Journal of Pharmaceutical Analysis 13 (2023) 683–688

Contents lists available at ScienceDirect

Journal of Pharmaceutical Analysis

journal homepage: www.elsevier.com/locate/jpa

Original article

Identification, structure elucidation and origin of a common pyridinium-thiocyanate intermediate in electrospray mass spectrometry among the benziamidazole-class proton pump inhibitors

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ARTICLE INFO

Article history:
Received 25 December 2022
Received in revised form
5 April 2023
Accepted 18 April 2023
Available online 21 April 2023

Keywords:
Proton pump inhibitor
Mass spectrometry
Electrospray ionization
Pseudo-molecular ion
Pyridinium thiocyanate

ABSTRACT

During the analysis of benziamidazole-class irreversible proton pump inhibitors, an unusual mass spectral response with the mass-to-charge ratio at $[M+10]^+$ intrigued us, as it couldn't be assigned to any literature known relevant structure, intermediate or adduct ion. Moreover, this mysterious mass pattern of $[M+10]^+$ has been gradually observed by series of marketed proton pump inhibitors, viz. omeprazole, pantoprazole, lansoprazole and rabeprazole. All the previous attempts to isolate the corresponding component were unsuccessful. The investigation of present work addresses this kind of signal to a pyridinium thiocyanate mass spectral intermediate **(10)**, which is the common fragment ion of series of labile aggregates. The origin of such aggregates can be traced to the reactive intermediates formed by acid-promoted degradation. These reactive intermediates tend to react with each other and give raise series of complicated aggregates systematically in a water/acetonitrile solution by electrospray ionization. The structure of the corresponding pyridinium thiocyanate species of omeprazole (**10a**) has been eventually characterized with the help of synthetic specimen (**10a′**). Our structural proposal as well as its origin was supported by in situ nuclear magnetic resonance, chemical derivatization and colorimetric experiments.

1. Introduction

The irreversible proton pump inhibitors (PPIs) of the benziamidazole-class are group of acid-sensitive prodrugs, which have been proved to be effective at suppressing gastric acid secretion by binding to the cysteines of the H^+/K^+-ATPase covalently. The pharmacophores of PPIs are pyridyl, methylsulfinyl and benzimidazole, which connect to each other as in timoprazole. This basic structural framework has been maintained through the development of PPIs (Table 1).

The two base moieties in the molecule endow PPIs with two pKas and therefore PPIs are to be activated first through doubled protonation processes (Fig. 1). The secondary protonation on the benzimidazole triggers an intramolecular rearrangement, namely Truce-Smiles rearrangement [1,2]. Through a spiro intermediate, a pyridinium sulfenic acid (**2**) will be formed. This active species can either react directly or via a cyclic pyridinium sulfenamide intermediate (**3**) with the cysteine and form a disulfide adduct (**4**) [3,4]. The covalent binding on the Cys321, Cys813 and Cys822 within the proton pumps as to on the Cys892 outside the proton pumps will eventually block the proton pump [5,6]. The substituents on the pyridine and benzimidazole rings may have a certain impact on the activation of the PPIs. All the irreversible PPIs are pharmacologically acid-sensitive. Due to the acid-labile character of PPIs, the corresponding protolytic substance is a matter of concern in pharmaceutical production [7].

Recently, a feeble signal with mass-to-charge ratio (m/z) at 355.1227 in the samples of omeprazole (**1a**) aroused our

Peer review under responsibility of Xi'an Jiaotong University.
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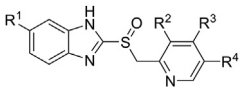
https://doi.org/10.1016/j.jpha.2023.04.011

Table 1
Frameworks of the marketed irreversible proton pump inhibitors (PPIs, **1**).



