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Journal of Chromatography A, 1116 (2006) 57–68
JOURNAL OF CHROMATOGRAPHY A
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Separation and characterization of silybin, isosilybin, silydianin and silychristin in milk thistle extract by liquid chromatography–electrospray tandem mass spectrometry

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Received 3 January 2006; received in revised form 2 March 2006; accepted 7 March 2006
Available online 24 April 2006

Abstract

A selective and sensitive liquid chromatography/tandem mass spectrometry (LC/MS/MS) method has been developed for the characterization of silymarin in commercially available milk thistle extract. In this study, six main active constituents, including silydianin, silychristin, diastereomers of silybin (silybin A and B) and diastereomers of isosilybin (isosilybin A and B) in silymarin, were completely separated on a YMC ODS-AQ HPLC column using a gradient mobile phase system comprised of ammonium acetate and methanol/water/formic acid. Identification and characterization of the major constituents were based not only on the product ion scan, which provided unique fragmentation information of a selected molecular ion, but also on the specific fragmentation of multiple reaction monitoring (MRM) data, which confirmed the retention times of LC chromatographic peaks. The method was applied in the analysis of human plasma samples in the presence of silymarin and appeared to be suitable for the pharmacokinetic studies in which the discrimination of silymarin constituents is essential.
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Keywords: Silymarin; Milk thistle; Silybin; Isosilybin; Diastereomers; LC/MS/MS

1. Introduction

Silymarin, derived from the milk thistle plant, *Silybum marianum*, has been used for centuries as a natural remedy in the treatment of hepatitis and cirrhosis, as well as in the protection of the liver from toxic substances [1–5]. Recent reports have demonstrated that silymarin also has exceptionally high anti-tumor promoting activity [5–8]. The main bioactive constituents in silymarin are composed of a mixture of flavonolignans, silychristin, silydianin, silybin and isosilybin [9–12]. In nature, silybin and isosilybin have been reported to contain two groups of diastereoisomeric flavonolignans, silybin A and silybin B, and isosilybin A and isosilybin B, respectively [13–15]. The chemical structures of the six main active constituents in *Silybum marianum* are shown in Fig. 1. Among commercial products currently available, silymarin, the standardized extract obtained from the dried fruits of *Silybum marianum* contains approximately 70% to 80% of the silymarin complex and approximately 20% to 30% of a chemically undefined fraction comprised mostly of polymeric and oxidized polyphenolic compounds [12].

The different biological activities have been reported for these six main components in silymarin [16–22]. Moreover, controversy also surrounds the activity of these compounds in animal studies. Many pharmacological studies on silymarin conducted using standardized plant extract fail to identify the manufacturing source of silymarin and quantitate silymarin contents and its individual active components in the extract [12,22–24], making the evaluation of dose-exposure relationships impossible and likewise ensure that dose relationships continue to be poorly-defined. Therefore, there is a pressing need for the development of a sensitive and selective analytical method to characterize and quantify each component of silymarin in commercial products, and furthermore, to quantify these active components in biologic matrices. This is especially true in light of recent requests to define exposure-response relationships for silymarin. Such relationships could be used

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doi:10.1016/j.chroma.2006.03.053

Fig. 1. Chemical structures of the main active constituents in *Silybum marianum*: silychristin, silydianin, diastereomers of silybin (silybin A and B), and diastereomers of isosilybin (isosilybin A and B).

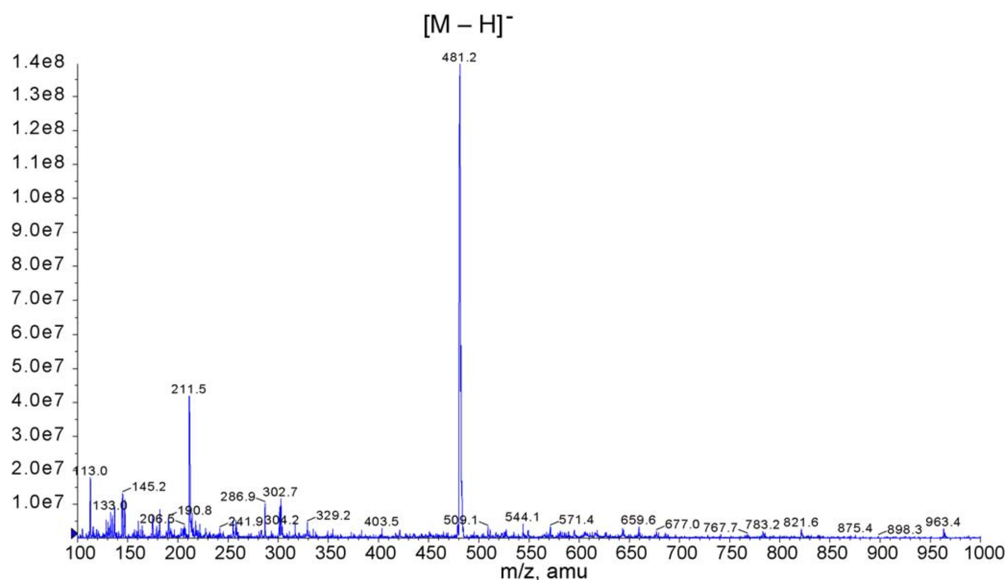

Fig. 2. Q1 full scan mass spectrum of silymarin in a commercial milk thistle seed extract by negative TurboIonSpray ionization.

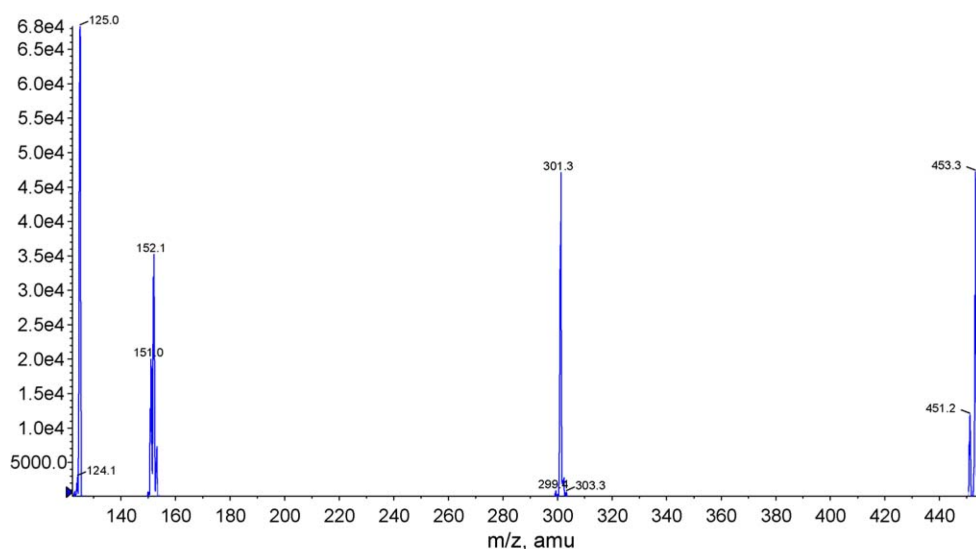

Fig. 3. Product ion spectrum of $[M-H]^-$ at m/z 481 at a collision energy of −42 V for commercial milk thistle standardized extract. Other conditions as described in Section 2.

to design more informative clinical pharmacology trials to finally assess whether the exposure of silymarin constituents can indeed be correlated with clinical outcomes [25].

