separations

MDPI

Article

Unveiling Novel Chaotropic Chromatography Method for Determination of Pralidoxime in Nerve Agent Antidote Autoinjectors

Bohyun Shin [1], Hyung-seung Kim [2], Ji-Youn Lee [1], Sumin Seo [1], Cho Hee Jeong [1], Eunbin Bae [1], Jiyu Kim [1], Hyojeong Lee [1], Donghee Lee [1], Dong-Kyu Lee [1,*] and Sang Beom Han [1,*]

[1] Department of Pharmaceutical Analysis, College of Pharmacy, Chung-Ang University, 84 Heukseok-ro, Dongjak-gu, Seoul 06974, Republic of Korea; qhgus4510@naver.com (B.S.); velvetrose0805@naver.com (J.-Y.L.); ssm1302@naver.com (S.S.); wjdchgml@cau.ac.kr (C.H.J.); eunbin0807@cau.ac.kr (E.B.); bbds1904@cau.ac.kr (J.K.); jung7513_@naver.com (H.L.); dheehee1023@naver.com (D.L.)

[2] Division of Forensic Toxicology and Chemistry, Defense Institute of Forensic Science, Ministry of National Defense, Seoul 04383, Republic of Korea; yknowkhs@mnd.go.kr

* Correspondence: leedk@cau.ac.kr (D.-K.L.); hansb@cau.ac.kr (S.B.H.); Tel.: +82-2-820-5854 (D.-K.L.)

Abstract: Pralidoxime chloride, a highly hydrophilic antidote, cannot be effectively separated by reverse-phase high-performance liquid chromatography (RP-HPLC), unless the mobile-phase composition is varied. However, the use of ion-pairing reagents for pralidoxime separation is hindered by the persistent contamination of the stationary phase or chromatography system inside the HPLC system. Thus, this study aimed to develop a simple, rapid, and robust method based on RP-HPLC to determine pralidoxime chloride in antidote autoinjectors using a chaotropic salt as the mobile-phase additive. The use of UV detection at 270 nm allowed for the simultaneous detection of pralidoxime chloride and the internal standard, pyridine-2-aldoxime. The addition of chaotropic salts ($NaPF_6$, $NaBF_4$, and $NaClO_4$) and an ionic liquid ([EMIM]PF_6) increased the retention time of pralidoxime chloride. Among them, $NaPF_6$ exhibited the highest capacity factor in the reverse-phase C18 column. Increasing the salt concentration increased the capacity factor and the number of theoretical plates. Analytical method validation was performed to assess the linearity, accuracy, precision, recovery, and repeatability, according to the Ministry of Food and Drug Safety guidelines. Additionally, this newly developed method exhibits an adequate separation capability, making it a potential substitute for the current method employed in the United States/Korean Pharmacopoeia, and it ensures the necessary durability to maintain the robustness and reliability of the analytical system.

Keywords: pralidoxime chloride; antidote autoinjector; highly hydrophilic; chaotropic salts; $NaPF_6$

Citation: Shin, B.; Kim, H.-s.; Lee, J.-Y.; Seo, S.; Jeong, C.H.; Bae, E.; Kim, J.; Lee, H.; Lee, D.; Lee, D.-K.; et al. Unveiling Novel Chaotropic Chromatography Method for Determination of Pralidoxime in Nerve Agent Antidote Autoinjectors. *Separations* **2024**, *11*, 82. https://doi.org/10.3390/separations11030082

Academic Editors: Xingchu Gong, Shengqiang Tong and Guanglei Zuo

Received: 29 January 2024
Revised: 28 February 2024
Accepted: 4 March 2024
Published: 7 March 2024

1. Introduction

Pralidoxime hydrochloride is a biochemical remedy for organophosphate poisoning, which causes clinical symptoms [1]. To manage such poisoning, an intravenous or intramuscular injection of atropine and pralidoxime is adopted, along with symptomatic care and supportive therapy [2]. This antidote reactivates the cholinesterase enzyme, effectively counteracting the toxic effects in patients exposed to poisons that inhibit acetylcholinesterase by covalent binding to the serine residue [3,4]. The strategic application of emergency antidotes combined with fundamental medical interventions, including ensuring airway patency, providing oxygen, and implementing artificial respiration, can significantly enhance survival [5,6]. These treatments address both muscarinic and nicotinic symptoms, including paralysis of the muscles and diaphragm, involuntary contractions, heart muscle spasms, and central nervous system disturbances such as coma and seizures [7].

