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Research Article

Direct-Injection ESI-MS/MS Method for Studying Fragmentation Pathways of Prospidium Chloride

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Abstract

Objective: Investigation of the ionization and fragmentation behavior of precursor ions enables improvement of qualitative analytical approaches for pharmaceutical substances without using chromatographic separation. Identification of diagnostic product ions additionally facilitates the development of quantitative mass-spectrometric methods based on MRM/SRM transitions and expands libraries of diagnostic mass ions for the studied drugs.

Materials and Methods: Mass spectra of precursor and product ions of prospidium chloride were obtained using a High-Performance Liquid Chromatograph—Triple Quadrupole Mass Spectrometer LCMS-8050 equipped with electrospray ionization (ESI) and direct sample introduction (DIP).

Results: Five precursor ions at m/z 187.2, 205.2, 373.3, 409.2 and 740.2 were detected and characterized. The most intense precursor ions at m/z 205.2 and 409.2 were selected for collision-induced dissociation in the collision cell. MS/MS spectral analysis enabled elucidation of fragmentation pathways and the identification of a diagnostic product ion at m/z 164.4.

Conclusion: The precursor ions reported here, described for the first time, can be used to broaden existing normative documentation for prospidium chloride by providing a non-chromatographic method for authenticity testing. Elucidation of fragmentation mechanisms reveals diagnostic product ions that retain core structural elements of the prospidium chloride molecule and are suitable for development of quantitative mass-spectrometric assays.

Keywords: Prospidium chloride, DIP-ESI-MS/MS, fragmentation mechanism, development of analytical methods

INTRODUCTION

Mass spectrometry is a leading analytical technique for characterization of pharmaceutical substances, and development of analytical workflows based on direct sample introduction (DIP) remains an important task in contemporary pharmaceutical chemistry and medical sciences [1, 2]. Limitations of chromatographic-mass spectrometric hybrid methods include reduced throughput and potential bias from matrix effects that arise from interactions between solvents and sample components [3, 4]. For example, the diastereomers ephedrine and pseudoephedrine have identical molecular formulas, similar bonding sequences, comparable pKa values and high polarity, differing only in stereochemistry; this makes chromatographic separation challenging even with enantioselective columns. Tandem mass spectrometry with direct injection and low collision energy has been shown to differentiate such diastereomers, resolve coelution issues, and enable assessment of relative content [5]. Accordingly, the DIP approach offers several advantages, including short analysis times (2-5 minutes), reduced matrix effects, and high sensitivity [6].

Modern antineoplastic therapy comprises diverse chemical classes, with a substantial portion represented by alkylating cytostatics. Their pharmacological effect is based on formation of covalent bonds via a donor-acceptor mechanism between an electron-deficient carbocation (originating from a chloroalkyl fragment of the drug) and nucleophilic sites in DNA—commonly the N7 atom of guanine or the N3 atom of adenine in tumor cell DNA [7]. Despite therapeutic benefits, alkylating

agents are associated with high systemic toxicity, creating demand for drugs with improved tolerability and fewer adverse effects. For these reasons, prospidium chloride (prospidine, PrsCl2) was chosen as the study object due to its comparatively favorable safety profile within this pharmacological class.

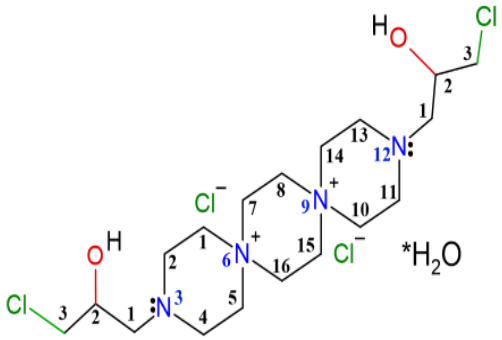
Current quality-control approaches for PrsCl2 include various analytical techniques; however, ionization behavior and fragmentation mechanisms of the molecule are not fully characterized, and there is a lack of rapid, high-precision mass-spectrometric methods for purity assessment and quantitation. Determination of fragmentation pathways enables isomer identification, qualitative analysis without reference standards or chromatographic systems, identification of diagnostic fragment ions (DFIs) for building PrsCl2 DFI libraries, optimization of MRM transitions used in quantitative MS assays, and investigation of drug metabolites [8 - 11].

The objective of the present study was therefore to establish reproducible fragmentation patterns of $PrsCl_2$ under DIP-ESI-MS/MS conditions to enable identification of the active pharmaceutical ingredient (API) using diagnostic fragment ions and to construct a spectral library for the compound.

MATERIALS AND METHODS

2.1. Object of research

The object of this study was the antineoplastic active pharmaceutical ingredient $PrsCl_2$ monohydrate $PrsCl_2 \cdot H_2O$ (3,12 - Bis (3 - chloro - 2 - hydroxypropyl) - 3,12 - diaza - 6,9 - diazoniadispiro [5.2.5.2] hexadecane dichloride monohydrate; Prospidine; $C_{18}H_{36}Cl_4N_4O_2 \cdot H_2O$; Mr = 500.3). The material was supplied by the scientific and technological park "Unitechprom BSU", Republic of Belarus (batch 271222; expiry date December 2027); declared API content w = 100.5% on a dry-weight basis (Figure 1).



3,12-Bis(3-chloro-2-hydroxypropyl)-3,12-diaza-6,9-diazoniadispiro [5.2.5.2] hexadecane dichloride monohydrate [12]

Figure 1: Chemical structure of $PrsCl_2$.

$PrsCl_2 \cdot H_2O$ is a white crystalline powder with hygroscopic properties; it is readily soluble in water and practically insoluble in 96% ethanol and chloroform. The studied drug displays the lowest toxic action among agents of its pharmacological class according to published intraperitoneal LD_{50} data in rats (see Table 1).