A number of methods utilizing high-performance liquid chromatography (HPLC) separation with ultraviolet (UV) detection [11,15,26–30], column-switching with electrochemical detection for stereoselective separation of silybin in human plasma [15] or mass spectrometry (MS) detection for quantitation of the six active constituents in silymarin [26,29], and capillary electrophoresis for partial separation of the six constituents in

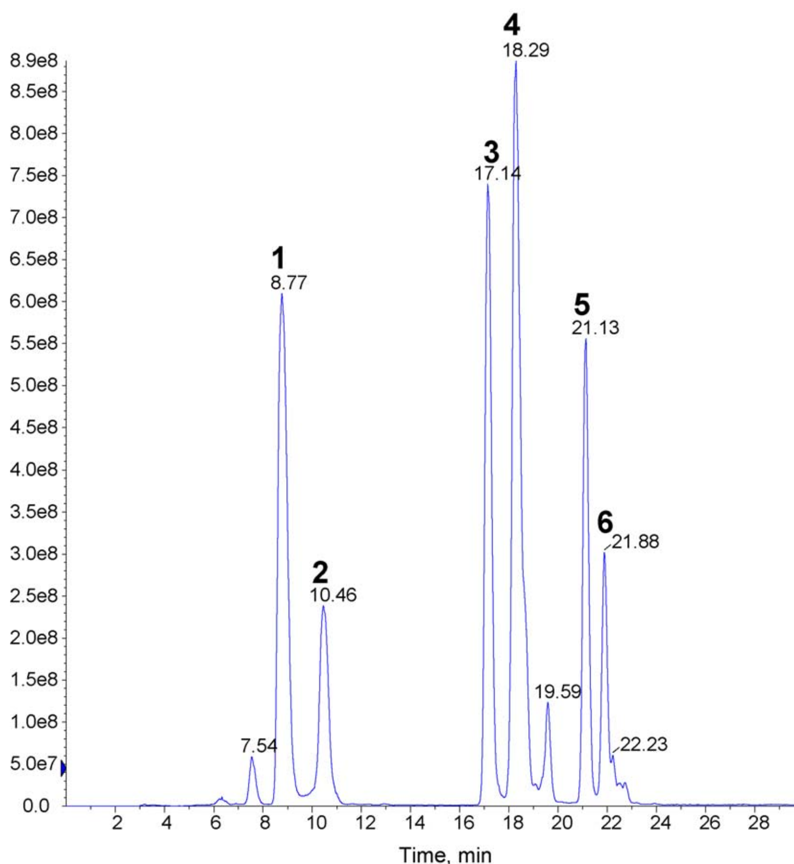

Fig. 4. A representative chromatogram of product ion scan at m/z 481 from the commercial standardized extract. The eluted peaks were tentatively assigned based on retention time (min): peak 1 (8.77), peak 2 (10.46), peak 3 (17.14), peak 4 (18.29), peak 5 (21.13) and peak 6 (21.88).

silymarin [11] have been reported for the determination of the major active constituents in *Silybum marianum*, the majority of which suffer from the incomplete separation of silychristin, silydianin, and the two diastereoisomers of silybin and isosilybin. Only one HPLC method with UV detection, reported by Ding et al. [26], achieved complete separation for the six components; the method used a methanol and solvent mixture (water:dioxane = 9:1) as the mobile phase, and is incompatible with mass spectrometry detection and limited in term of sensitivity by UV detection. Therefore, the development of a specific and sensitive LC/MS/MS method becomes critical to characterize and quantitate all six active components in milk thistle extract.

In another study, Khan et al. reported using electrospray (ESI)–MS/MS to study molecular weights and fragmentation patterns of silymarin after liquid-liquid extraction [31]. Because there was no separation prior to the MS/MS analysis, the fragmentation pattern proposed in the study was from a mixture of all constituents in silymarin instead of individual constituents. The fragmentation pathway of the six individual active components in silymarin has not been reported previously under collision-induced dissociation (CID). The objective of our present study was to develop an LC/MS/MS method which combines the separation potential of the HPLC method and the structural characterization capacity of ESI–MS/MS to identify and characterize the six key bioactive constituents in silymarin.

2. Experimental

2.1. Reagents and materials

Milk thistle dietary supplements standardized extract from Natural Resource Products (Mission Hills, CA, USA) were