Pralidoxime, which is used in intramuscular pressure-type antidote autoinjectors, is a critical countermeasure in military scenarios involving exposure to chemical weapons [8].

These autoinjectors are specifically engineered for optimal performance under severe conditions and are vital for an immediate response to nerve agents such as tabun, sarin, somans, and VX [9]. In such demanding military environments, maintaining the long-term stability of pralidoxime is paramount. However, the chemical structure and composition of pralidoxime chloride make it prone to degradation by hydrogen and hydroxyl ions, leading to its relatively lower stability in solution than atropine, as indicated in various studies [10,11]. Environmental factors, notably the pH and ambient temperature, are critical for determining the stability of pralidoxime chloride. In particular, it is known to be more unstable under alkaline conditions, where various degradation products increase due to consecutive dehydration reactions catalyzed by OH^- and subsequent hydrolysis reactions. There are also studies reporting differences in the extent of the decomposition of pralidoxime depending on the storage temperature [10,12–16]. Consequently, rigorous quality control of the content and degradation products is essential to ensuring that pralidoxime stored over extended periods in autoinjectors maintains efficacy and safety.

Pralidoxime chloride contains a quaternary methylated nitrogen atom that imparts a permanent positive charge to the molecule (Figure 1). Its aromatic pyridinium structure induces the maximum UV absorption at λ_{max} 293 nm under acidic conditions. In the analysis of highly polar ionic compounds, such as pralidoxime chloride, the use of RP-HPLC remains a critical issue for chemical, biomedical, pharmaceutical, and environmental applications. Highly hydrophilic analytes are weakly retained and can elute near the column dead time (t0) under traditional reverse-phase conditions [17]. This reduces the chromatographic resolution, owing to complex matrix interference, and baseline disturbances at t0 can adversely affect the accurate quantification of the object to be analyzed [10,18]. For the HPLC separation of highly polar molecules, analytical methods such as the derivatization of the corresponding compounds, using a hydrophilic interaction chromatography (HILIC) column as a stationary phase, or adding ion-pairing reagents to the mobile phase are typically employed [19–24].

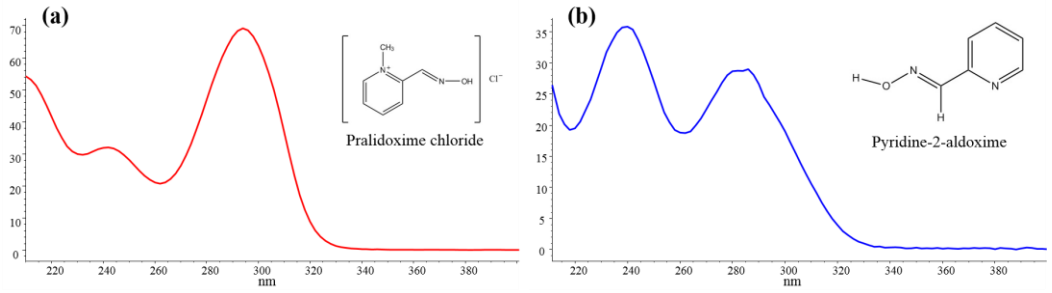

Figure 1. UV absorbance spectra of (**a**) pralidoxime chloride and (**b**) pyridine-2-aldoxime.

To solve these problems, cation-exchange chromatography, size exclusion chromatography, ion-pair chromatography (IPC), reverse-phase chromatography, normal-phase chromatography, capillary electrophoresis, and several other analytical methods have been reported [25–29]. Due to the highly polar nature of the analytes, IPC methods using various ion-pairing reagents are the most frequent and common chromatographic techniques for pralidoxime separation, covering more than 50% of all column chromatography methods. Cation exchange (CEX) has also been effectively utilized, and in some rare instances, separation based on Sephadex has also been achieved. However, such separation techniques have faced challenges in broader applications to diverse samples, including biological samples. In that regard, the combination of reverse-phase stationary phases and various mobile-phase additives has begun to gain attention [4].