Table 1: Toxicity, carcinogenicity and molecular complexity of alkylating antineoplastic agents.

Drug (marketing year) Molecular weight (g·mol^{-1}),	Toxic dose (LD_{50}), mg·kg^{-1}	Carcinogen Classification*	Complexity**
Busulfan (1954) 246,3	18 [13]	Group 1: Carcinogenic to humans	185
Chlorambucil (1957) 304,2	14 [14]	Group 1: Carcinogenic to humans	250
Mechlorethamine (1958) 156,1	0,860 [15]	Group 2A: Probably carcinogenic to humans	43,7
Melphalan (1964) 305,2	4,48 [16]	Group 1: Carcinogenic to humans	265
Carmustine (1977) 214,05	17,42 [17]	Group 2A: Probably carcinogenic to humans	162
Cyclophosphamide (1959, шире 1970) 261,1	40 [18]	Group 1: Carcinogenic to humans	218
Uramustine (1970) 252,1	1,25 [19]	Group 2B (Possibly carcinogenic to humans)	277
Mannomustine (1970) 378,11	56 [20]	Group 3: Not classifiable as to its carcinogenicity to humans	227
Prospidium chloride (1970-1980) 500,3	1200 [12]	Not classified	280
Ifosfamide (1988) 261,08	140 [22]	Group 3: Not classifiable as to its carcinogenicity to humans	218

* The International Agency for Research on Cancer (IARC) https://www.iarc.who.int/

** "Complexity" is a numerical measure of molecular structural complexity that includes parameters such as number of sp^3 carbon atoms, number of stereocenters, presence of non-aromatic rings and heavy atoms, ring complexity and chain branching. [23, 24]

The presented data confirm the comparatively favorable safety profile of $PrsCl_2$ relative to other drugs in this pharmacological group and justify the relevance of developing analytical quality-control methods for this substance.

2.2. Method of research

Mass spectra of precursor and product ions were acquired on a High-Performance Liquid Chromatograph - Triple Quadrupole Mass Spectrometer LCMS-8050 equipped with electrospray ionization (ESI), a UFsweeper III collision cell, and a direct sample-introduction (DIP) capability. The instrument mass range covered m/z 2-2000; mass resolution R < 0.7 u FWHM and adjustable to 0.5 u; maximum scan speed up to 30,000 u/s (0.1 u step: 300,000 data points/s). Sample injection volume was 5 µL and the total analysis time was 5 minutes. Experiments were performed with aqueous solutions at a concentration of 0.05 $mg \cdot mL^{-1}$.

2.2.1. Study of design

The experimental design used the 3-quadrupole LCMS-8050 platform to investigate fragmentation pathways of $PrsCl_2$. The triple-quadrupole architecture is well suited for fragmentation studies due to its two-stage mass-filtering capability (Figure 2).

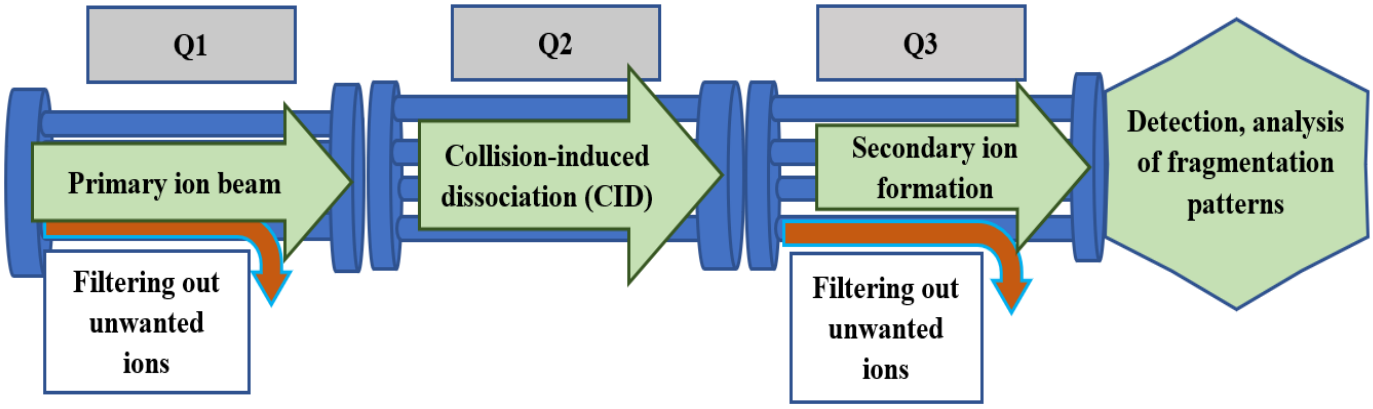


Figure 2: Schematic of PrsCl2 analysis on the LCMS-8050: Q1 - first quadrupole (precursor selection), Q2 - collision cell, Q3 - mass filter (product ion analysis).

Based on instrument capabilities, the experiment comprised two sequential steps: (1) acquisition of full MS spectra in Scan mode to identify characteristic precursor ions of $PrsCl_2$, taking into account their isotopic patterns; selected specific precursor ions were then chosen for the next stage; (2) acquisition of MS/MS (Product Ion Scan) spectra for the selected precursors to record all product ions formed under collision-induced dissociation (CID).

Sample preparation and measurement conditions were defined to produce reproducible MS and MS/MS spectra for pathway elucidation of API fragmentation, detection of possible related impurities, and characterization of complex mixtures containing $PrsCl_2$.

