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Preliminary Communication

Separation, Identification and Structural Elucidation of a New Impurity in the Drug Substance of Amlodipine Maleate Using LC-MS/MS, NMR and IR

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Abstract. Amlodipine maleate is a maleate salt of 3-ethyl 5-methyl 2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate. An unknown impurity at m/z 392.2 for $[M+H]^+$ ion has been detected during the accelerated stability analysis (40 °C/75 % RH) of amlodipine maleate drug substance by reverse-phase high performance liquid chromatography–mass spectrometry (RP-HPLC-MS). MS and MS/MS spectra of amlodipine maleate and unknown impurity are obtained using HPLC-MS/MS equipped with positive electrospray ionization (ESI). The nuclear magnetic resonance (NMR) and infrared (IR) spectra of the unknown impurity are recorded after isolation of the impurity by preparative HPLC. Based on MS, NMR and IR spectral data, the structure of the unknown impurity was proposed as 5-ethyl-7-methyl-6-(2-chlorophenyl)-8-methyl-3,4,6,7-tetrahydro-2*H*-1,4-benzoxazine-5,7-dicarboxylate.

Keywords: amlodipine maleate, new impurity, separation and identification, structural elucidation, HPLC-MS/MS, NMR and IR studies

INTRODUCTION

Amlodipine maleate is an anti-depressant drug, classified as a long-acting dihydropyridine type calcium channel blocker with chemical name, 2-[(2-aminoethoxy) methyl]-4-(2-chloro phenyl)-3-ethoxy carbonyl-5-methoxy carbonyl-6-methyl-1,4-dihydro pyridine benzene sulphonate,[1,2] (Figure 1). This drug blocks the calcium entry into the muscle cells, causes the blood vessel to relax and then lowering the blood pressure. So, it is used in the management of hypertension, chronic stable angina pectoris and Prinzmetal's variant.[3] It inhibits the trans-membrane influx of calcium ions into vascular smooth muscle and cardiac muscle.[4–6] It has long elimination half life and large volume of distribution. The synthesis of amlodipine maleate in consideration is based on Hantzch cyclization to substituted dihydropyridine ring with triphenylmethyl protected amino group. This protecting group is easily removed by excess of benzene sulphonic acid in an appropriate solvent to form amlodipine benzensulphonate.[7]

All drugs contain impurities but investigation of impurity profile of an active pharmaceutical ingredient is of crucial importance for medical safety reasons. The impurities formed typically at higher levels are named as primary impurities and considered by regulatory authorities.[8,9] The secondary impurities usually formed at very low levels such as ppm or ppb are more important for pharmaceutical and forensic areas. In general, drug impurities in excess of 0.1 % should be identified and quantified by selective methods.[8,10,11] The development of selective methods for the determination of impurities is one of the most important fields of analysis in pharmaceutical industry. Therefore, much effort is necessary to focus on minimizing the impurities during the

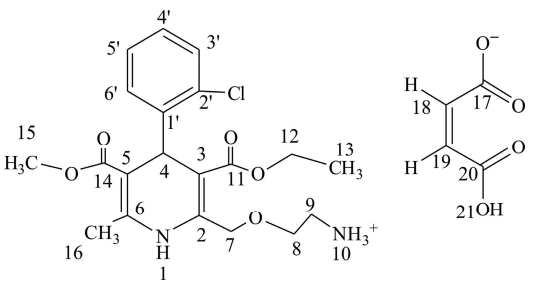

Figure 1. Structure of amlodipine maleate.

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development of synthetic technologies. To achieve this, identification and origin of each impurity is essential.