Incorporating an ion-pairing reagent as an additive can resolve retention challenges in the chromatographic analysis of polar pralidoxime hydrochloride [30,31]. At a specific buffer solution pH, these reagents form a neutral complex with the analyte, thereby augmenting retention and facilitating more effective separation. Ion-pairing reagents are characterized by their amphiphilic nature, possessing both long alkyl chains and charged

functional groups. Upon introduction into the mobile phase, these reagents effectively transform the matrix into a pseudo-ion-exchange surface. In RP-HPLC, the primary function of ion-pairing reagents is to intensify the interaction between the stationary phase and the analyte, consequently enhancing retention, selectivity, and separation efficiency. However, although ion-pairing reagents offer significant advantages for pralidoxime separation, their use is limited by the continuous contamination of the stationary phase or internal parts of the HPLC system.

In this study, we proposed a new RP-HPLC method for highly polarized pralidoxime separation using a chaotropic salt as a mobile additive. To evaluate the effect of the chaotropic salt as a mobile-phase additive, the developed method was compared with the current official methods of the USP [32] and Korean Pharmacopoeia (KP) [33]. The proposed method was validated by assessing its linearity, accuracy, precision, and recovery. As a result, this new analytical method showed better efficiency in pralidoxime chloride analysis than the two official methods and could be successfully applied to the quantitative analysis of pralidoxime autoinjectors.

2. Materials and Methods

2.1. Reagents and Chemicals

Pralidoxime chloride (1-methylpyridinium-2-aldoxime chloride, >98%) was purchased from TCI Chemicals (Tokyo, Japan). Pyridine-2-aldoxime (pyridine-2-carboxaldoxime, 99%), used as an internal standard (IS), was purchased from Alfa Aesar (Haverhill, MA, USA).

HPLC-grade mobile-phase solvents (water and acetonitrile) were purchased from Fisher Scientific (Waltham, MA, USA).

The reagents used as mobile-phase additives (tetraethylammonium chloride (>98%) and 1-ethyl-3-methlimidazolium hexafluorophosphate ([EMIM]PF_6, >98%)) were purchased from TCI Chemical (Tokyo, Japan). Sodium hexafluorophosphate ($NaPF_6$, 98%), sodium tetrafluoroborate ($NaBF_4$, >98%), sodium perchlorate ($NaClO_4$, >98%), sodium 1-octanesulfonate, and phosphoric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrochloric acid and methanol were purchased from DAEJUNG (Siheung, Republic of Korea). A polytetrafluoroethylene (PTFE) membrane syringe filter (0.2 μm) was obtained from Whatman (Maidstone, UK).

2.2. Preparation of Standard Solutions and Sample

Standard stock solutions of pralidoxime chloride (1000 mg/kg) and pyridine-2-aldoxime (1000 mg/kg) were prepared by dissolving them in deionized water and methanol. Standard solutions were prepared by diluting the stock solution with the mobile phase. The standard solutions were stored at 4 °C. Working solutions were prepared daily by diluting the standard stock solutions. Antidote autoinjectors were obtained using a KMARK-1 kit from the Defense Institute of Forensic Science, Ministry of National Defense, Korea. The autoinjector pralidoxime chloride sample was obtained by injecting a syringe into a sealed headspace vial with a lid and was used after dilution. Then, 1 mL of the injection solution containing pralidoxime chloride was transferred into a 50 mL volumetric flask and filled with distilled water. A 1 mL aliquot of this solution was pipetted into a 25 mL volumetric flask and diluted to volume with deionized water. Subsequently, the diluted injection solution was diluted 10 times with the mobile phase and then filtered with a 0.2 μm PTFE syringe filter.

2.3. Instruments and Equipment

The liquid chromatography system was an HPLC system equipped with an Agilent 1260 Infinity (Agilent, Santa Clara, CA, USA), binary pump VL (G1312C), degasser (G1322A), autosampler (G1329B), diode array detector (DAD) VL (G1315D), and thermostatted column compartment (G1316A). The system was controlled using an Agilent ChemStation (ChemStation for LC 3D systems; Rev. B.04.03-SP1, Agilent, Santa Clara, CA,

USA). A Waters XSELECT HSS T3 C 18 column (150 mm × 4.6 mm, 5 µm) and Phenomenex Luna C18 column (150 mm × 4.6 mm, 5 µm) were used for separations.