		R^1	R^2	R^3	R^4
Omeprazole (**1a**)/Esomeprazole	1988 SE 2000 GB	MeO–	Me–	MeO–	Me–
Pantoprazole (**1b**)	1994 DE	CF_2HO–	MeO–	MeO–	H–
Lansoprazole (**1c**)/Dexlansoprazole	1991 FR 2009 US	H–	Me–	CF_3CH_2O–	H–
Rabeprazole (**1d**)	1997 JP	H–	Me–	$MeO(CH_2)_3O$–	H–
Ilaprazole (**1e**)	2008 CN	1-Pyrrolyl–	Me–	MeO–	H–
Leminorazole (**1f**)	2010 JP	MeO–	Me_2N–	H–	H–

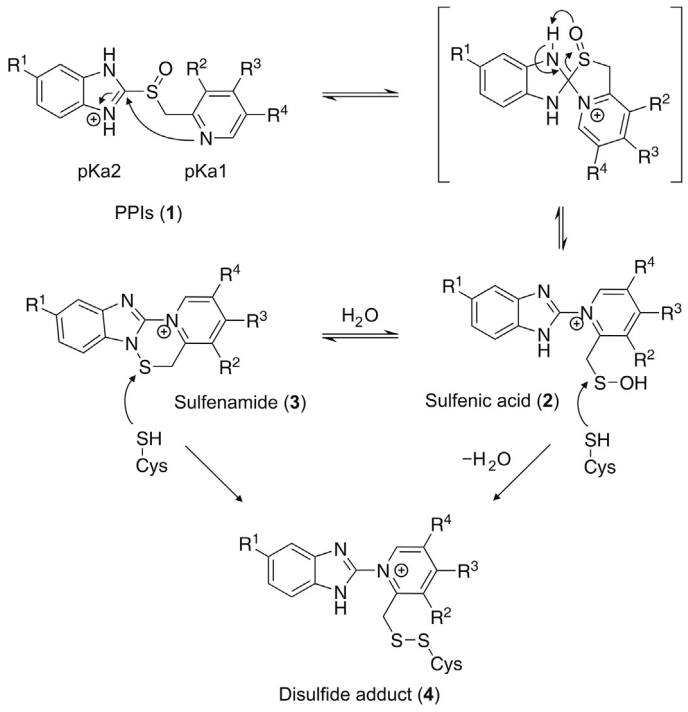


Fig. 1. Mechanism of acid activation of proton pump inhibitors (PPIs) [1].

attention (Fig. S1A). Considering the protonic adduct ion of omeprazole $[M+H]^+$ is to be found at *m/z* 346.12, $[M+10]^+$ appears not to be an common adduct ion of ammonium or alkali metals. A reasonable molecular formula fitting to *m/z* 355.12 is $[M-O+C+N]^+$ at *m/z* 355.1223. The degree of unsaturation of this molecular formula reminds us of a cyanide group (−CN). According to the best of our knowledge, no cyanide containing related substance or intermediate in PPIs has been reported. The corresponding component has been subsequently isolated via preparative liquid chromatography (LC). Interestingly, the mass spectral response of $[M+10]^+$ was just minority in the residue after evaporating the solvent. It seems that the related substance was labile. All the attempts of isolation and characterization of this species remained unsuccessful. In the present study, quadrupole time-of-flight mass spectrometry (Q-TOF MS), nuclear magnetic resonance (NMR) analytical methods combined with solid phase extraction (SPE) separation technique, chemical derivatization and colorimetric strategies were applied for investigating this phenomenon. A pyridinium thiocyanate species, a common fragment ion derived from hydrolyzed PPIs, has been identified and the origin of such species has been discussed.

2. Materials and methods

2.1. Materials

PPIs (Omeprazole, pantoprazole, lansoprazole and rabeprazole), derivatization reagents (cysteamine, 5,5-dimethyl-1,3-cyclohexanedione), pyridyl and benzimidazole building blocks (2-(chloromethyl)-3-methyl-4-(2,2,2-trifluoroethoxy)pyridine hydrochloride, 5-methoxy-2-thio-1H-benzimidazole, 5-(difluoromethoxy)-2-mercapto-1H-benzimidazole) of analytical grade were purchased from Macklin Biochemical Co., Ltd. (Shanghai, China). High-performance liquid chromatography (HPLC)-grade acetonitrile and methanol were from Fisher Scientific Co., Ltd. (Shanghai, China). All other reagents and solvents were of analytical grade.