In the literature, several articles have been reported on quantitative determination of amlodipine maleate using different analytical techniques including, spectrophotometry,[12] high performance liquid chromatography (HPLC),[13] gas chromatography GC,[14] GC-mass spectrometry (MS)[15], LC-MS[16–18] and LC-nuclear magnetic resonance (NMR) spectroscopy.[17–19] But the articles dealing with the identification and structural characterization of the impurities are scarce. One of the articles dealing with the sources of an impurity, 4-(2-chlorophenyl)-3-ethoxycarbonyl-5-methoxycarbonyl-6-methyl-2-[(2-phthalimidoethoxy)methyl]-1,4-dihydropyridine formed during synthesis of amlodipine besylate has been reported.[20] Sudhakar *et al*. reported the identification and characterization of potential impurities in the bulk drug of amlodipine maleate using LC–MS.[21]

The present study describes the identification of a new impurity formed during the stability analysis of amlodipine maleate drug substance at accelerated conditions (40 °C, 75 % RH, as per ICH guidelines)[22] by reversed-phase HPLC–MS. The impurity was isolated by preparative HPLC and then characterized by MS, NMR and IR spectra to elucidate its structure.

EXPERIMENTAL

Materials

Amlodipine maleate samples and known impurities (isopropyl ester and oxazine) were supplied by Neuland Laboratories (Hyderabad, India). HPLC grade acetonitrile (ACN) was obtained from Merck Co (Darmstadt, Germany) and formic acid was purchased from Sigma-Aldrich (Steinheim, Germany). Deionized water was purified by using a Milli-Q purification system (Millipore, Bedford, USA).

Sample Preparation

A sample solution of amlodipine maleate was prepared at a mass concentration, $\gamma = 0.1$ mg mL^{-1} in 50 : 50 mixture of aqueous formic acid (pH = 3.0) and ACN.

HPLC-MS

Analyses were performed using a HPLC system consists of an Agilent 1100 series Vacuum degasser, G1311A Quaternary pump, G1329A Auto sampler with column oven and G1316A PDA Detector. Separation was achieved on C18 150 mm × 4.6 mm, 3 µm column (Alltech Altima) at the flow rate of 1.5 mL min^{-1}. Column temperature was 35 °C. The mobile phase consists of A (50 %) and B (50 %); A was aqueous formic acid (pH = 3.0) and B was pure ACN. The total run time was 30 min. The injection volume was set to 20 µL.

MS and MS/MS experiments were carried out with an API 3000 LC-MS/MS (Applied Biosystems instrument, Concord, Ontario, Canada) interfaced with an ESI (electrospray ionization) ion source. ESI source parameters were: vaporizer temperature, 325 °C; nebulizer gas at 12 bar; curtain gas at 11 bar; and ion spray voltage at 3500 V. Nitrogen was used as collision gas and optimized to 35 eV. The ESI source was operated in the positive ion mode. The data was acquired using Analyst software.

Preparative HPLC Conditions

A Shimadzu LC-8A preparative HPLC equipped with SPD-10A VP UV-Vis detector (Shimadzu, Kyoto, Japan) was used for the isolation of the unknown impurity. Intersil ODS (i.d 250 mm × 20 mm) preparative column packed with 8 µm particle size was used. The flow rate was 30 mL min^{-1} and the detection was carried out at 220 nm. Pump mode was isocratic and mobile phase used was a mixture of buffer (aqueous formic acid (pH = 3.0) and ACN in the ratio of 50 : 50.

NMR Spectroscopy

The ^{1}H and ^{13}C NMR experiments were carried out on a Bruker AVANCE-300 (Bruker Ag Industries, Faellanden, Switzerland). The instrument was equipped with Z-gradient coil at 298 K. For ^{1}H NMR experiments, the sample solution was prepared by dissolving 7 mg of isolated unknown impurity in 1.0 mL of $CDCl_3$. ^{13}C NMR experiments were performed by dissolving 35 mg of sample in 1 mL of $CDCl_3$. Data was collected and processed by XWIN-NMR software. The ^{13}C NMR and DEPT experiments were performed with a spectral width of 16.500 kHz using 64 k data points. The two dimensional experiments were performed using Bruker standard pulse sequences and parameters. ^{1}H and ^{13}C chemical shifts were reported on the δ scale in ppm, relative to tetramethylsilane (δ 0.00) and $CDCl_3$ (δ 77.0), as internal standards, respectively.