2.4. Chromatographic Conditions

Reverse-phase liquid chromatography with isocratic elution was initially employed to explore various mobile-phase additives, including $NaBF_4$, $NaClO_4$, $[EMIM]PF_6$, and $NaPF_6$. The retention effects of the additives were analyzed by determining the capacity factors (k′) and theoretical plates (N) of pralidoxime. Following the selection of the most effective additive, its concentration was varied between 10 and 50 mM to compare the retention. The optimal mobile phase was a mixture of acetonitrile and water containing 30 mM $NaPF_6$ in a 10:90 v/v ratio. The chromatographic conditions were a column temperature of 30 °C; an injection volume of 5 µL; and a flow rate of 1.0 mL/min for an 8 min duration. The UV absorbance spectra of pralidoxime chloride and pyridine-2-aldoxime were analyzed and compared (Figure 1) at a detection wavelength of 270 nm.

We conducted a chromatographic analysis of pralidoxime following the analytical methods outlined in the USP and KP. In accordance with the quantification test method for the pralidoxime monograph in the USP, a C18 column was used, and the mobile phase included sodium 1-octanesulfonate and tetraethylammonium chloride. Additionally, following the analytical method outlined in the KP, a pralidoxime analysis was conducted using a C18 column with tetraethylammonium chloride and phosphoric acid in the mobile phase.

3. Results and Discussion

3.1. Advancing Pralidoxime Separation Using Chaotropic Salt as a Mobile-Phase Modifier

The use of chaotropic ions with the capacity for dispersive interactions as mobile-phase modifiers is a prevalent strategy in analyzing polar analytes [34]. Their mechanism of action is based on charge delocalization, which is responsible for their ability to disrupt the water solvation shell of the analytes and introduce chaos into the organized ionic solution. The disruption of the solvation shells of the analytes not only exposes their more hydrophobic parts to interactions with the side chains of the stationary phase, thereby increasing retention, but also counteracts the role of the shells in suppressing interactions with the stationary phase, which otherwise leads to rapid elution [35]. In particular, employing chaotropic ions is advantageous for analyzing analytes that incorporate a nitrogen atom (primary, secondary, or tertiary) within their molecular structure, particularly in cases where the analytes exhibit the characteristics of weakly basic compounds.

A comparison of mobile-phase additives was conducted to derive an efficient separation of pralidoxime. Initially, the additives capable of ensuring a sufficient capacity factor were examined. Three inorganic salts, PF_6^-, ClO_4^-, and BF_4^-, and an ionic liquid with a chaotropic salt ($[EMIM]PF_6$) are recognized as excellent chaotropic agents for separating polar compounds. This separation is achieved via the interaction of cations and anions with the stationary phase and amine solute, respectively. The experimental results show that the capacity factor of pralidoxime chloride increased the most when $NaPF_6$ was added (Figure 2a). Figure 2a shows the capacity factor of pralidoxime chloride with 30 mM of mobile-phase additives ($NaPF_6$, $NaBF_4$, $NaClO_4$, and $[EMIM]PF_6$) in water and acetonitrile (90:10, v/v). The retention time of pralidoxime chloride in the chaotropic salt increased in the order of $PF_6^- > ClO_4^- > BF_4^-$, and the retention of the compound was better with the chaotropic salt $NaPF_6$ than with the ionic liquid $[EMIM]PF_6$. Chromatograms were compared when 30 mM of various additives was used (Figure 2b). The chromatograms of each chaotropic salt show that the additive that increased pralidoxime retention the most was $NaPF_6$, followed by $[EMIM]PF_6$ (Figure 2b).

Notably, for pyridine-2-aldoxime, in contrast to pralidoxime, a marked decrease in the retention time was observed. This phenomenon can be ascribed to variations in the basic ionizable groups inherent to each molecule [36]. Pyridine 2-aldoxime, characterized by its secondary amine structure, is distinct from pralidoxime chloride, which contains a

quaternary ammonium cation in its pyridinium ring. Quaternary ammonium ions exhibit a pronounced enhancement in ion pairing with chaotropic salts, leading to greater retention in the reverse-phase stationary phase. In contrast, the secondary amine in pyridine-2-aldoxime exhibits a lower positive charge, which causes the chaotropic salt to interact more with the polar mobile phase than with the analyte, thus increasing its affinity for the polar mobile phase and consequently decreasing its retention time in the stationary phase [37,38].