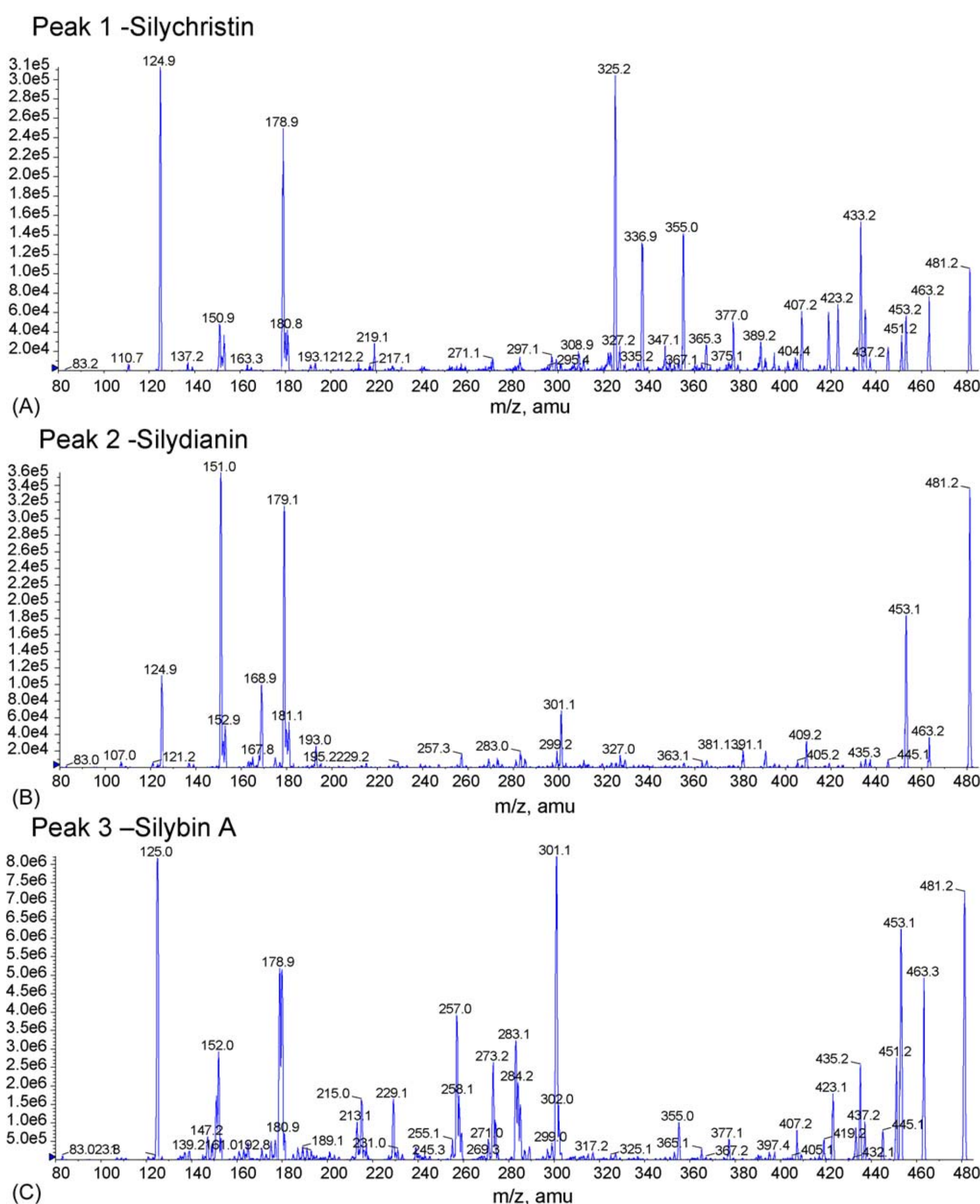

Fig. 5. Product ion spectra of commercial standardized extract with a molecular mass of 482. Peaks 1–6 corresponded to (A) silychristin; (B) silydianin; (C) silybin A; (D) silybin B; (E) isosilybin A; and (F) isosilybin B, respectively.

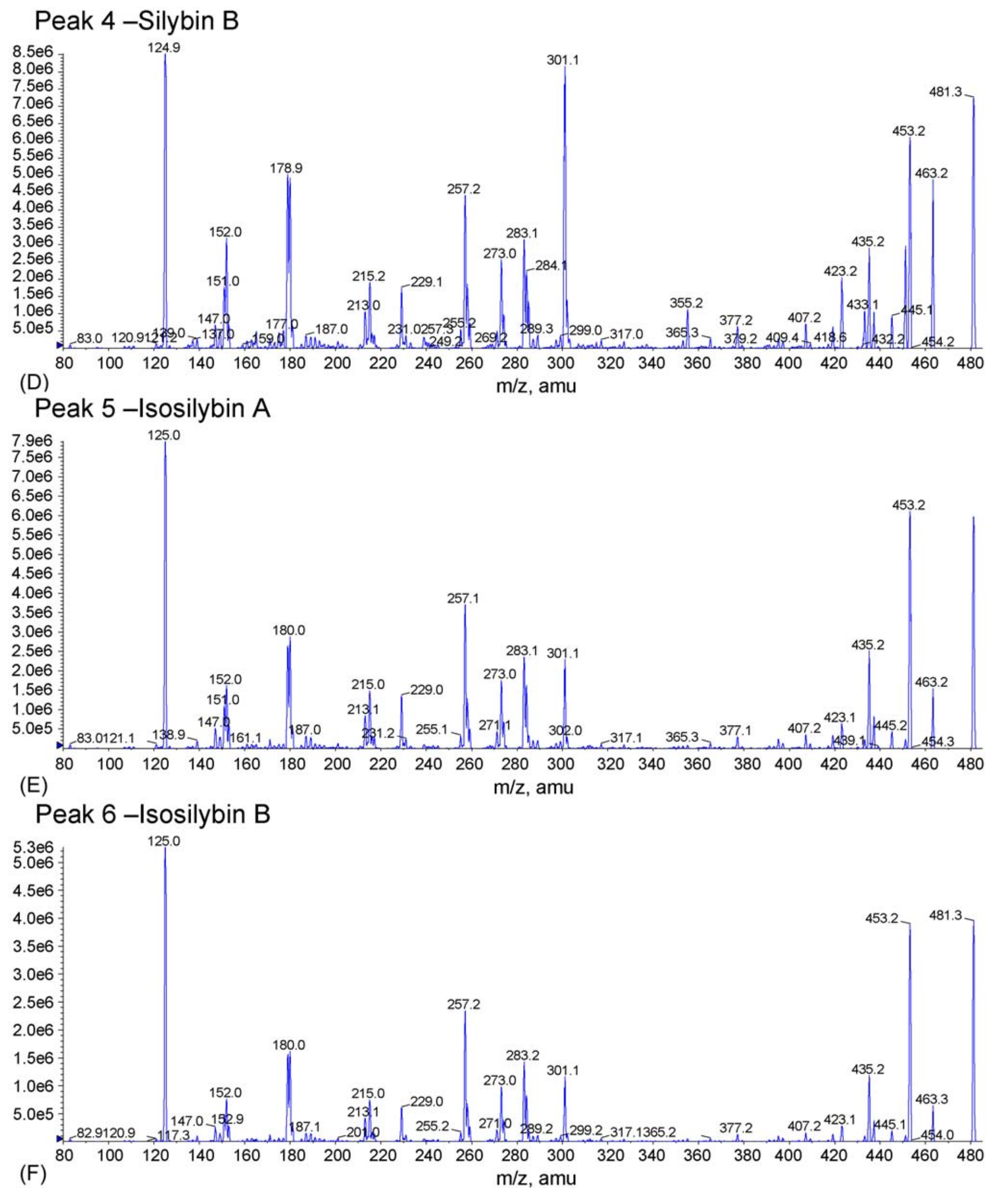

Fig. 5. (*Continued*).

purchased locally in Philadelphia, PA, USA. Silychristin, silydianin and silybin (A and B) were obtained from ChromaDex Inc. (Santa Ana, CA, USA). Silybin was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). All solvents used were of HPLC grade and purchased from Fisher Scientific (Pittsburgh, PA, USA). Reagent grade formic acid (96%), ammonium acetate and ethyl acetate were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). Extraction cartridges (Oasis HLB 1 cc, 30 mg) were obtained from Waters Corp. (Milford, MA, USA). Water was purified by a MilliQ water system (Millipore, Bedford, MA, USA).