2.2. MS

MS was performed on a triple TOF 5600 and 6600 mass spectrometer (SCIEX, Toronto, Canada), each equipped with a TurboIonSpray™ ESI source. Data acquisition was achieved using Analyst software 1.6.1 and 1.5.2. In fragmentation experiments, analyte solutions were diluted thousand times with diluents and infused into the MS through a syringe pump at 10 μL/min. Optimized MS parameters were as follows: Source temperature, 500 °C; ion spray voltage, 5500 V; nebulizer, heater, and curtain gas (N_2), 50, 50, 20 psi, respectively. Declustering potential (DP), collision energy (CE), and air flow (AF2) values were tuned manually in each experiment.

2.3. Degradation and derivatization protocol

To a solution of 1 mg/mL PPI (water/acetonitrile, 4/1), 1 μL formic acid was added and the mixture was incubated for 30 min at ambient temperature. Obvious color change from pale yellow to dark brown could be observed by the samples of omeprazole, lansoprazole and ilaprazole. For derivatization reactions, an acetonitrile solution of 1 mg/mL corresponding derivatization reagent (cysteamine, 5,5-dimethyl-1,3-cyclohexanedione) was added to the aforementioned degradation solution and incubated for 15 min at ambient temperature.

2.4. SPE purification and NMR

Pre-condition of the SPE cartridges (Agilent Bond Elut™ C18, 500 mg, 3 mL; Folsom, CA, USA): flush the SPE cartridges sequentially with 2 mL acetonitrile (1 mL each time) and 2 mL water (1 mL each time), and excess solvent was removed under vacuum. Load the degradation sample to the pre-conditioned SPE cartridge and wash the cartridge with 2 mL water (1 mL each time), and excess solvent was removed under vacuum. Elute the loaded SPE cartridge with 1 mL d_4-methanol for the direct NMR experiments. The 1D

and 2D NMR experiments were performed on Bruker™ 400 MHz and 600 MHz spectrometer (Billerica, MA, USA).

3. Results and discussion

3.1. MS responses of $[M+10]^+$ among the PPIs

The species of *m/z* 355.12 should be a relevant substance of omeprazole (**1a**), since a benzimidazole product ion at *m/z* 149.07 can be found in the multiple reaction monitoring (MRM) experiment (Fig. S1B). At the beginning, the MS response at *m/z* 355.12 could be only occasionally observed. Investigations indicate water/acetonitrile mixed solvent and low pH value favor the transformation from $[M+H]^+$ to $[M+10]^+$. Moreover, this phenomenon could be reproduced by series of PPIs (Fig. S2).

3.2. Solvent effect and solvent adducts

Considering the acid-labile character of PPIs, the $[M+10]^+$ species is presumably related with the acid-promoted degradation reaction. The protonolysis experiments with omeprazole (**1a**) have been conducted in two kinds of mixed solvent systems respectively, viz. water/methanol and water/acetonitrile. The protolytic samples were then diluted 100 times and analyzed through syringe pump. A signal of the $[M+10]^+$ kind for **1a** at *m/z* 355.12 can be only obviously observed in water/acetonitrile diluents, regardless which kind of protolytic solvent has been applied.

Such a solvent specific mass spectral response gives an impression that the signal of $[M+10]^+$ might be an acetonitrile adduct ion [8]. A parallel experiment has been then conducted in water/d_3-acetonitrile. If the mass spectral response at *m/z* 355 was an acetonitrile adduct, a corresponding adduct ion of deuterium acetonitrile should be observed at *m/z* 358. The relative percentage signal intensity of *m/z* 355 in d_3-acetonitrile (Fig. S3A) is stronger than that in acetonitrile (Fig. S3B), but without the trace of *m/z* 358. Beyond that, the collision-induced dissociation (CID) of *m/z* 355.12 produces a fragment at *m/z* 328.12, which matches the cyclic pyridinium sulfenamide intermediate (**3a**). It hints that the molecular scaffold of omeprazole remains unchanged and the signal at *m/z* 355.12 couldn't be an acetonitrile adduct ion.

The hybrid solvent experiment has been then carried out with other PPIs. **1a**–**d** have been first acidolyzed in water/acetonitrile for 40 min at room temperature, followed by dilution 49 times with different diluents (water/methanol, water/ethanol or water/acetone) and analyzed through syringe pump. The MS responses of $[M+10]^+$ were faint in the diluents others than water/acetonitrile, while signals of **1a**–**d** or thioether **8a**–**d** in the mixture were not significantly affected by the solvent shifting. Moreover, solvent adducts have been observed, especially in water/acetone (Table S1). Since $[M+10]^+$ isn't an acetonitrile adduct but its occurrence is acetonitrile dependent, it is highly likely derived from some labile intermediates.