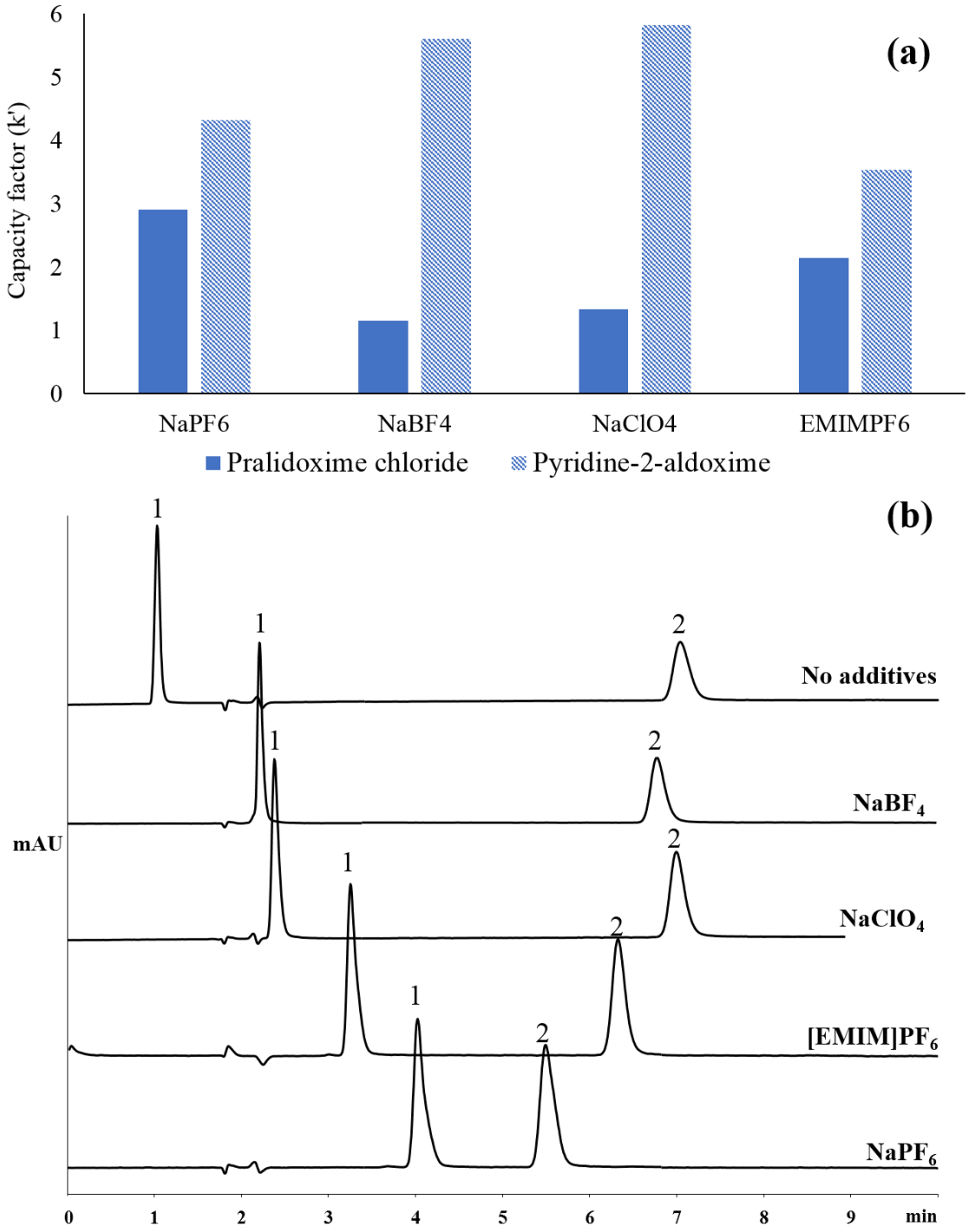

Figure 2. Comparison of (**a**) capacity factors and (**b**) chromatograms by mobile-phase additives (30 Mm). The front peak (1) is pralidoxime, and the latter peak (2) is pyridine-2-aldoxime.