FT-IR Spectroscopy

The FT-IR spectrum of the unknown impurity was recorded using Perkin-Elmer instrument, model-spectrum one FT-IR Spectrophotometer (Beaconsfield, UK). Taken dried KBr powder into a mortar and added 1 % of the unknown impurity sample, mixed and grinded to get a fine powder. A small portion of this sample was made as a pellet with the help of quick press KBr pellet kit (International Crystal Laboratories, Garfield, NJ 07026). The sample pellet was kept into the sample holder to obtain the IR spectrum

RESULTS AND DISCUSSION

Separation and Identification of Unknown Impurity

The amlodipine maleate samples were provided by Neuland laboratories. In their synthetic process two potential impurities which commonly occur in the drug substance were isopropyl ester impurity and oxazine impurity. However, in the present work, initially the amlodipine maleate drug substance was analyzed by reversed-phase HPLC with MS detection according to the HPLC-MS conditions mentioned in the experimental section. At the retention time (RT) of 7.15, a peak was detected and confirmed to be amlodipine. The RTs of the known impurities, isopropyl ester and oxazine were 9.6 and 17.2 min, respectively and those were not detected in the sample of amlodipine maleate drug substance. An unknown peak was detected at the RT of 18.34 min and was suspected as an unknown impurity with molecular mass 391 Da. The HPLC-MS chromatogram of the amlodipine maleate drug substance was shown in Figure 2a which presents the total ion count (TIC). Figure 2b presents the ESI-MS spectrum of the unknown impurity at RT of 18.34 min, shows the precursor ion peak at m/z 392.2 $[M+H]^+$. For further studies, the unknown impurity was isolated.

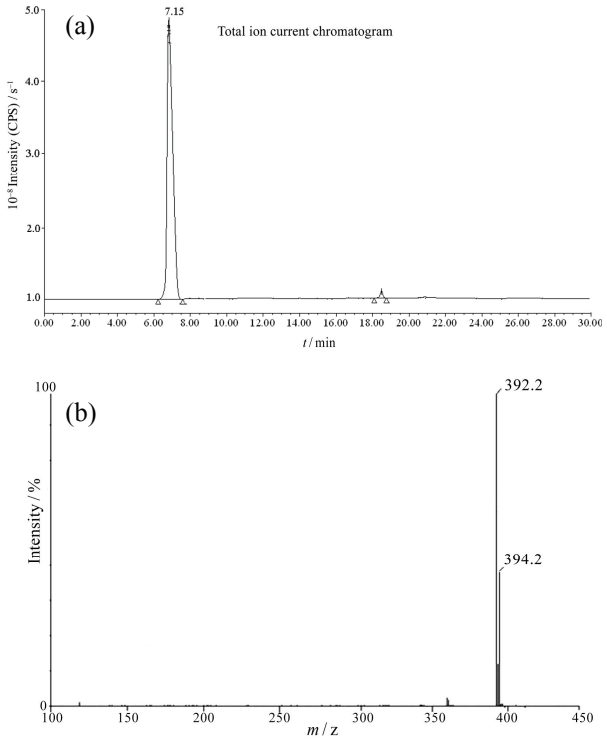


Figure 2. HPLC-MS chromatogram of amlodipine maleate drug substance sample (TIC) (a) and ESI-MS spectrum of the unknown impurity (b).