3.3. SPE enrichment and in situ NMR experiment

In order to prevent the potential decomposition during the separation or solvent evaporation procedure, SPE separation has been employed for the enrichment of the target substance. Washing the loaded SPE column with deuterated solvent, the eluent was directly ready for an NMR test. The 1D/2D NMR and TOF MS experiments point the main component in the eluent to a disulfide dimer **7a**, a literature known degradation product. Although the mass spectral response at *m/z* 355.12 is still to be detected in the eluent, no sign of cyanide could be observed by distortionless enhancement by polarization transfer (DEPT) NMR. Moreover, the color of the sample **7a** in NMR tube turned from yellow to black after several days deposing at ambition temperature. Retesting of the sample by NMR and MS indicates the disulfide dimer **7a** was transformed into thioether **8a** spontaneously and completely [9–13].

According to the previous work [3,14], the disulfide dimer **7a** is formed between two reactive intermediates, pyridinium sulfonamide **3a** and pyridinium thiole **6a** (Fig. 2). The mass spectral response at *m/z* 355.12 may suggest the participation of some short-life cyanide intermediate during the protonolysis. Consequently, an in situ protonolysis experiment has been conducted in NMR-tube, in order to capture some evidences of such a “transient” intermediate. With the decay of the omeprazole signals within 15 min at room temperature, only a reactive intermediate sulfenic acid **2a** has been recorded. There is no newly emerged sp-hybridized carbon signal in the NMR spectrum to be found. Based on the aforementioned observation, we had the assumption that the mass spectral response of $[M+10]^+$ was a product ion of some intermediates instead of intermediates themselves.

3.4. Series of double charged pyridinium salt dimers

It has been found that the signal of $[M+10]^+$ in the protolytic solution of **1** was always accompanied by series of double charged species, such as the disulfide dimer **7a**. The double charged species are to be recognized easily by MS from their 0.5 Da mass difference between the isotope signals. For example, the MS response of **7a** is to be found at *m/z* 329.12, its single charged form **7a′** is to be found at *m/z* 657.23. Beyond that, thiosulfinate intermediate **5a** at *m/z* 337.12 and 673.23 and trisulfide dimer **9a** at *m/z* 345.10 and 689.20 can also be found in the protolytic sample of omeprazole (Fig. 3). It has been noticed later that the pattern of $[\text{pyridinium dimer}]^{2+}$, $[\text{pyridinium dimer}+O]^{2+}$ and $[\text{pyridinium dimer}+S]^{2+}$ can be observed in the protolytic samples across the PPIs (Table S2).

Noteworthy, in the protolytic solution of **1a,** there are still series of pyridinium dimers with feeble intensity to be found at *m/z* 533.65, 536.22, 549.64, 552.20, 557.62, 560.18, etc. Without evidence from a secondary characterization method, the structures of these species remained unrevealed. However, it indicates that the pyridinium intermediates of PPIs tend to aggregate and form some complicated dimers systematically during the protonolysis. Such aggregates are labile under the electrospray ionization (ESI) or CID conditions. The signal of $[M+10]^+$ should be one kind of stable CID fragment ion in common.

3.5. Structure and mechanism proposal of $[M+10]^+$

All the aforementioned protolytic intermediates of omeprazole observed by NMR and MS contained the segment of 2-pyridiniobenzimidazolide. And the MRM transition of *m/z* 353.1 → 26.0 and 57.9 under negative mode suggests that this fragment contains a CN- and SCN-moiety (Fig. S1C). Following these structural clues, the pyridinium thiocyanate (**10**) for the $[M+10]^+$ emerged (Fig. 4). Consequently, the thiocyanate **10a′** in form of bromide salt has been synthesized and investigated (Fig. S4), which exhibits the exactly identical MS^2 fragmentation pattern to that of **10a**. The structure of **10a′** has been characterized by 1D/2D NMR. The sp-hybrid carbon of the thiocyanate group has been recorded by NMR at 112.5 ppm in $CDCl_3$. Unlike the insubstantial behavior of *m/z* 355.12 in the previous experiments, the synthesized thiocyanate **10a′** is relatively stable in water/methanol solution at low temperature. These results again confirmed our proposal that the mass spectral response of $[M+10]^+$ is an MS