3.2. Optimizing Chaotrope Concentrations for Enhanced Chromatographic Separation of Pralidoxime

To determine the optimal concentrations of the two additives $NaPF_6$ and $[EMIM]PF_6$, which showed the most efficient separation, a comparative analysis of the capacity factors and theoretical plate numbers of pralidoxime was conducted using concentrations ranging from 10 to 50 mM (Figure 3). An increase in the concentration of the mobile-phase additive was correlated with an increase in the capacity factor from 10 to 50 mM. However, the theoretical plate number increased progressively up to 40 mM, followed by a slight decrease at 50 mM. Pyridine-2-aldoxime, used as an IS, demonstrated a gradual decrease in retention time with increasing additive concentrations (Figure 3c). Considering the number of theoretical plates (N) and the resolution with pyridine-2-aldoxime, the optimal concentration was set to 30 mM $NaPF_6$ in water.

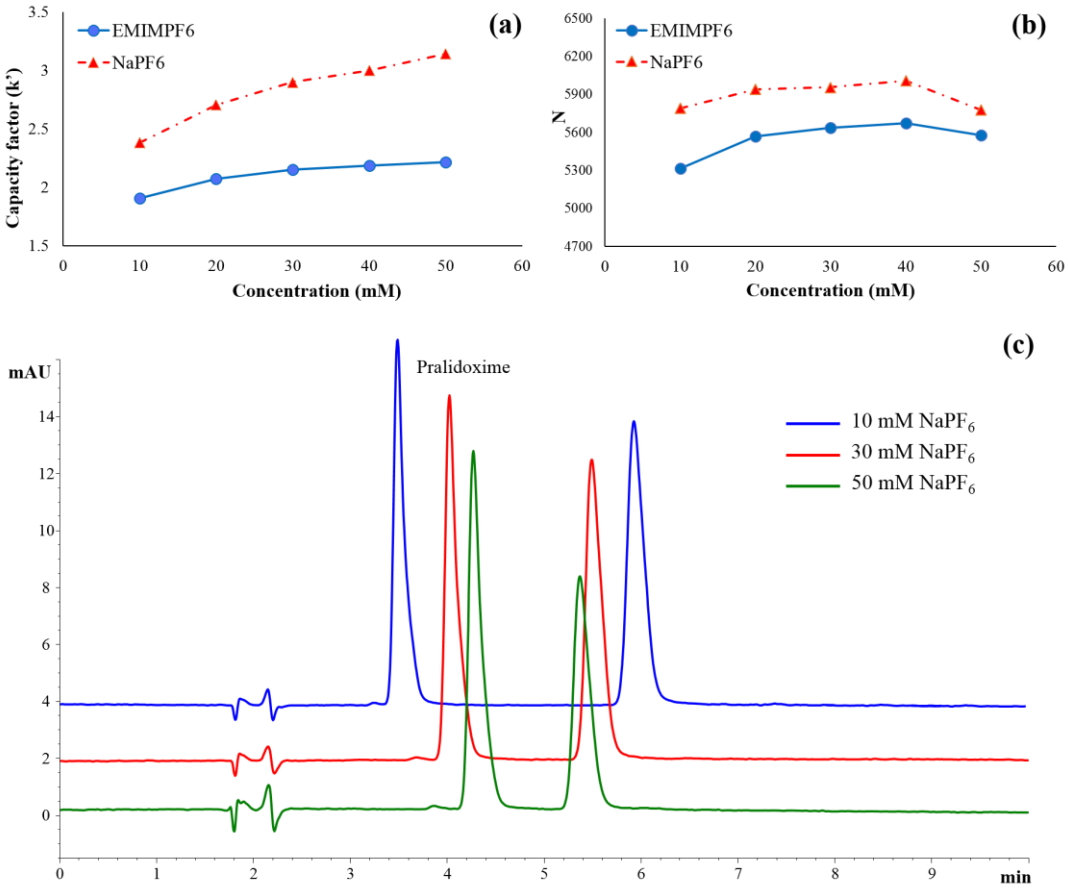

Figure 3. Comparison of (**a**) capacity factor of pralidoxime and (**b**) theoretical plate number of pralidoxime by chaotropic salt ($NaPF_6$) and ionic liquid ($[EMIM]PF_6$) concentration. (**c**) Comparison of chromatograms of $NaPF_6$ concentrations. The front peak is pralidoxime, and the latter peak is pyridine-2-aldoxime.