2.2. Sample preparation

For structure elucidation, the commercial milk thistle standardized extract (50 mg) was extracted with 4 mL of ethyl acetate by vortexing for 2 h at room temperature in a 13 mm × 100 mm glass tube. After centrifuging at 3000 rpm for 5 min, the upper organic layer was then transferred to LC injection vials. Human plasma was sampled from a healthy volunteer after oral administration of three capsules of milk thistle standardized extract (Natural Resource Products, 140 mg of milk thistle seed extract). Blood was collected after 14 h of interval. The plasma sample was stored at −80 °C prior to LC/MS/MS analysis. For analyzing the samples of human plasma, a 300 μL aliquot of human plasma was mixed with 300 μL of ethyl acetate in a 1.5 mL conical vial. The mixture was vortexed for 1 h and then centrifuged at 3000 rpm for 10 min. Three hundred microliters of the ethyl acetate phase was applied to an Oasis HLB cartridge previously conditioned with 1 mL methanol and 1 mL water. The cartridge was washed with 1 mL of methanol:water (10:90, v/v). The silymarin compounds were eluted with 1 mL methanol and the eluate was evaporated to dryness at 40 °C under nitrogen gas. The residue was reconstituted in 150 μL methanol.

A 2 μL aliquot of the resulting solution was injected onto the LC/MS/MS for analysis.

2.3. Chromatography

HPLC analysis was performed using a Waters 2690 HPLC system with a built-in autosampler (Water Corporation, Milford, MA, USA). HPLC separation was conducted on a YMC ODS-AQ C_{18} column (100 mm × 2.1 mm, 5 μ, 120 Å) (Water Corp., Milford, MA, USA) at a flow rate of 0.2 mL/min using a gradient mobile phase comprised of 5 mM ammonium acetate adjusted to pH 4.0 with formic acid (A) and methanol/water/formic acid (95:5:0.1, v/v/v) (B). The mobile phase was comprised of a 60:40 mixture of component A and B as the initial condition of each chromatographic run, increased to 65% B in a linear gradient over 25 min, and then returned to 40% B for next injection. The column and autosampler were maintained at 40 and 4 °C, respectively. An electronic valve actuator with a Rheodyne selector valve was used to divert the LC flow to waste when no data acquisition was taking place.

2.4. Mass spectrometry

LC/MS/MS analyses were performed on API 4000 tandem mass-spectrometer (Sciex, Toronto, Canada) using an ESI source with negative ion mode and the following conditions: Curtain gas, 10 psi; Gas 1 (nebulizer gas) 32 psi; Gas 2 (heater gas) 70 psi; CAD gas 4 psi; TurboIonSpray (IS) voltage −4500 V; Entrance potential (EP) −8 V; Collision cell exit potential (CXP) −10 V; Source temperature 500 °C; Declustering potential (DP) −80; and Dwell time 250 ms. The collision energy (CE) was optimized based on the individual fragmentation selected to obtain the most intense precursor to product ion transitions. Analytical data were acquired by Analyst software (version 1.4).

3. Results and discussion

3.1. Optimization of chromatographic conditions

Since the main active components, silydianin, silychristin, silybin (silybin A and B), and isosilybin (isosilybin A and B) are structural analogs with identical molecular weight, separation of these two bioactive isomers as well as their four possible natural diastereomers prior to MS detection was crucial for characterizing each component present in a mixture. Recently, an LC method with MS detection utilized formic acid, suitable for MS as a mobile phase modifier for analysis of silymarin, has been published [29]. However, the overlapping of target peaks hindered the accurate quantification of the active constituents. In addition, a single molecule ion, ($[M-H]^-$) at *m/z* 481 was used to monitor for all six components. Surprisingly, the elution

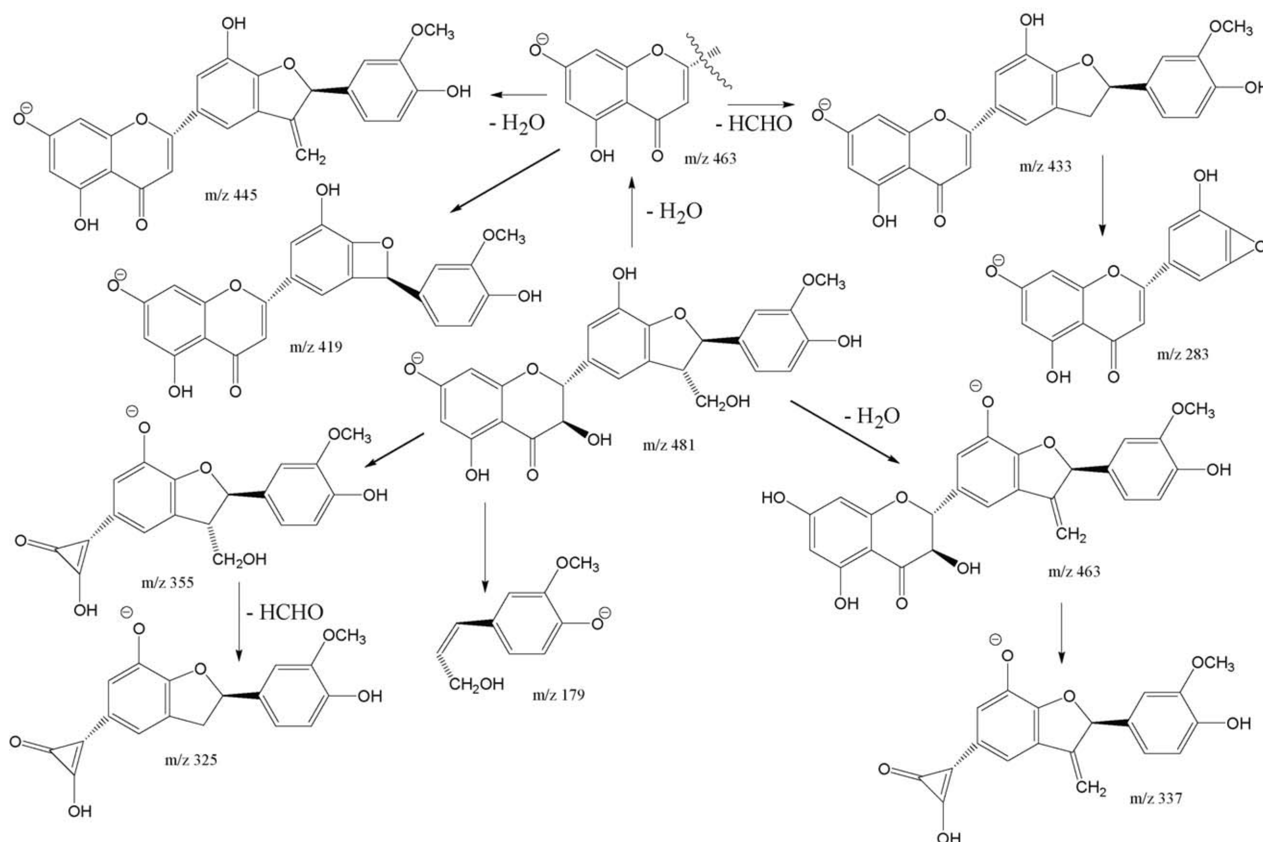

Fig. 6. Proposed fragmentation pathways (A) silychristin; (B) silydianin; (C) silybin; and isosilybin of *m/z* 481 ($[M-H]^-$).