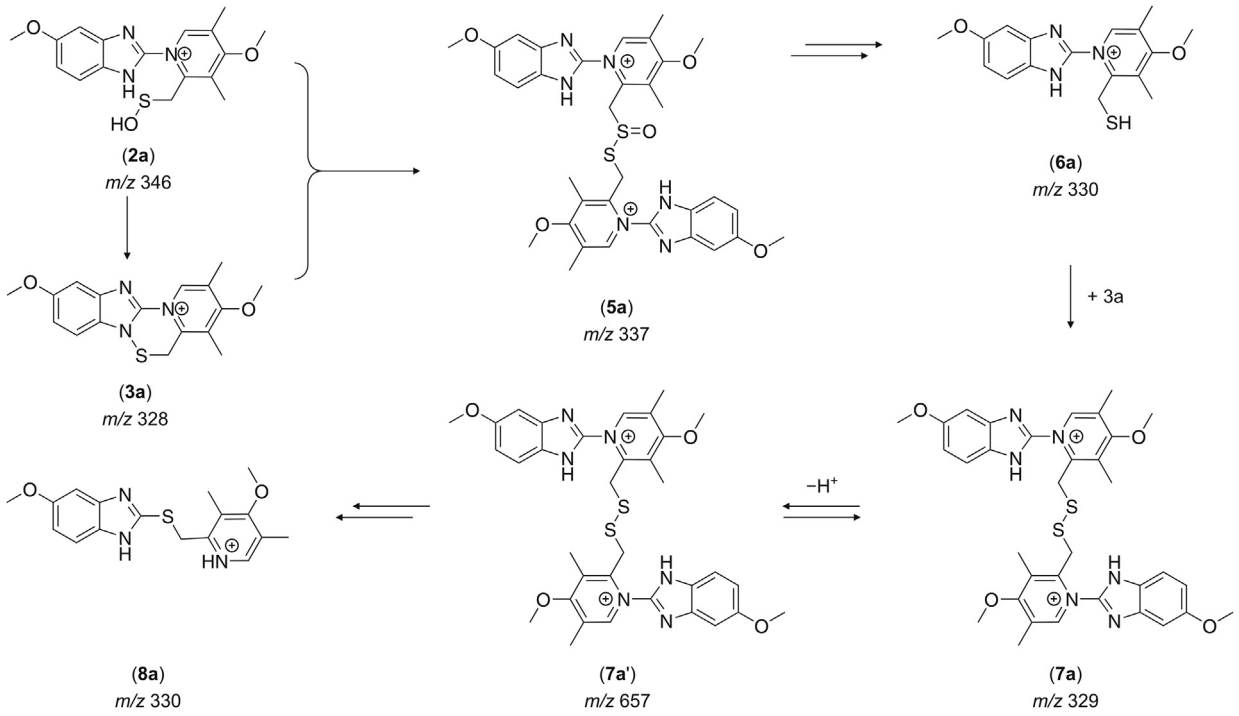


Fig. 2. Literature reported formation of disulfide dimer **7**.

fragment ion of some labile aggregates. This explains why no corresponding carbon signal of thiocyanate has been ever recorded by NMR in the previous examinations. It has been noticed that **10a′** decomposed slowly in aqueous solution at room temperature. Degradation product of **10a′** is 4-pyridone (**10a″**) (Fig. 4), which is formed by bromide-associated dealkylation similar to Michaelis-Arbuzov reaction.

NMR of **10a′**: ^{1}H NMR (600 MHz, $CDCl_3$): δ = 8.88 (s, 1H), 7.56 (d, *J* = 9.0 Hz, 1H), 7.09 (d, *J* = 2.4 Hz, 1H), 6.98 (dd, *J* = 2.4, 9.0 Hz, 1H), 4.59 (s, 2H), 4.30 (s, 3H), 3.80 (s, 3H), 2.61 (s, 3H), 2.60 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 172.9, 158.3, 149.1, 146.6, 142.0, 131.6, 128.0, 118.8, 115.7, 112.5, 97.0, 62.6, 56.0, 33.9, 16.0, 13.6. high-resolution mass spectrometry (HRMS) of $[M+H]^+$ theoretical 355.1223, found 355.1270.