2.2.3. Visualization of experimental data

Graphical representation and curve fitting of the experimental data were performed in OriginPro 2021 software (OriginLab Corporation, Northampton, MA, USA). Molecular mass calculations and chemical structure drawing were carried out using ACD/ChemSketch software (version 2020, Advanced Chemistry Development, Inc., Toronto, ON, Canada; ACD/Labs). The figures and schematic illustrations were generated and edited using Paint.net (version 4.0, dotPDN LLC)

RESULTS AND DISCUSSION

3.1. Relationship between toxicity and molecular complexity of alkylating cytostatics: structure-activity relationship analysis

A screening of alkylating cytostatics was performed to identify the agents with the lowest toxicity on the basis of the data summarized in Table 1. $PrsCl_2$ with a molecular mass of 482.3 Da, formally meets the Lipinski criterion (Mr ≤ 500 Da) [25]. It should be noted that exceeding this threshold is usually associated with reduced bioavailability and altered pharmacokinetic behavior of cytostatics [26]. The screening results for the studied alkylating agents are shown in the following diagram (Figure 3).

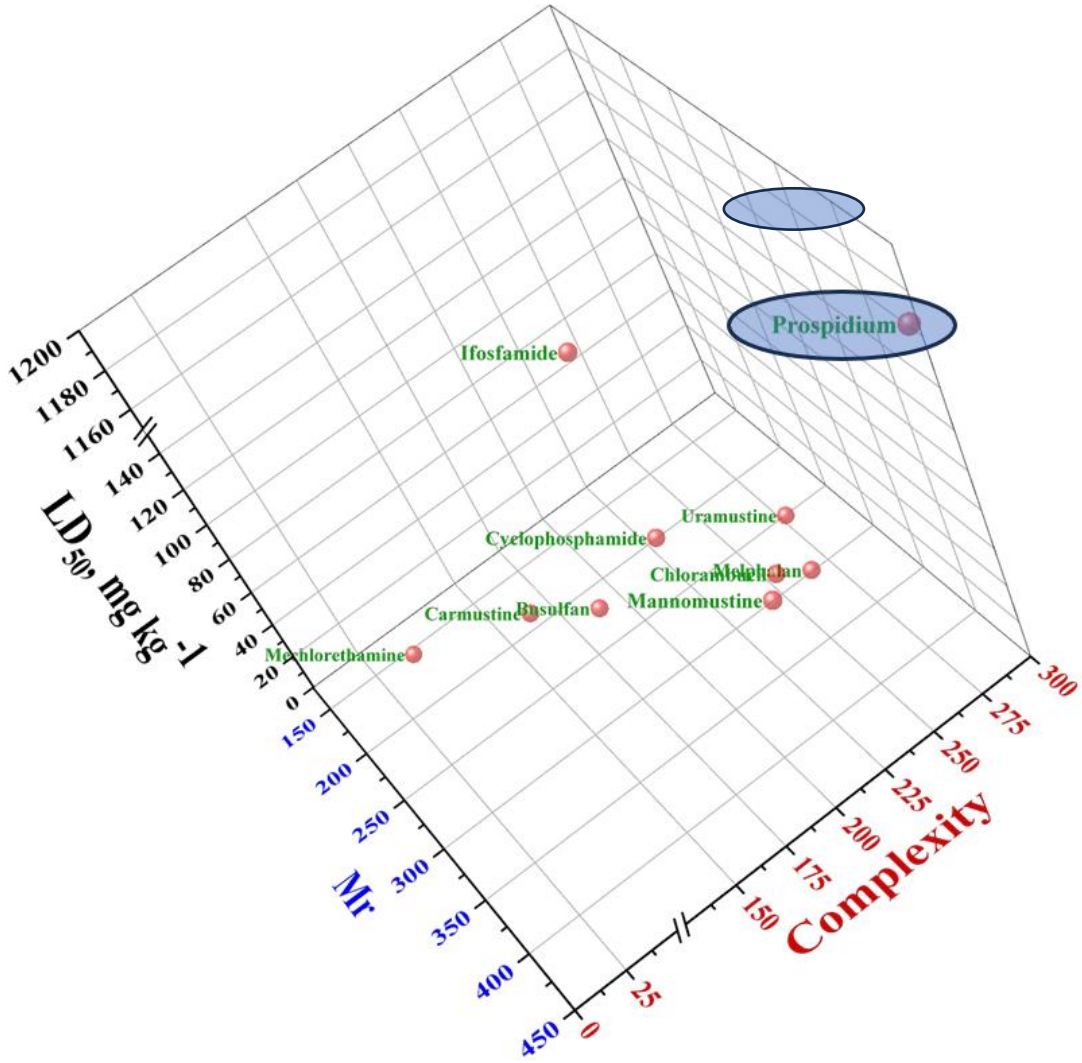


Figure 3. 3D diagram showing the relationship between molecular mass *x* and complexity *y* of alkylating cytostatic molecules; the *z* axis corresponds to the toxic dose LD_{50}.

An increase in molecular mass leads to greater molecular complexity and is generally associated with increased drug toxicity. However, the diagram shows that the studied $PrsCl_2$ occupies a distinct region within the group of alkylating cytostatics. This demonstrates the relevance of $PrsCl_2$ and the growing interest in it, which stimulates the development of new methods for quality control.

Identification of characteristic precursor ions of PrsCl2 using the DIP-ESI-MS method

This section presents the mass-spectrometric identification of $PrsCl_2$ through acquisition and analysis of precursor-ion MS spectra. After recording MS spectra in DIP-ESI-MS/MS mode for $PrsCl_2$, ions at *m/z 187.2, 205.2, 373.3, 409.2* and *740.2* were selected. Since the $PrsCl_2$ molecule contains the chlorine isotopes ^{35}Cl and ^{37}Cl, the MS spectra exhibit a characteristic isotopic distribution for all observed ions. The presence of the characteristic chlorine isotope pattern confirms the structure of the ions formed during ionization and links them to the studied sample. The observed ions arise from cleavage of ionic bonds with chloride ions, dehydrogenation, and rupture of the most labile bonds in the molecular framework.

