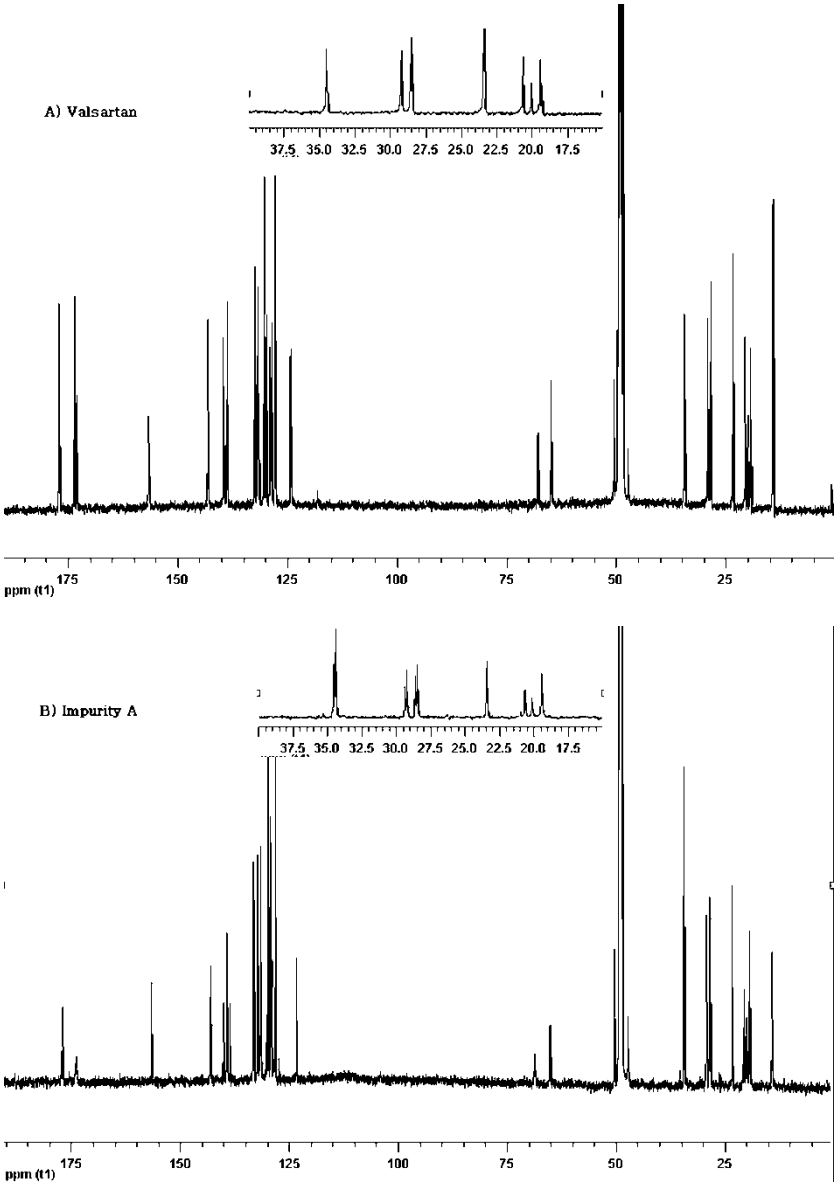


Figure 10. ^{13}C NMR spectra of (A) valsartan and (B) impurity A.

CONCLUSIONS

A combination of analytical and preparative LC was employed successfully to isolate three impurities in crude valsartan, which has not been previously

Table 1. NMR proton chemical shift data and assignments for valsartan and impurity A

Proton	Valsartan	Impurity A
5-H(CH_3,t)	0.78–0.83(0.93–0.96)	0.78–0.83(0.93–0.98)
4-H(CH_2,m)	1.22–1.28(1.35–1.41)	1.22–1.31(1.34–1.44)
3-H(CH_2,m)	1.49–1.55(1.64–1.67)	1.47–1.59(1.60–1.72)
2-H(CH_2,m)	2.28–2.35(2.19–2.23)	2.24–2.41(2.58–2.72)
6-H(CH,d)	4.57–4.59(4.13–4.15)	4.54–4.62(4.03–4.14)
7-H(CH,m)	2.46–2.56(2.59–2.69)	2.41–2.58(2.58–2.72)
8,9-H(CH_3,dd)	0.83–0.86(0.99–1.01)	0.82–0.91(0.98–1.07)
11-H(CH_2,AB coupling)	4.64–4.78	4.62–4.83
Aromatic proton(A&B ring)	7.01–7.25(7.52–7.69)	6.98–7.32(7.53–7.80)
NH(C ring)	4.60	
N–CH3(S)		3.36(3.37)

Table 2. NMR carbon chemical shift data and assignments for valsartan and impurity A

Carbon	Valsartan	Impurity A
5-C	14.2	14.2
4-C	23.3(23.4)	23.4
3-C	29.2	29.3(29.2)
2-C	34.5(34.4)	34.5(34.3)
1-C	176.9	176.9
6-C	67.9	68.6
7-C	28.4(28.5)	28.6
8,9-C	19.4(19.2), 20.6(20.0)	19.4, 20.5
10-C	177.1	177.1
11-C	64.9	65.1
Aromatic ring(A&B ring)	124.1–156.7	123.2–156.6
C═N(C ring)	173.6(172.9)	173.9
N–CH3		34.3

reported. The C_{18} and CN stationary phases exhibited complementary selectivity for the separation of the impurities. A degradation study of the impurity provided very valuable information concerning the structure of the impurity A. Mass spectrometry and nuclear magnetic resonance spectroscopy were the ultimate tools in the structural elucidation. The other two impurities need further characterization.

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