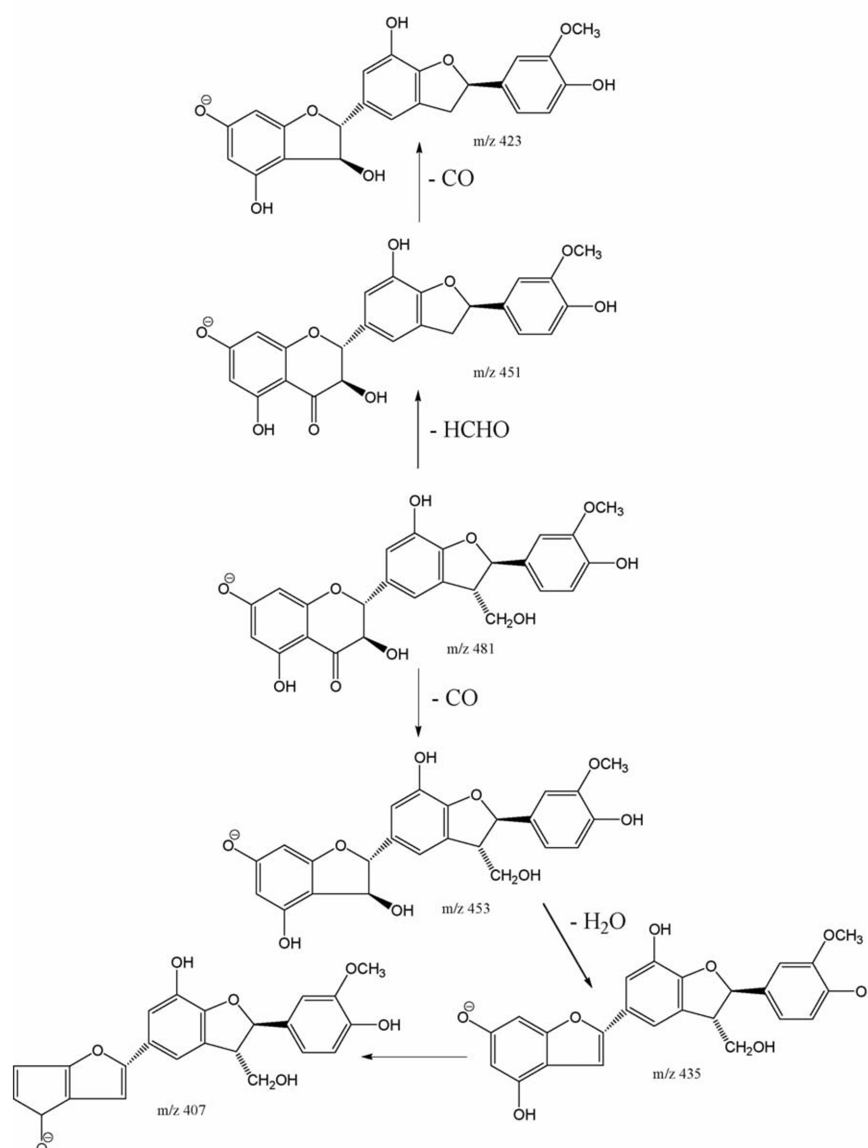

Fig. 6. (*Continued*)

orders assigned for the silychristin and silydianin were later than silybin A, silybin B, isosilybin A, and isosilybin B; even reversed-phase HPLC columns were used for all published methods [25–29,31]. Therefore, an LC method coupled to MS/MS to characterize and confirm all elution peaks appears to be critical.

Different type of HPLC columns and various HPLC conditions were evaluated in this study and the best chromatographic conditions for the separation of the compounds was a YMC ODS-AQ column with mobile phase consisting of 5 mM ammonium acetate and methanol/water/formic acid. Using the HPLC method described, we were able to efficiently separate all active constituents in silymarin. In addition, several unknown peaks were also observed. The separation could be further improved by utilizing longer columns. However, the resolution obtained was adequate for characterizing the active components in silymarin in the present study. The method also enables a quick screening of plasma samples, clinically or non-clinically, for a pharmacokinetic study.

3.2. Elucidation of LC/MS and LC/MS/MS spectra for silymarin

The high selectivity of LC coupled to MS/MS utilizing CID technique is a superior technique for the identification of flavonoid isomers and diastereomers in complex mixtures. Khan et al. have previously reported using ESI–MS/MS to study the fragmentation patterns of silymarin [31]. Besides that the fragmentation pattern proposed in the study was from a mixture of the combined six active constituents in silymarin, our point of view for the fragmentation pathway is different from that report. Ding et al. [26] also reported utilizing LC/MS spectra to differentiate silybin A and silybin B from isosilybin A and isosilybin