Because the analysis was performed on a triple quadrupole mass spectrometer with limited mass accuracy, all *m/z* values should be regarded as approximate, and their use in this work is justified by the study of ionization and fragmentation of the API rather than by exact mass determination; this is consistent with the known unit mass resolution limitation of quadrupole analyzers [27].

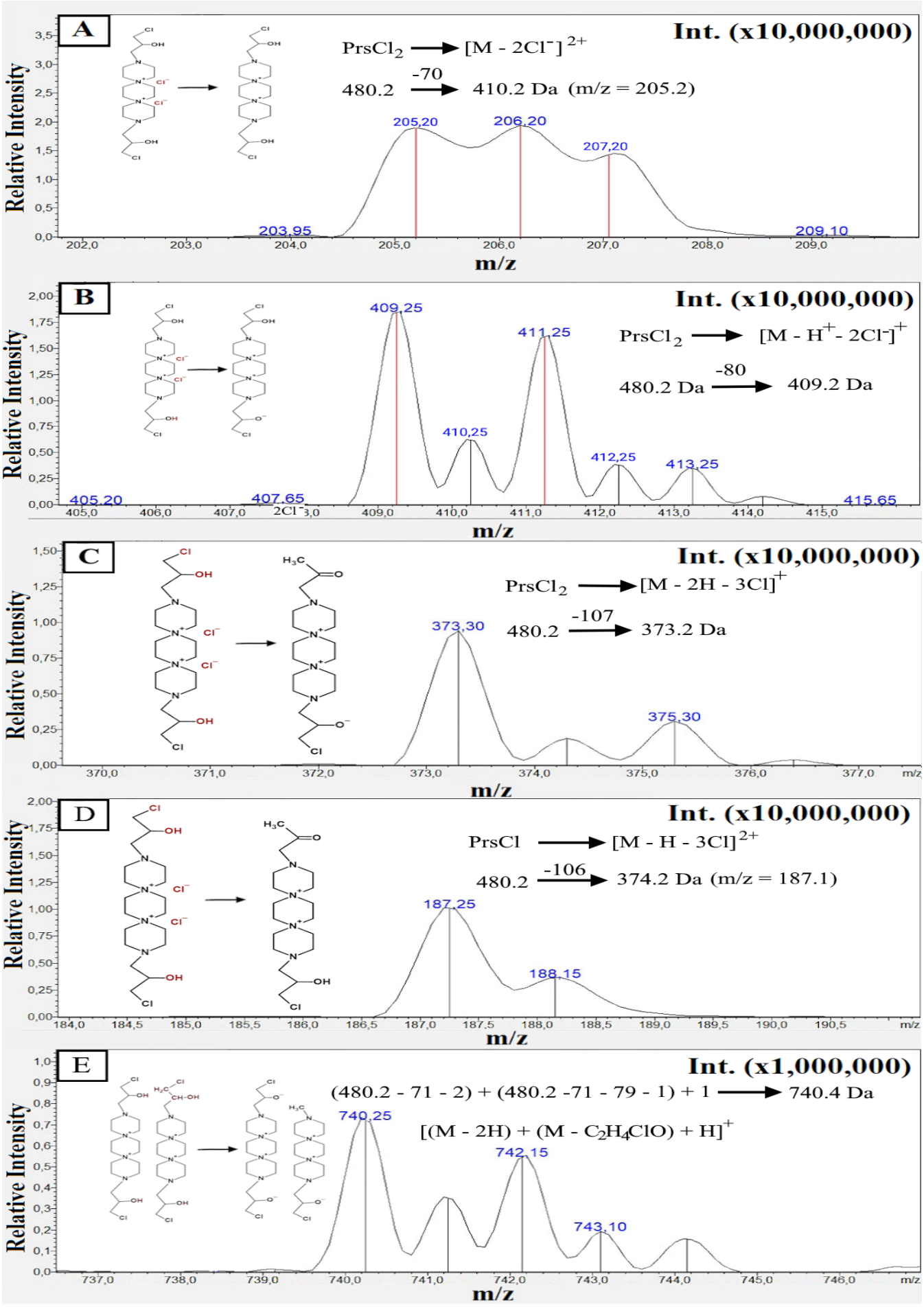


Figure 4: Ionization pathways of $PrsCl_2$ leading to precursor ions: A - *m/z 205.2*, B - *m/z 409.2*, C - *m/z 373.3*, D - *m/z 187.2*, E - *m/z 740.2*.

The monoisotopic mass of $PrsCl_2$ [$C_{18}H_{36}Cl_4N_4O_2$] is Mi = 480.2 Da. Under ESI in positive-ion mode, the most probable ionization pathway involves dehalogenation of the $PrsCl_2$ molecule with formation of a dication through cleavage of two ionic -N^+-Cl^- bonds. Thus, the mechanism of formation of the doubly charged ion at *m/z 205.2* is as follows: [M - $2Cl^-$]$^{2+}$ [480.2 Da - 70 = 410.2 Da], with the gross formula [$C_{18}H_{36}Cl_2N_4O_2$]$^{2+}$ (Figure 4A).

The next, and most stable, ion retaining the original structural framework is the ion at *m/z 409.2*. This ion is singly charged, and the proposed mechanism of its formation is cleavage of two ionic -N^+-Cl^- bonds together with deprotonation of one hydroxyl group in the $PrsCl_2$ molecule: [M - 2Cl- - H^+] $^+$ [480.2 Da - 71 = 409.2 Da], with the gross formula [$C_{18}H_{35}Cl_2N_4O_2$]$^+$ (Figure 4B).