Isolation of the Unknown Impurity

To increase the amount of unknown impurity the amlodipine maleate was exposed to 105 °C for 3 days. The unknown impurity was then isolated from the crude sample by preparative HPLC conditions given in the experimental section. The unknown impurity was detected at the retention time of 7.65 min, the corresponding preparative HPLC chromatogram shown in Figure 3 and this unknown impurity peak was confirmed by MS. The collected fractions having > 95 % of the unknown impurity were polled together, concentrated on rotavapor under vacuum to remove solvent. The obtained unknown impurity was in off-white color having chromatographic purity of 96.5 %. The purity of the unknown impurity was obtained after analyzing the pure impurity by HPLC using the LC conditions as mentioned above. The purity was calculated by the following formula:

Purity of the impurity = 100 – (% of impurities found in HPLC + weight loss observed in TGA)

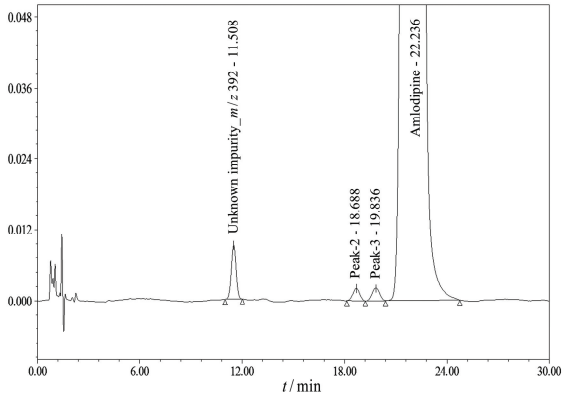
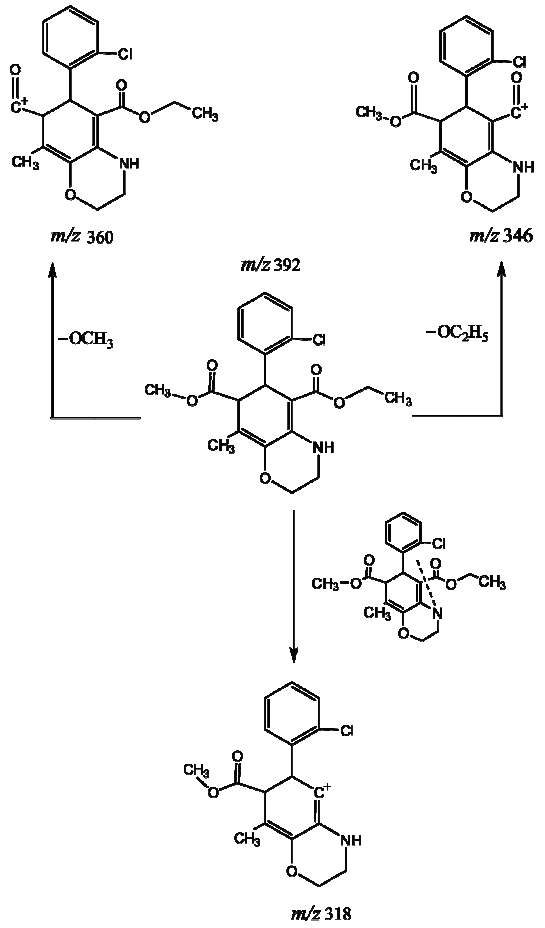


Figure 3. Preparative HPLC chromatogram for the unknown impurity.

Structure Elucidation of the Unknown Impurity

Using HPLC-MS/MS in product ion mode, the MS/MS spectrum of the unknown impurity in amlodipine maleate drug substance eluting at retention time 18.34 min was recorded and presented in Figure 4a. The MS/MS spectrum of the unknown impurity (Figure 4a) shows a precursor ion peak at m/z 392.2 $[M+H]^+$ indicating the molecular weight of the compound is 391. The MS/MS fragments of the unknown impurity were detected at m/z 360.2, 346.5, 318.1 and 286.2. The MS fragmentation pathways of amlodipine have been described by previous studies. [23–25] The MS/MS fragments of the unknown impurity were different from the fragments of amlodipine (MS/MS spectrum of the amlodipine was shown in Figure 4b), indicating that the decomposition pathway of the unknown impurity was different from the amlodipine. The MS data including precursor and product ions of amlodipine and unknown

impurity was presented in Table 1. The MS/MS fragments of the unknown impurity at m/z 360.2 and 346.5 were due to loss of methoxy and ethoxy moieties, respectively. The fragment at m/z 318.6 was due to the loss of ethyl ester moiety and the fragmentation pathway of the unknown impurity was presented in Scheme 1. The MS spectrum of the unknown impurity shows the characteristic $^{35/37}$Cl isotopic pattern indicating that the presence of one chlorine atom in its structure.