NMR of **10a″**: ^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.78 (s, 1H), 7.58 (d, *J* = 9.0 Hz, 1H), 7.14 (d, *J* = 2.4 Hz, 1H), 6.99 (dd, *J* = 2.4, 9.0 Hz, 1H), 4.46 (s, 2H), 3.87 (s, 3H), 2.26 (s, 3H), 1.91 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 178.7, 157.6, 144.3, 141.7, 138.5, 126.0, 124.2, 117.5, 115.7, 114.2, 97.6, 56.0, 32.9, 14.0, 12.4. HRMS of $[M+H]^+$ theoretical 341.1067, found 341.1096.

3.6. Colorimetric analysis

Colorimetric analysis experiments have been also carried out for the determination of cyanide. Hereby, the colorimetric test kit VISOCOLOR® ECO Cyanide (Macherey-Nagel, Düren, Germany) was employed. The solution system applied in the colorimetric test appears to influence the results dramatically. In the water/methanol solution, all the colorimetric tests of cyanide were negative. Meanwhile, several cyanide test results in a water/acetonitrile solution were positive, which turned to be false positive results later when compared with the control group. According to literature and product instruction, the cyanide analysis with the chloramine T/isonicotinic acid/1,3-dimethyl barbiturate system is susceptible to the interference of thiole, thiocyanate, halide ions, etc [15,16]. Since no cyanide ion in **1a** or in its protolytic solution has been observed, the thiocyanate **10a** should be a rearrangement product formed in situ during the CID process. The same solvent effect observed by the protonolysis and the colorimetric tests indicates that water/acetonitrile solution system benefits the formation of such aggregates.

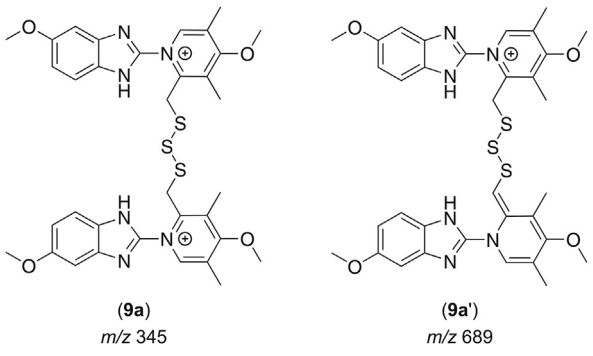


Fig. 3. Trisulfide dimers **9a** and **9a′**.

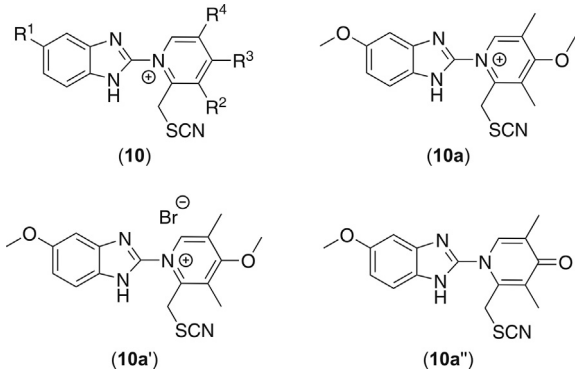


Fig. 4. Structure proposal of *m/z* 355 (**10a**) and synthesized sample (**10a′**).

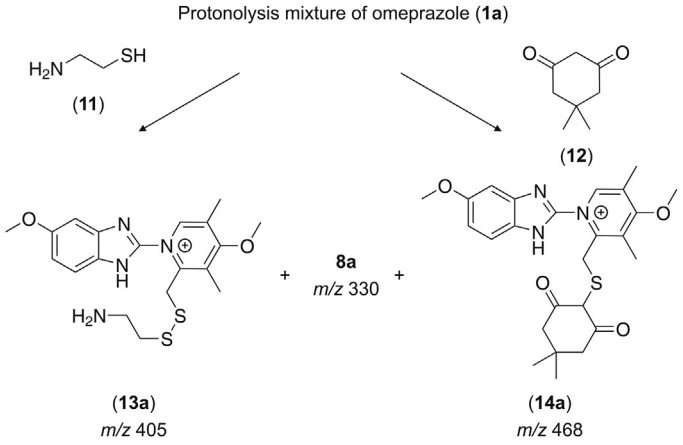


Fig. 5. Chemical derivatization of the **1a** protolytic sample.