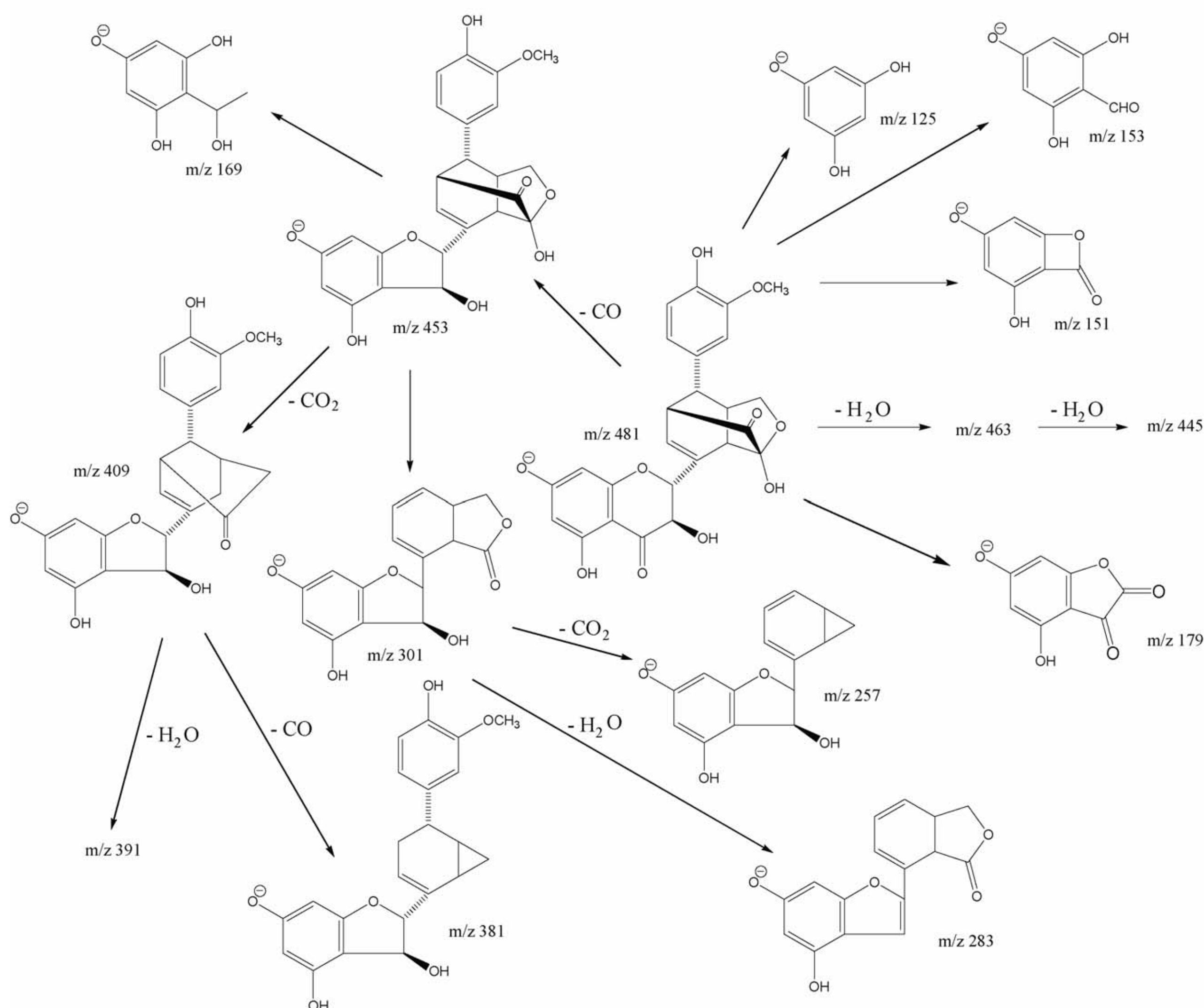

Fig. 6. (*Continued*)

B. But the aim of the study isn't to investigate the fragmentation pathways of silymarin.

The following strategies were used to identify the active principle constituents in silymarin. First, a mass spectrometric full scan was initially performed for the commercial milk thistle extract to identify the related molecular ions. Secondly, the combination of HPLC separation and MS/MS capability using product ion scan with different collision energies of −25, −30, and −35 V were performed for the parent molecule ion to characterize the fragment ions of each component. These unique product ion spectra could lead to the confirmation on the possibility of the structures. Thirdly, the use of HPLC separation together with MS/MS detection in MRM mode, which allows remarkable linkage of parent ion and product ion, was performed to further confirm the identity of each component. In this case, the HPLC retention times and intensity of MRM peaks were used to differentiate the structural isomers of the active constituents in silymarin. Finally, the characteristics of product ion spectra and the LC/MS peak intensities under MRM mode as well as the HPLC retention times of the individual active component were compared with those of reference standards to authenticate each major active component in the commercial product.

A negative ion ESI–MS full scan was performed using the first quadruple (Q1) for silymarin with the scan range from *m/z* 100 to 1000 (Fig. 2). Ionization of commercial milk thistle extract without HPLC separation in the negative ion mode generated predominantly an abundant ion at *m/z* 481 ions ($[M-H]^-$), which corresponded to the molecular mass of silychristin, silydianin, silybin and isosilybin (chemical formula $C_{25}H_{22}O_{10}$). The product ion spectrum for silymarin ($[M-H]^-$) was obtained by conducting the CID (MS/MS) experiment of the molecular ion. The product ion spectrum for the molecular ion of *m/z* 481 is shown in Fig. 3, which shows four main fragment ions at *m/z* 453, 301, 152 and 125. Since the extract of milk thistle consists of six components, the fragment ions shown in the product ion spectrum were contributed from one or more major components present in the extract. Scanty information from individual components rendered a need for a separation method.

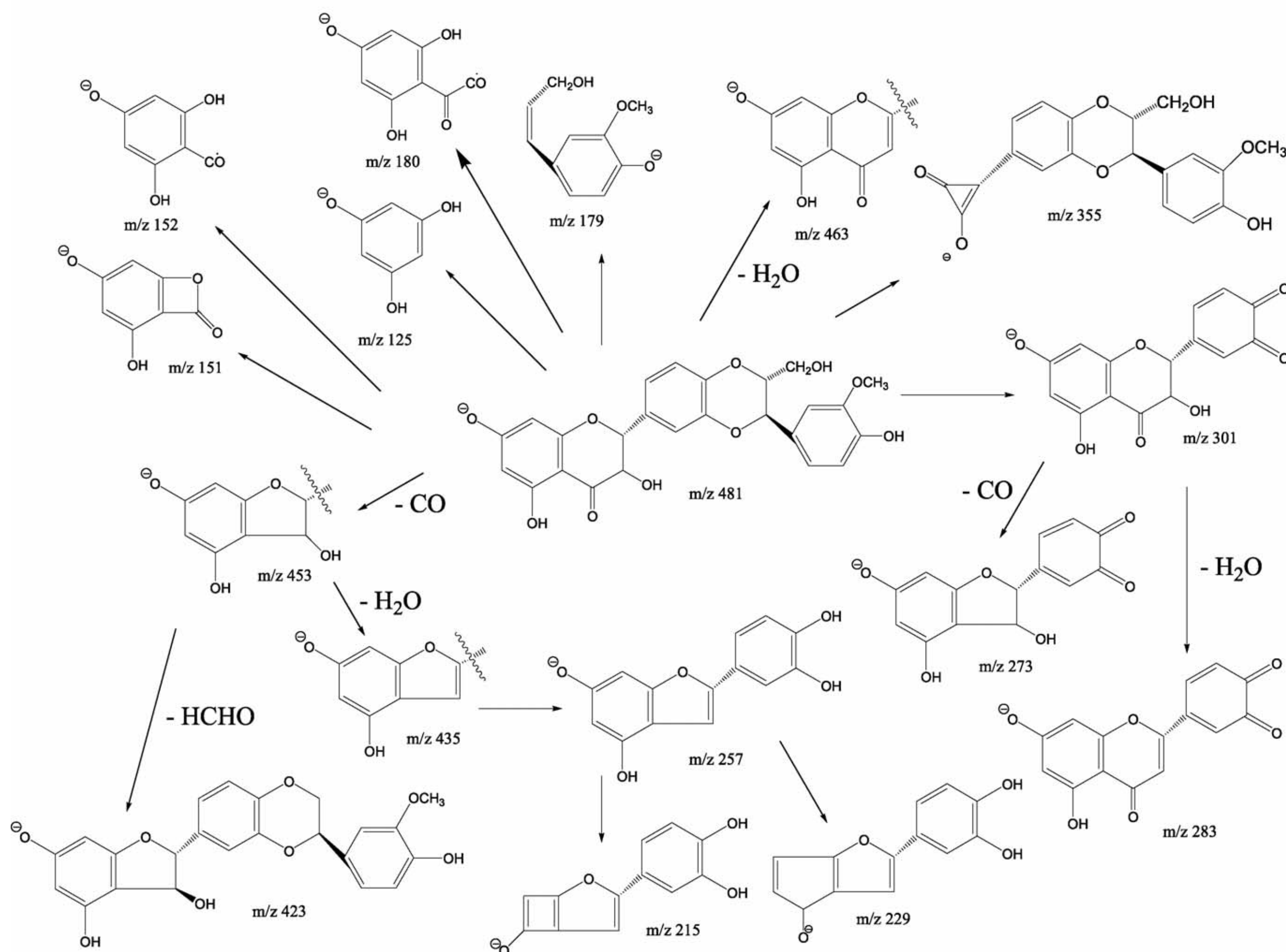

Fig. 6. (*Continued*).