Scheme 1. The proposed fragmentation pathway of the unknown impurity.

The ^{1}H and ^{13}C NMR assignments of amlodipine maleate and its unknown impurity were presented in Table 2. In the ^{1}H NMR of the unknown impurity, no signal was observed at 6.01 ppm, indicating the absence of maleate salt. The proton signals at the positions of H-8 and H-9 were observed between 3.5 and 4.0 ppm in

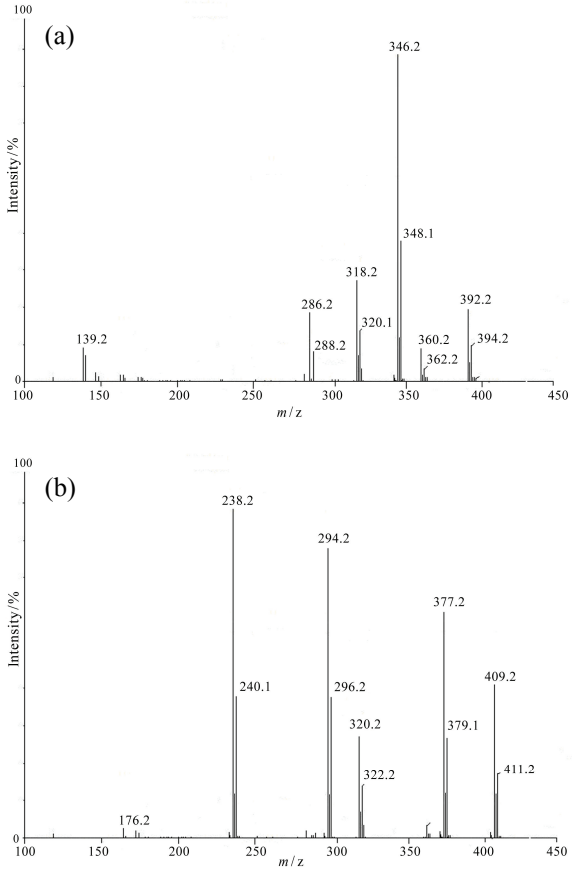


Figure 4. MS/MS spectra of unknown impurity (a) and amlodipine (b).

the unknown impurity, but in the amlodipine maleate those protons were detected between 2.8 and 3.4 ppm and also one exchangeable proton signal was found at 9.13 ppm. The methyl protons in the unknown impurity were detected at the position of H-15 (1.54 ppm) but in amlodipine maleate those protons were observed at H-16 (2.32 ppm), suggesting that shielded proton in the unknown impurity at that position. In the NOE experiment, the intensity enhancement was observed for the signal at 3.05 ppm (H-5) on irradiation of H-15 proton (1.54 ppm) in the unknown impurity. This observation has led to confirm the formation of a ring.

In the ^{13}C NMR study of amlodipine, the signals for the carbons at the positions, C-5, 6, 7, 8 and 9 were observed at 104.5, 144.1, 72.8, 68.3 and 40.5 ppm, respectively, where as in the unknown impurity, C-5, 6, 7, 8, and 9 were found at 50.5, 113.5, 142.3, 38.5 and 63.5 ppm, respectively. The HSQC experimental showed a

Table 1. MS data of precursor and product ions

Compound	LC-MS, RT / min	Precursor ion, m/z	Fragment ions, m/z
Amlodipine maleate	7.15	409 $[M+H]^+$	377, 320, 294 and 238
Unknown impurity	18.34	392 $[M+H]^+$	360, 346, 318 and 286

cross peak for the signal at 3.05 ppm. Furthermore, a HMBC cross peak connected the signal at 3.05 ppm to the C-6 and C-13 (Figure 5). The extra quaternary carbon was assigned to C-7 due to a correlation with H-15 in HMBC and the key correlations were observed in the HMBC spectrum (Figure 5).