The previously undescribed singly charged ion of $PrsCl_2$ at *m/z 373.3*, showing a chlorine isotope pattern that confirms its relationship to the API structure, is formed through dehalogenation via cleavage of two ionic -N^+-Cl^- bonds, deprotonation, dehydrogenation of one -OH group, elimination of H^+ to form a double bond at the second OH group, and cleavage of the C-Cl bond in the side alkyl fragment [M - 2H - 3Cl]$^+$ [480.2 Da - 107 = 373.2] (Figure 4C). The gross formula of this ion is [$C_{18}H_{34}ClN_4O_2$]$^+$.

Due to the low resolving power of the mass spectrometer, no chlorine isotope pattern is observed for the previously undescribed doubly charged ion at *m/z 187.2*. It is formed by a mechanism analogous to that of the preceding ion: dehalogenation by cleavage of two ionic -N^+-Cl^- bonds, elimination of H^+ to form a double bond in one -OH group, and cleavage of the C-Cl bond in the side alkyl fragment [M - H - 3Cl]$^{2+}$ [480.2 Da - 106 = 374.2] (Figure 4D). The gross formula is [$C_{18}H_{35}ClN_4O_2$]$^{2+}$.

In the high-mass region of the MS spectrum, an ion at *m/z 740.2* is detected, corresponding to a singly charged dimer. Its formation likely proceeds as follows: two $PrsCl_2$ molecules dissociate to form dications [M - $2Cl^-$]$^{2+}$, and subsequent association of the two dications yields a conglomerate with a mass of 820.4 Da and a charge of +4. The next step in ion formation involves reduction of Coulombic strain through destruction of the -C_2H_4ClO side chain (79 Da) and dehydrogenation of hydroxyl groups to compensate for charge [(M - 2H^+) + (M - C_2H_4ClO - H^+) + 2H]$^+$ [(480.2 - 70 - 2) + (480.2 - 70 - 79 - 1) + 2 = 740.4] (Figure 4E).

Ionization of $PrsCl_2$ is characterized by the formation of two ions of highest intensity: the doubly charged cation [M - $2Cl^-$]$^{2+}$ at *m/z 205.2* and the singly charged deprotonated ion [M - 2Cl^- - H^+]$^+$ at *m/z 409.2*. These data confirm the structure of the bis-quaternary salt of $PrsCl_2$ and can be used for fragmentation studies and MS/MS analysis. In addition, the present work provides an MS method for authenticity testing of the API in $PrsCl_2$ substance without reference standards or chromatographic systems.

Identification method

The spectrum of the test sample should contain fourteen main peaks (m/z (I, %)): 205.2 (100%), 206.2 (85-100%), 207.2 (64-68%), 409.2 (100%), 410.2 (25-35%), 411.2 (85-100%), 373.3 (100%), 374.3 (20-35%), 375.3 (30-35%), 187.2 (100%), 188.2 (30-37%), 740.2 (100%), 741.2 (30-37%), 742.2 (80-87%).

Test solution: dissolve 0.50 mg of the sample substance in 10.00 mL of water.

Injection volume: 5 µL.

Ionization and detection conditions: capillary voltage 2500 V; drying gas flow 10 L/min; nebulizer pressure 40 psig; drying gas temperature 300°C; mass range 50-1000 m/z; fragmentor value 50.

Note: peak intensities may vary depending on the mass spectrometer configuration.

Fragmentation mechanisms of $PrsCl_2$ ions using the DIP-ESI-MS method

The recorded MS/MS spectra of $PrsCl_2$ show a complex but reproducible ion pattern that reflects both the features of primary ionization and subsequent fragmentation in the collision cell. Analysis of the data made it possible to identify the most significant ions and their precursor-to-product transitions for quantitative analysis and for construction of the DFI library.

To obtain the MS/MS spectra, first quadrupole (Q1) was set to transmit only ions of a selected m/z value. In the second quadrupole (Q2), or collision cell (CID), the precursor ions fragmented under the action of an inert gas. The third quadrupole (Q3) was operated in scan mode to detect all product ions formed from the selected precursor ion and to study their fragmentation patterns. The study focused on the highest-intensity ions at *m/z 409.2* and *205.2.* Because the *m/z 205.2* ion is doubly charged, its fragmentation should proceed through charge separation; therefore, due to electrostatic repulsion, it undergoes collision-induced dissociation to form singly charged fragments (Figure 5A). This explains the presence of numerous low-mass but highly intense fragments in its MS/MS spectrum. In contrast, the *m/z 409.2* ion is singly charged, which results in weaker intramolecular interactions and MS/MS fragments differing from the precursor by the loss of water or one chloride ion.