An HPLC method using mass spectrometric friendly mobile phase has been developed by this laboratory to completely separate the major peaks present in commercial milk thistle extract. Under these HPLC conditions using the product ion scan of molecular ion, *m/z* 481, the possible prominent components in silymarin were eluted at 8.77 (peak 1), 10.46 (peak 2), 17.14 (peak 3), 18.29 (peak 4), 21.13 (peak 5) and 21.88 (peak 6) min with two minor peaks eluted at 7.54 and 19.59 min (Fig. 4). Peaks 1–4 were preliminarily identified as silychristin, silydianin, and silybin A and B, respectively, by using the reference standards and comparing their HPLC retention times as well as relative intensities of their fragment ions. Presumably peaks 5 and 6 are isosilybin A and B, but since there are no reference standards available, peaks 5 and 6 were temporarily not confirmed.

The criteria for the identification and characterization of silymarin products were based on the comparison of the fragment ions and their relative abundances from product ion spectra as well as retention times from HPLC separation with those of reference standards. The comparison of the LC/MS/MS product ion spectra is shown in Fig. 5 for the peaks 1–6 eluted from HPLC with ESI–MS detection at the collision energy of −30 V. The fragment ions and their relative abundances for six major components were virtually used for comparison. Product ion spectra for the HPLC retention times at peak 1 (t_R = 8.77 min) and peak 2 (t_R = 10.46 min) are shown in Fig. 5A and B, respectively. Product ion spectra for the HPLC retentions of peak 3 (t_R = 17.14 min), peak 4 (t_R = 18.29 min), peak 5 (t_R = 21.13 min), and peak 6 (t_R = 21.88 min) are shown in Fig. 5C–F, respectively. The product ion spectra were obtained under the same CID conditions for the isomers. Fragment ions for peaks 1 and 2 were significantly different from those of peaks 3, 4, 5 and 6. In contrast to peaks 1 and 2, the fragment ions shown in product ion spectra for peaks 4–6 are very similar albeit their relative intensities show some differences.

The HPLC retention time and the fragment ions generated from molecular ion are summarized in Table 1 for the six HPLC eluted compounds. Since the fragmentation pattern and intensity of MS/MS spectra were different at various collision energies, these product ion data were obtained by the combination of three product ion spectra with different CID energies (−25, −30 and −35 V). For the comparison of the product ion spectra for peaks 1 and 2, ions with negligible intensities from peak 1 but signifi-

Table 1
Product ions of m/z 481 for individual peak

Peak 1 Silychristin $t_R = 8.77$	Peak 2 Silydianin $t_R = 10.46$	Peaks 3 & 4 Silybin (A & B) $t_R = 17.14$ & 18.29	Peaks 5 & 6 Isosilybin (A & B) $t_R = 21.13$ & 21.88
481	481	481	481
463	463	463	463
453	453	453	453
451	–	451	–
435	435	435	435
433	433	433	–
423	–	423	423
419	–	–	–
–	409	–	–
407	–	–	–
–	391	–	–
–	381	–	–
355	–	355	–
337	–	–	–
325	–	–	–
–	301	301	301
–	283	283	283
–	–	273	273
–	257	257	257
–	–	229	229
–	–	215	215
–	–	213	213
–	–	180	180
179	179	179	179
–	169	–	–
153	153	–	–
152	152	152	152
151	151	151	151
125	125	125	125

cant in peak 2 were m/z 409, 391, 381, 301, 283, 257 and 169. In contrast, the significant ions observed in the product ion spectrum of peak 1, but not in peak 2, were m/z 451, 423, 419, 407, 355, 337 and 325. Based on comparison of the product ion spectra and the retention times from HPLC with those of reference standard, peak 1 and peak 2 were confirmed as silychristin and silydianin, respectively. The structure of each compound and its possible fragmentation pathway are illustrated in Fig. 6. The proposed fragmentation pathway of the $[M-H]^-$ ion of silychristin and silydianin are shown in Fig. 6A and B, respectively. Thus far, the separation of silychristin isomers by HPLC has not been reported. Further development is in progress to resolve and characterize these isomers using LC/MS/MS technology.

The fragment patterns of the compound eluted in peaks 3 and 4 as well as in peaks 5 and 6 were almost identical indicating that the chemical structures are very similar for the compounds eluted in peaks 3 and 4, and peaks 5 and 6. The major distinction between peaks 3, 4 and peaks 5, 6 is the difference of peak intensity for the unique fragment ion at m/z 301 and the absence of fragment ions of m/z 451, 433, and 355 in the product ion spectrum of peaks 5 and 6. The relative abundance of m/z 301 for silybin A or B (peaks 3 and 4) is at least three-fold higher than those observed in the product ion spectrum of isosilybin A or B (peaks 5 and 6). On the other hand, no observable difference was seen in the relative intensity of fragment ion of m/z 301 between peak 3 and peak 4 or between peak 5 and peak 6. A possible explanation for this phenomenon is the different location of 4-hydroxy-3-methoxyphenyl moiety at C-α and C-β (see Fig. 1); this may sterically hinder the bond cleavage between flavonoid and lignan moieties that leads to the di-keto fragment ion which of the intensity is significantly high in silybin A and B. The proposed fragmentation pathway of the $[M-H]^-$ ion of silybin or isosilybin are shown in Fig. 6C. Interestingly, the formation of radical negative ion as evidenced by the presence of m/z 152 and 180 in the product ion spectra was more favorable in the fragmentation pathway of the $[M-H]^-$ ion in peak 3-6 compared to those in peak 1–2.

Based on the fragmentation behavior (Fig. 6), it is clear that the four components (peak 3–6) can be classified into two groups, that is peaks 3 and 4 as one group and peaks 5 and 6 as the other group. These four peaks were further confirmed by comparing both the product ion spectra and the HPLC retention time with the reference standards. Peak 3 and peak 4 are identified as diastereomers for silybin. Because there were two diastereomers of silybin (A and B) and two diastereomers of isosilybin (A and B) present in the silymarin [13–15], and the identical fragmentation patterns should be observed in the product ion scans for silybin (A and B) and isosilybin (A and B) in this study, peaks 5 and 6 were then assigned as diastereomers of isosilybin A and B. Since the product ion spectra of diastereomers as well as the molecular masses for all isomers were found to be essentially identical, it is crucial to rely on HPLC separation prior to MS/MS analysis for the characterization of diastereomer components.