Table 2. NMR spectral data of amlodipine maleate (AM) and its unknown impurity (UI)

Position	^{1}H		^{1}H NMR δ/ppm (multiplicity[(a)], J/Hz)		^{13}C		DEPT	
	AM	UI	AM	UI	AM	UI	AM	UI
1	NH	NH	7.82(s)	9.13 (s,br)	–	–	–	–
2	–	–	–	–	144.3	145.7	–	–
3	–	–	–	–	101.6	85.6	–	–
4	1H	1H	5.39(s)	5.12 (d,1.1)	38.2	35.4	CH	CH
5	–	1H	–	3.05 (d,1.0)	104.5	50.5	–	CH
6	–	–	–	–	144.1	113.5	–	–
7	2H	–	4.71(d,15.5) 4.82(d,15.5)	–	72.8	142.3	CH_2	–
8	2H	2H	3.41(m)	4.12, 4.02 (m,m)	68.3	38.5	CH_2	CH_2
9	2H	2H	2.88(t,5.1)	3.41,3.57 (m,m)	40.5	63.5	CH_2	CH_2
10	NH	–	8.35(s)	–	–	168.7	–	–
11	–	2H	–	3.54 (m)	167.5	58.7	–	CH_2
12	2H	3H	4.02(m)	1.10 (t,7.2)	58.4	13.8	CH_2	CH_3
13	3H	–	1.16(t,7.2)	–	14.5	171.5	CH_3	–
14	–	3H	–	3.31 (s)	166.8	51.5	–	CH_3
15	3H	3H	3.61(s)	1.54 (s)	50.2	16.8	CH_3	CH_3
16	3H	–	2.32(s)	–	18.6	–	CH_3	–
17	–	–	–	–	167.9	–	–	–
18	1H	–	6.01(s)	–	134.5	–	CH	–
19	1H	–	6.01(s)	–	134.5	–	CH	–
20	–	–	–	–	167.9	–	–	–
21	OH	–	–	–	–	–	–	–
1’	–	–	–	–	143.2	138.7	–	–
2’	–	–	–	–	131.4	135.8	–	–
3’	1H	–	7.20(dd,1.3,7.8)	–	129.8	129.8	CH	–
4’	1H	–	7.02(td,7.3)	–	126.4	126.4	CH	–
5’	1H	–	7.11(td,7.5)	–	126.0	125.4	CH	–
6’	1H	–	7.35(dd,1.5,7.5)	–	130.4	128.4	CH	–

[(a)] multiplicity of signals: s=singlet; d=doublet; t=triplet; m=multiplet; br=broad; dd=doublet of a doublet, td=triplet of doublets.

Table 2. NMR spectral data of amlodipine maleate (AM) and its unknown impurity (UI)

$\tilde{\nu}$/cm^{-1}		Assignment		Mode of Vibration	
AM	UI	AM	UI	AM	UI
3328	3275	N–H/O–H	N–H	Stretching	Stretching
1696,1683	3052	C=O	Aromatic C–H	Stretching	Stretching
1644,1604	2986,2951	C=C/C=N	Aliphatic C–H	Stretching	Stretching
1484,1435	1730	Aliphatic C–H	C=O	Bending	Stretching
1287	1673,1647, 1585	S=O	C=C	Asymmetric Stretching	Stretching
1212	1448,1431	O=C–O of ester	Aliphatic C–H	Stretching	Bending
1187	1287	O–C–C of ester	C–N	Stretching	Stretching
1105	1248	S=O	C–O–C	Symmetric Stretching	Asymmetric Stretching
755,735	1174	Aromatic C–H	O–C–C of ester	Bending Stretching	Stretching
622	1043	C–S	C–O–C		Symmetric Stretching
	783,759		Aromatic C–H		Bending