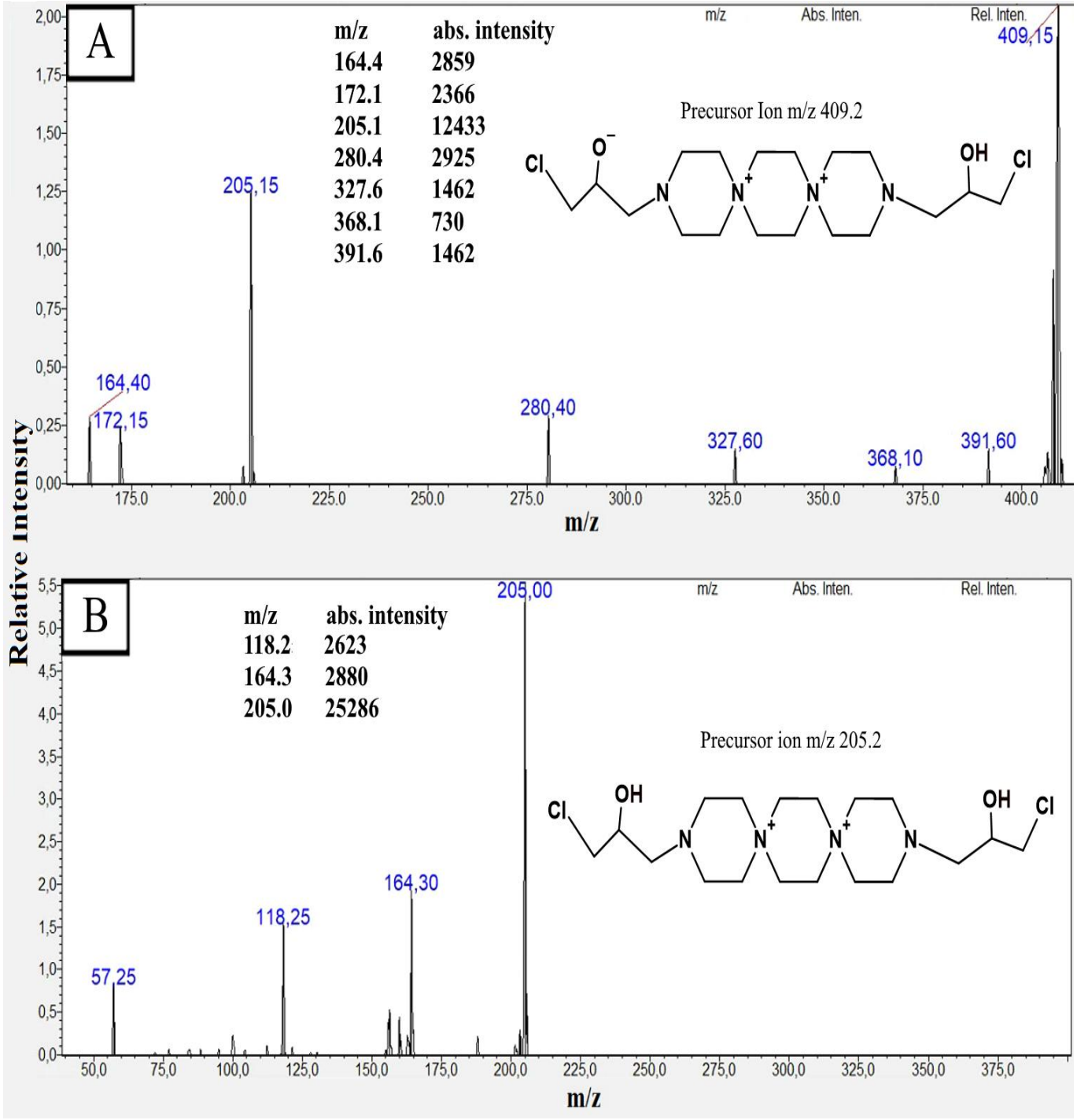


Figure 5. MS/MS spectra of $PrsCl_2$ solution for the selected ion at m/z 409.25 (A) and for the selected ion at m/z 205.11 (B), obtained by fragmentation in the CID cell.

According to the obtained data, the main fragmentation pathways for both precursor ions are dehydration of the alkyl fragment of the ion (-18 Da) and degradation of the side-chain 3-chloro-2-hydroxypropyl fragments, cleavage of the spiro-core at the nitrogen spiro atoms (N6 and N9) (see Fig. 1).

Fragmentation of the precursor ion at *m/z 409.2* begins with dehydration of the alkyl fragment to form the singly charged ion at *m/z 391.6* [M - H_2O] [409.2 - 18 = 391.2], with the proposed gross formula $[C_{18}H_{33}Cl_2N_4O]^+$.

The fragment ion at *m/z 368.1* is formed by dehydroxylation of one -OH group at position 3 of the side chain, together with cleavage of C-N bonds in the cyclic system and loss of methyl groups from nitrogen atoms N3 and N6. Thus, the proposed mechanism is [M - C_2HO] [409.2 - 41 = 368.2]. The proposed gross formula is $[C_{16}H_{34}Cl_2N_4O]^+$.

Table 2. Fragmentation transitions and proposed decomposition pathways of the precursor ions of $PrsCl_2$ at *m/z 205.2* and *409.2*.

Precursor ion, m/z	Product ion, m/z Brutto-formula	Abs. intensity	Δm (Da) Proposed neutral loss	Proposed fragmentation pathway
409.2 Brutto-formula $[C_{18}H_{35}Cl_2N_4O_2]^+$	391.6 $[C_{18}H_{33}Cl_2N_4O]^+$	1462	-18 - H_2O	Dehydroxylation of the -OH group of the side alkyl fragment
	368.1 $[C_{16}H_{34}Cl_2N_4O]^+$	730	-41 - C_2HO	Cleavage of N-C bonds in the cyclic system with loss of methyl groups from nitrogen atoms N3 and N6, dehydroxylation of the -OH group of the alkyl fragment
	327.6 $[C_{17}H_{35}N_4O_2]^+$	1462	-82 - CCl2	Cleavage of the C-Cl bond and the C-C bond between carbons 1 and 2 in the alkyl fragments, with dehydrogenation of one -OH group
	280.4 $[C_{16}H_{32}N_4]^+$	2955	-129 - $C_2H_3Cl_2O_2$	Cleavage of N-C bonds in the cyclic system at N3 and N6 with loss of the -CH_2-CH_2- fragment of the ring, cleavage of C-Cl bonds and dehydroxylation of the -OH groups of the alkyl fragments
	172.1 $[C_{17}H_{33}ClN_4O]^{2+}$	2366	-65 - CH_2ClO	The deprotonated oxygen -O- forms a double bond with the C3 carbon of the side alkyl chain, followed by cleavage of the C-C bond between carbons 2 and 3 of the second side alkyl chain and cleavage of the C-O bond at carbon 3
	164.4 $[C_{17}H_{35}N_4O_2]^{2+}$	2859	-81 - CCl_2 +	Cleavage of C-Cl and C-C bonds at positions 1 and 2 of the alkyl fragments
205.2 Brutto-formula $[C_{18}H_{36}Cl_2N_4O_2]^{2+}$	164.4 $[C_{17}H_{36}N_4O_2]^{2+}$	2880	-81 - CCl_2	Cleavage of C-Cl and C-C bonds at positions 1 and 2 of the alkyl fragments
	118.2 $[C_6H_{18}N_2]^+$	2623	-292 -$C_{12}H_{18}Cl_2N_2O_2$	Asymmetric cleavage of the central piperazine ring to form a singly charged fragment