After obtaining structural information using LC/MS/MS with product ion scan, an LC/MS/MS experiment with MRM can be subsequently employed to screen and confirm the isomers or diastereomers present in a complex mixture by the difference of HPLC retention time and MRM peak intensity. MRM mode with specific precursor ion and fragment ion linked together offers benefits of filtering out the interferences and providing unambiguous identification for individual components with higher sensitivity. The LC/MS/MS chromatograms under MRM mode is illustrated in Fig. 7 for screening silymarin in human plasma. With MRM transition set at m/z 481 → 481 using low CID energy (−5 V) all six silymarin related components are detected (Fig. 7A), which showed the proposed sensitive and selective LC/MS method can be used for monitoring six active silymarin in human plasma. In contrast, the HPLC separation utilizing column-switching with sensitive electrochemical detection [15] has been used only for monitoring of silybin (A and B) in human body fluid. By selecting MRM transition at m/z 481 → 325 (Fig. 7B) only the relatively significant silychristin (peak 1) and minor silydianin (peak 2) were observed, whereas silybin and isosilybin were invisible. Likewise monitoring the transition of m/z 481 → 169 can specifically determine only the presence of silydianin (Fig. 7C). Since m/z 301 was a predominant fragment ion in the product ion spectra of silybin A and B, MRM with transition at m/z 481 → 301 produced two distinct peaks corresponding to silybin A and B (Fig. 7E). Similar results were also observed in the commercial extract sample using MRM approaches.

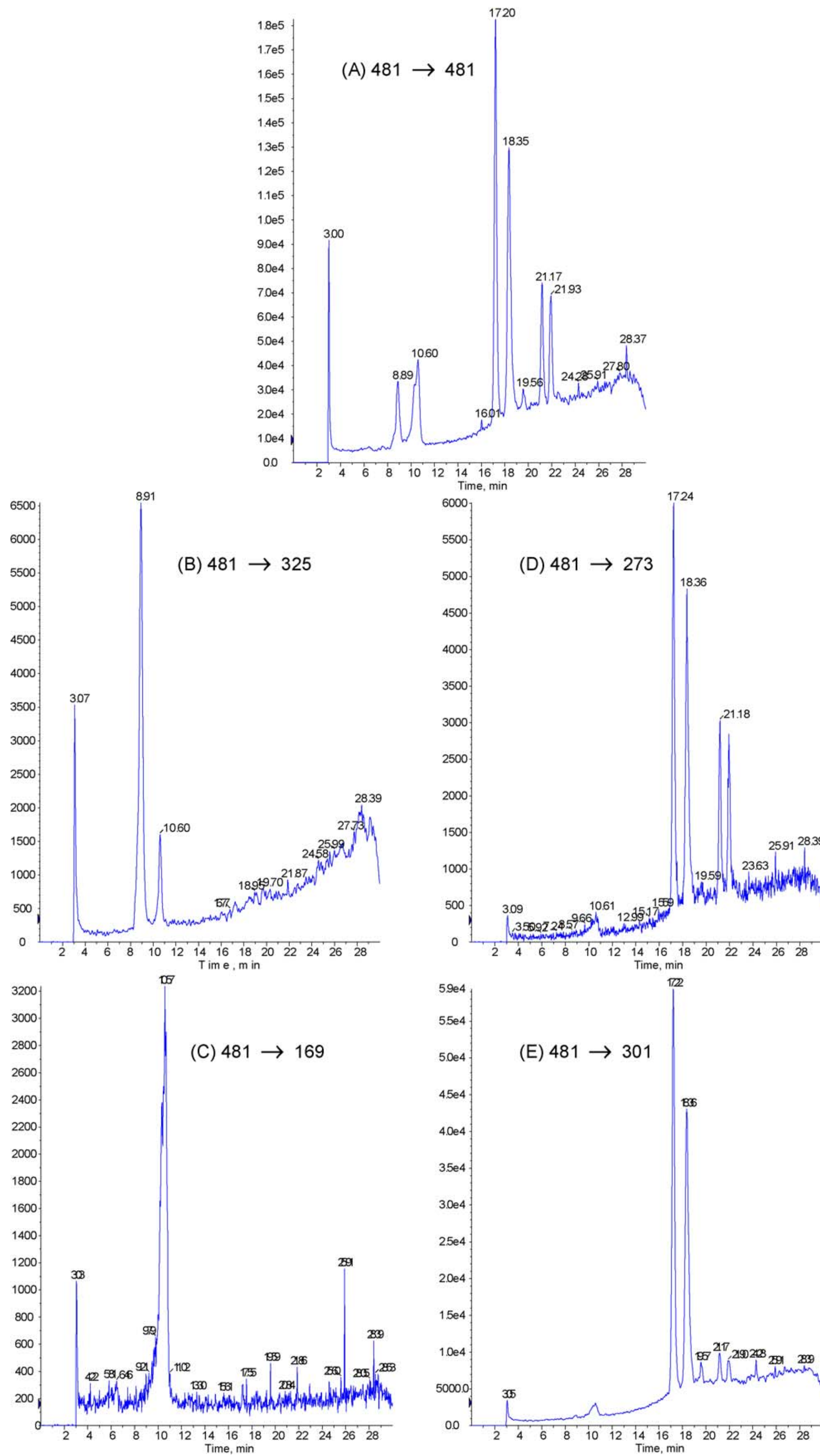

Fig. 7. MRM ion chromatogram of silymarin in human plasma sample. Mass chromatograms for the MRM transitions at m/z (A) 481 → 481; (B) 481 → 325; (C) 481 → 169; and (D) 481 → 301.

4. Conclusions

In the present study, an HPLC–ESI–MS/MS method has been developed and applied for the identification and characterization of active components in silymarin in commercial standardized extract. The new LC method described here completely resolved bioactive constituents, including the two sets of diastereomers. The LC/MS/MS method with both product ion scan and MRM approaches is a useful tool for identifying and elucidating the fragmentation pathways of active components in silymarin. As a result, the LC/MS/MS method could provide a base for the quality control of commercial milk thistle standardized extract. In addition, the MRM mode utilized in LC/MS/MS is more selective and sensitive than previous methods published. The established method has been successfully applied to the analysis of active components in silymarin in human plasma and will be eventually employed to support pharmacokinetic characterization of silymarin constituents in healthy volunteers and patients. Further work is now in progress to characterize silymarin content in various commercial products as well as to develop proper procedures to quantitate bioactive components of silymarin in biologic matrices. Summarily, our research can provide a base for the quality control of commercial milk thistle standardized extract and the chemical basis of its biological activities.

Acknowledgements

The authors express their gratitude to Drs. Peter C. Adamson and Jeffrey M. Skolnik for critically reviewing this manuscript. This project is funded, in part, under a grant with the Pennsylvania Department of Health. The Department specifically disclaims responsibility for any analyses, interpretations or conclusions.

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