3.7. Chemical derivatization for MS

In order to verify the origin of the thiocyanate **10a**, chemical derivatization experiments have been performed. Two derivatization reagents, cysteamine **11** and dimedone **12**, have been chosen, which are capable of capturing reactive intermediates such as disulfide, sulfenic acid, thiole as well as thiocyanate [4,16–20]. After protonolysis for 30 min, when the mass spectral response of omeprazole **1a** or sulfenic acid **2a** at *m/z* 346.12 disappeared, derivatization reagents were added to the reaction mixture. Along with the vanishing of **10a** at *m/z* 355.12, derivatization products **8a**, **13a** and **14a** have been detected by MS (Fig. 5). In both of the experiments, thioether **8a** was the main component in the reaction mixture after the derivatization (Fig. 6). The formation of disulfide **13a** is literature known, as to the formation of thioether **8a** through a further reaction between disulfide **13a** and cysteamine **11.** However, if **10a** does exist as a real entity in the solution, the consumption of **10a** at *m/z* 355.12 during the derivatization must lead to the release of cyanide. Actually, cyanide could be found neither in cysteamine nor in dimedone treated sample by MS under negative mode. The absent of the cyanide fragment after derivatization further supports our hypothesis that the **10** is merely a MS species.

The MS^2 patterns of the derivatization products **13a** and **14a** are quite similar to that of **10a**. The signal intensity of their common sulfonamide fragment **3a** is more intensive by **13a** than by **14a** or **10a** (Figs. S5A–C). The different MRM transition efficiency of sulfonamide **3a** can be addressed to the different bond dissociation energies between the S–S bond (65 kcal/mol) by **13a** and the S–C bond (72 kcal/mol) by **10a** and **14a**. This result matches our structural proposal of **10a** perfectly.

The derivatization experiments have been then carried out with the synthesized thiocyanate **10a′** (Figs. S5D–G). The reaction between **10a′** and dimedone **12** give thioether **14a** as the only product. The derivatization product profiles of **10a'** can be well explained by the nucleophilic reactivity of cysteamine and dimedone. In comparison, the nucleophilicity of cysteamine is better than dimedone. Differential product profiles correctly match the electrophilic reactivity of the thiocyanate group. Accordingly, the complicated product profiles by **1a** can be acutely explained as derivatization products of series of reactive intermediates, such as **5a**, **7a** or **9a**. Therefore, the disappearance of *m/z* 355.12 should be rather attributed to the consumption of these reactive intermediates, namely its precursor species.

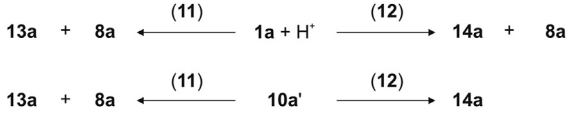


Fig. 6. Differential derivatization product profiles of protolytic sample and **10a′**.

3.8. Precursor ion scan

By the precursor ion scan of **10a**, series of precursor ions have been identified, such as *m/z* 764.85, 769.61 and 991.90 (Fig. S6A). The structures of these precursor ions remained unclear, but all of these precursor ions can give rise of *m/z* 355.12 as the only product ion under a low CE by MRM experiment. These results support the proposal that the signal of $[M+10]^+$ is one kind of common CID fragment of some labile aggregates. In order to confirm this speculation, hybrid experiments have been carried out with synthesized structural analogues. Structural analogues with partially identical moieties are likely to aggregate together during ESI process. If such metastable aggregates do exist, one kind of hybrid precursor ion must exist, when two kinds of PPIs analogues have been mixed together and hydrolyzed.