The next two product ions, the singly charged *m/z 327.6* ion and the doubly charged *m/z 164.4* ion, differ structurally by the hydrogen of the hydroxyl -OH group at position 3, which accounts for the difference in charge state. Owing to the structural similarity of these ions, the formation mechanism is analogous, namely dehalogenation of one side alkyl chain through cleavage of the C-Cl bond and cleavage of the C-C bond between carbons 1 and 2 of the second alkyl chain, accompanied by loss of the -CH_2-Cl fragment. Thus, the fragmentation mechanism of the precursor ion at *m/z 409.2* to form product ions *m/z 327.6* and *164.4* is as follows: [M - $CHCl_2$]+ [409.2 - 82 = 327.2] for the singly charged ion and [M - CCl_2]$^{2+}$ [409.2 - 81 = 328.2] for the doubly charged ion, respectively. The gross formulas are $[C_{17}H_{35}N_4O_2]^+$ and $[C_{17}H_{36}N_4O_2]^{2+}$. The ion at *m/z 164.4* is also observed in the MS/MS spectrum of the precursor ion at *m/z 205.2*.

The product singly charged ion at *m/z 280.4* is formed by the following fragmentation mechanism: cleavage of C-N bonds in the piperazine cyclic structure at nitrogen atoms N3 and N6, accompanied by loss of the -CH_2-CH_2- fragment of the ring, dehalogenation via cleavage of the C-Cl bond, and dehydratation of the OH groups of the alkyl fragment of the molecule. [M - $C_2H_3Cl_2O_2$]$^+$ [409.2 - 129 = 280.2]. The gross formula of the resulting product ion is $[C_{16}H_{32}N_4]^+$.

The product doubly charged ion at *m/z 172.1* is formed through the following fragmentation mechanism: the deprotonated oxygen -O- forms a double bond with the C3 carbon of the side alkyl chain, followed by cleavage of the C-C bond between carbons 2 and 3 of the second side alkyl chain and cleavage of the C-O bond at carbon 3 [M - CH_2ClO]$^{2+}$ [409.2 - 65 = 344.2]. The gross formula is $[C_{17}H_{33}ClN_4O]^{2+}$.

The next fragmentation pathway of the precursor ion at *m/z 205.2* is due to deep fragmentation of the cyclic system: asymmetric cleavage of the central piperazine ring, cleavage of C-C bonds between carbons 4 and 5, 7 and 8, and 16 and 15. Subsequent cleavage of the C-N bond at nitrogen N3 in the third position is accompanied by loss of the cyclic -CH_2- fragment. Simultaneous cleavage of the C-C bond in the side alkyl fragment leads to loss of the chlorohydroxyethyl fragment -CH-(OH)-CH_2-Cl. Accordingly, the proposed fragmentation mechanism is [M - $C_{12}H_{18}Cl_2N_2O_2$]$^+$ [409.2 - 292 = 118.2]. The gross formula of the ion is $[C_6H_{18}N_2]^+$.

On the basis of the obtained MS/MS spectra, the fragmentation scheme shown in Figure 6 is proposed.