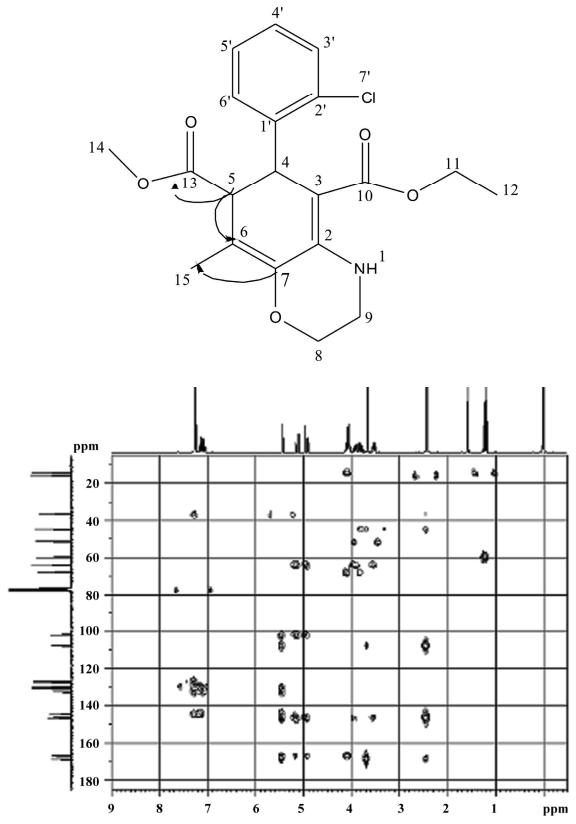


Figure 5. HMBC spectrum of the unknown impurity.

The FT-IR spectral assignments of the amlodipine maleate and the unknown impurity were given in Table 3. In the FT-IR spectrum of the unknown impurity, the band at 3275 cm^{-1} indicates the presence of the N-H group and was appeared as a sharp signal. The carbonyl group (C=O) stretching vibration of unknown impurity was observed at 1730 cm^{-1} and the same was detected at 1696 and 1683 cm^{-1} in amlodipine maleate. The other characteristic frequencies observed were correlated with the structural moieties of the unknown impurity mentioned in Table 3.

According to the NMR and IR spectra of the unknown impurity, it was found that the unknown impurity did not have either maleate salt or amino ethoxy group of the amlodipine maleate, which has undergone cyclization to form six-membered ring. Finally, by using NMR and IR measurements and MS fragmentation pattern, the unknown impurity was confirmed as 5-ethyl-7-methyl-6-(2-chlorophenyl)-8-methyl-3,4,6,7-tetrahydro-2*H*-1,4-benzoxazine-5,7-dicarboxylate (Figure 5).

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SAŽETAK

Odvajanje, identifikacija i strukturna karakterizacija novog onečišćenja u lijeku amlodipin maleatu pomoću metoda LC-MS/MS, NMR i IR

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Amlodipin maleat je sol 3-etil-5-metil-2-[(2-aminoetoksi)metil]-4-(2-klorfenil)- 6-metil-1,4-dihidropiridin-3,5-dikarboksilata i maleinske kiseline. Pomoću spregnute tehnike tekućinske kromatografije visoke djelotvornosti obrnutih faza i spektrometrija masa (RP-HPLC-MS) detektirano je nepoznato onečišćenje pri *m/z* 392,2 za ion $[M+H]^+$ tijekom analize stabilnosti (40 ^oC / 75 % RH). Spektri MS i MS/MS amlodipin maleata i nepoznatog onečišćenja snimljeni su ionizacijskom tehnikom elektroraspršenja (ESI) u pozitivnom modu. Nakon izolacije onečišćenja pomoću preparativne HPLC metode snimljeni su infracrveni (IR) i spektri nuklearne magnetne rezonancije (NMR). Na temelju analize MS, NMR i IR spektara predloženo je onečišćenje 5-etil-7-metil-6-(2-klorfenil)-8-metil-3,4,6,7-tetrahidro-2*H*-1,4-benzoksazin-5,7-dikarboksilat.