First, omeprazole **1a** and its analogue **1g** (Fig. 7) have been investigated separately. The corresponding $[M+10]^+$ fragments in the protolytic samples have been observed, i.e. **10a** at *m/z* 355.12 and **10g** at *m/z* 409.09 (Figs. S6A and B). Their precursor ions have been collected individually. Then, **1a** and **1g** have been mixed and hydrolyzed. The precursor ion scans of **10a** and **10g** in the mixed protolytic sample are basically consistent with their individual collected scans, but with several additional signals (Figs. S6C and D). Among these newly emerged signals, a common precursor ion at *m/z* 818.7 has been identified, whose MS^2 fragments contained *m/z* 355.12 and 409.09 simultaneously (Fig. S6E). The result of mixed protolytic experiment by pantoprazole **1b** and its analogue **1h** (Fig. 7) was quite the same. By the precursor ion scan of $[M+10]^+$ fragments of **10b** (*m/z* 393.08) and **10h** (*m/z* 377.09), a hybrid precursor ion at *m/z* 824.1 has been identified. The hybrid experiments have been then extended across all of the PPIs. Two series of hybrid precursor ions with the same pattern have been identified, namely, $[\text{set } \mathbf{x}+\text{set } \mathbf{y}+54]^+$ and $[\text{set } \mathbf{x}+\text{set } \mathbf{y}-28]^+$. The observed common hybrid precursor ions of the mixed PPIs in Fig. S7 were listed in Table 2. The presence of systematical hybrid precursor ions is an auxiliary evidence for the existence of such a metastable aggregation state. More importantly, the origin of the $[M+10]^+$ fragments **10** turned out to be the product ions thereof and formed in situ by CID, instead of deriving from some cyanide impurity.

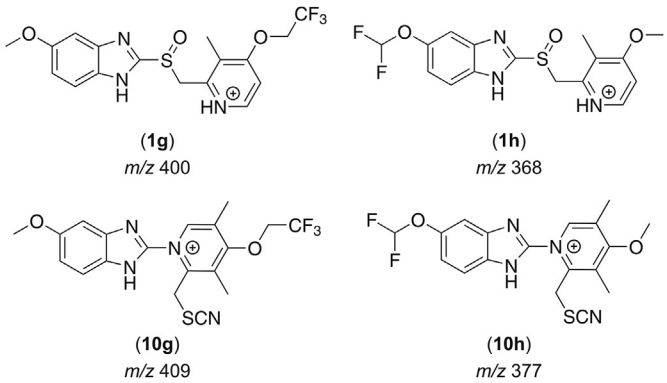


Fig. 7. Synthesized derivates and the corresponding $[M+10]^+$ fragments.

Table 2
Hybrid precursor ions between **10**s.

Hybrid precursor ions between **10**s out of the set **x** and **y**		set **x**		
		*m/z*369.13 (**10d**)	*m/z*379.08 (**10c**)	*m/z* 393.08 (**10b**)
set **y**	*m/z*355.12 (**10a**)	*m/z*778.43[a], 696.38[b]	*m/z*788.43[a], 706.39[b]	*m/z*802.44[a], 720.40[b]
	m/z 393.08 (**10b**)	*m/z*816.45[a], 734.40[b]	*m/z*826.45[a], 744.41[b]	–
	*m/z*379.08 (**10c**)	*m/z*802.44[a], 720.40[b]	–	–

[a] [set x+set y+54]$^+$; [b] [set x+set y−28]$^+$.

4. Conclusion

A common mass spectral response in art of $[M+10]^+$ in series of protolytic samples of PPIs has been identified as pyridinium thiocyanate **10** after a comprehensive assessment. This structure proposal has been confirmed with the synthesized derivate of omeprazole (**10a**′). When fitting together the puzzle of the solvent effect, colorimetric analyses, chemical derivatization and precursor ion scan, the origin of these species becomes distinct. It turns to be a product ion of labile pyridinium aggregates formed in situ by CID. This explanation is consistent with all the experimental observations. The discovery of such common mass spectrometry intermediates may not only ease the anxiety of pharmaceutical companies, but also provide insight into the genesis of “ghost signals” by ESI-MS.

CRediT author statement

Dong Sun: Conceptualization, Methodology, Writing - Original draft preparation; Chunyu Wang: Investigation; Yanxia Fan: Supervision; Jingkai Gu: Resources, Writing - Reviewing and Editing, Funding acquisition.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This study is supported by the National Natural Science Foundation of China (Grant Nos.: 82030107 and 81872831) and the National Science and Technology Major Projects for significant new drugs creation of the 13th five-year plan (Grant Nos.: 2017ZX09101001 and 2018ZX09721002007).

Appendix A Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpha.2023.04.011.

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