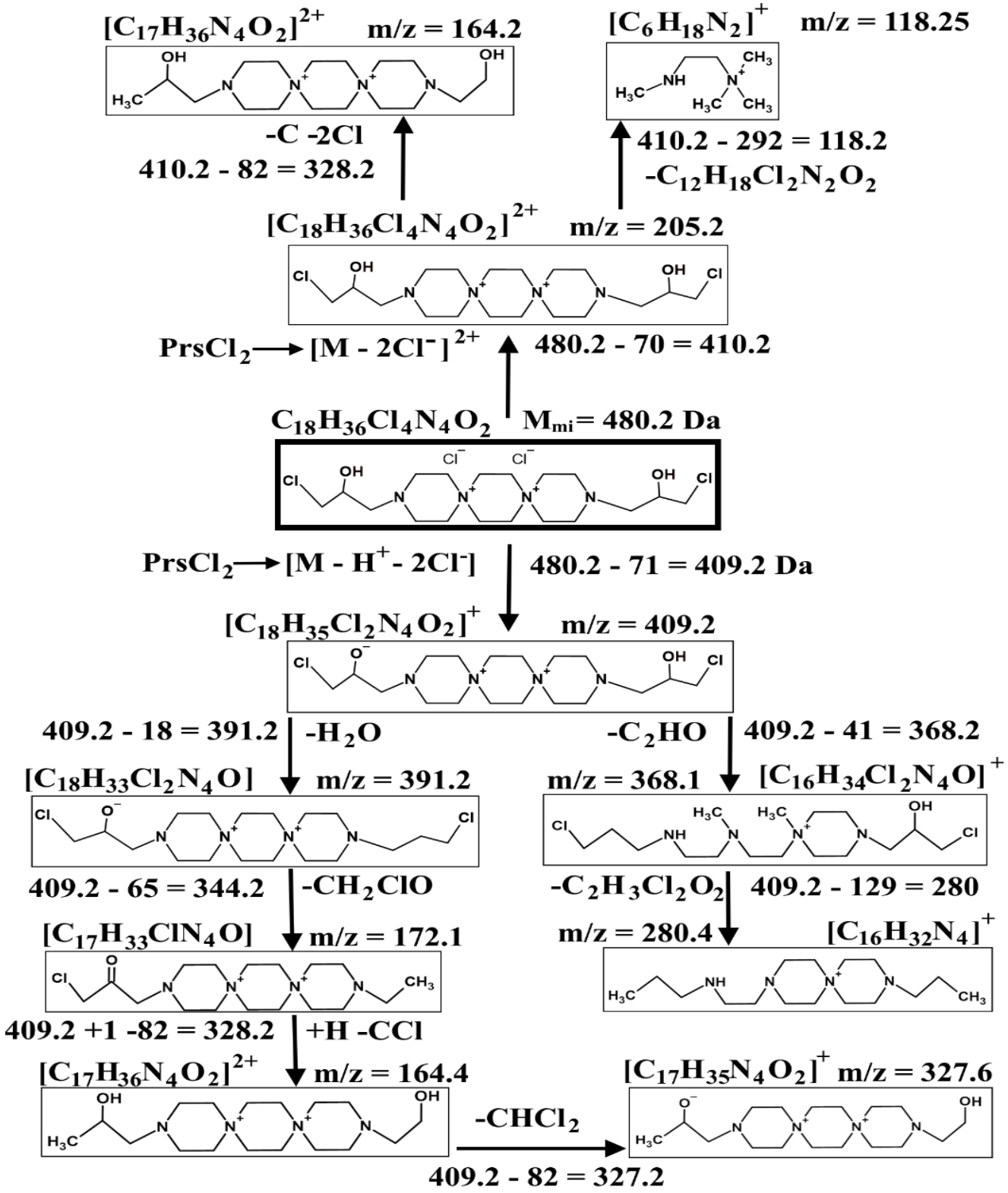


Figure 6: Fragmentation pathways of the ions at *m/z 205.2* and *409.2* of $PrsCl_2$ in DIP-ESI-MS/MS mode.

The fragmentation scheme of the ions at *m/z 205.2* and *409.2* under ESI-MS/MS conditions illustrate the proposed pathways of product-ion formation, neutral losses and structural fragments generated by collision-induced dissociation.

The presence in the MS/MS spectra of both *m/z 205.2* and *409.2* of the same doubly charged product ion at *m/z 164.4* allows it to be identified as the most stable fragment of the $PrsCl_2$ molecule and proposed as a DFI. Based on the fragmentation mechanism involving dehalogenation of one side alkyl chain and cleavage of the C–C bond between carbons 1 and 2 of the second alkyl chain, the ion at *m/z 164.4* represents the retained tripiperazine structure characteristic of the $PrsCl_2$ molecule and distinguishes it from possible impurities with different structures. Therefore, considering all characteristics of the ion at *m/z 164.4*, the MRM transitions [409.25 -> 164.4] and [205.11 -> 164.4] can be proposed for a quantitative $PrsCl_2$ assay.

Overall, the MS/MS data indicate a robust fragmentation behavior of $PrsCl_2$, which makes it possible to use the demonstrated MS and MS/MS spectra, as well as the precursor and product ion data, both for identification purposes through spectral library matching and for quantitative mass spectrometry using MRM/SRM transitions.

CONCLUSION

In this study, MS spectra of $PrsCl_2$ substance were obtained and used to propose an ionization mechanism. The $PrsCl_2$ molecule contains two charged N+ quaternary onium centers stabilized by chloride anions, so the main ionization pathway involves cleavage of N+–Cl- ionic bonds, formation of a charged dicationic species, and subsequent ionization accompanied by deprotonation with charge loss or formation of double bonds. The resulting method for authenticity testing of the $PrsCl_2$ API in aqueous solutions can be considered a practical output of this work.

Analysis of the MS/MS spectra of the selected ions at *m/z 205.2* and *409.2* made it possible to establish precursor-ion fragmentation regularities, identify the most labile regions of the molecule, and compile a table of fragment ions needed to build a DFI library for the studied sample. The most characteristic fragment for both precursor ions was the doubly charged ion at *m/z 164.4*, containing the tripiperazine cyclic core, which makes it a promising target for further developments in quality control of $PrsCl_2$ substance.

Conflicts of Interest: The authors declare that they have no conflicts of interest.

Contributors: All authors have read and approved the final manuscript.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Abbreviations

DIP	**Is a specialized sampe introduction device used in mass-spectrometry;**
$PrsCl_2$	Prospidin, prospidium chloride;
API	It is a chemical or biological substance that provides a therapeutic effect;
MRM	Multiple Reaction Monitoring transitions represent an analytical approach in mass spectrometry that monitors the specific fragmentation of precursor ions into product ions;
LD_{50}	Median lethal dose is the average dose of a substance required to kill half the members of a tested population. It serves as an indicator of the hazard level for toxic and moderately toxic substances;
Q1, Q2, Q3	First, second and third quadrupole;
ESI-MS/MS	Electrospray ionization tandem mass spectrometry is an advanced analytical technique that combines a soft ionization method (ESI) with a two-stage mass analyzer (MS/MS);
Mi	Monoisotopic mass is the molecular mass of a substance calculated by summing the exact atomic masses of the most naturally abundant stable isotopes for each chemical element in the molecule;
DFI	Diagnostic Fragment Ions represent specific ions generated via molecular fragmentation upon tandem mass spectrometry.

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