The utilization of chaotropic ions is particularly advantageous for weakly basic analytes containing nitrogen atoms with primary, secondary, or tertiary structures. Their mechanism of action is based on charge delocalization, which is pivotal in disrupting the water solvation shell surrounding the analytes and is responsible for dampening the interactions between the analyte and the stationary phase. This disruption leads to a chaotic state in the organized ionic solution. Concurrently, this process exposes the more hydrophobic regions of the analyte molecules, facilitating their interactions with the side chains of the stationary phases, thereby enhancing retention [39].

The use of the ionic liquid ([EMIM]PF_6) led to the effective separation of pralidoxime. However, certain constraints associated with its application restrict its utilization as a mobile-phase additive, despite its effectiveness in separating pralidoxime chloride. In an isocratic elution employing ion-pairing reagents in the mobile phase, challenges emerge with the retention of lipophilic matrix components, such as residual proteins; phospholipids; and various exogenous substances, including poisons and drugs [4]. These components adhere to the hydrophobic stationary phase and fail to elute effectively, owing to the suboptimal proportion of organic modifiers in the mobile phase. Consequently, this leads to potential column contamination in subsequent analyses, potentially impacting the chromatographic process by altering the retention characteristics and introducing analytical interference. Furthermore, IPC faces compatibility issues with mass spectrometry detection, which are attributed to the presence of high concentrations of nonvolatile salts in the mobile phase. Therefore, the majority of analyses utilizing IPC are conducted using UV detection, typically in the mid- to late-200 nm wavelength range. Ensuring the selectivity and resolution of the interfering elements is crucial for enhancing the analytical reliability of these methods. Additionally, the use of ionic liquids presents minor drawbacks, such as increased viscosity and elevated costs. Consequently, chaotropic salts emerge as a favorable alternative to ionic liquids in this context.

3.3. Comparison with Current Official Analytical Method of Pralidoxime in Pharmacopoeia

The developed method was compared with the official analytical procedures described by the USP and KP (Table 1). In accordance with the procedures prescribed in the USP, the pralidoxime chloride content was determined using IPC with a C18 column. The mobile phase consisted of acetonitrile, sodium 1-octanesulfonate, and tetraethylammonium chloride in water (17:83, v/v), adjusted to pH 4.3 with hydrochloric acid. Absorbance was measured at 270 nm. In accordance with the procedures prescribed in the KP, a solution of tetraethylammonium chloride and phosphoric acid in water and acetonitrile (48:52, v/v) was used. Ion-pairing reagents were used in the official methods to increase the retention time and suppress column residual silanols to improve the peaks. Quaternary ammonium salts were added to the mobile phase for the analysis of the acidic samples, and sodium alkanesulfonate was added for the analysis of the basic samples. Pralidoxime is a basic sample of the R-N+ form, so its retention can be increased by using sodium alkanesulfonate (R'-SO_3-) to form a neutral ion pair in RP-HPLC. The additives used in the USP method were tetraethylammonium chloride and sodium octane sulfonate (a type of alkane sulfonate). As shown in Figure 4a, pralidoxime retention significantly increased. However, only tetraethylammonium chloride, an ion-pairing reagent primarily used for acidic samples, was used in the KP method. Nevertheless, as shown in the experimental results (Figure 4b), it did not affect the retention of pralidoxime.

Table 1. Comparison of the proposed method with the current official methods.

Analyte	Rt (min)	k′	T_f	Width	Resolution	N
USP method [a]	6.55	5.35	1.16	0.156	10.21	11,968
KP method [b]	1.12	0.07	1.69	0.072	3.17	1543
Proposed method [b]	4.05	2.92	1.68	0.152	6.14	5795

Rt, retention time; k′, capacity factor; T_f, tailing factor; N, theoretical plate number. [a] Column: XSELECT HSS T3 (5 µm, 4.6 mm × 150 mm). [b] Column: Luna C18 (5 µm, 4.6 mm × 150 mm).

Compared to the KP method, the proposed method demonstrates an improved capacity factor, a higher theoretical plate number, and a better resolution with pyridine-2-aldoxime. The proposed method shows a slight decrease in retention time compared to the USP. However, in terms of the durability of equipment use, the new method employing chaotropic salt offers high value for such applications.

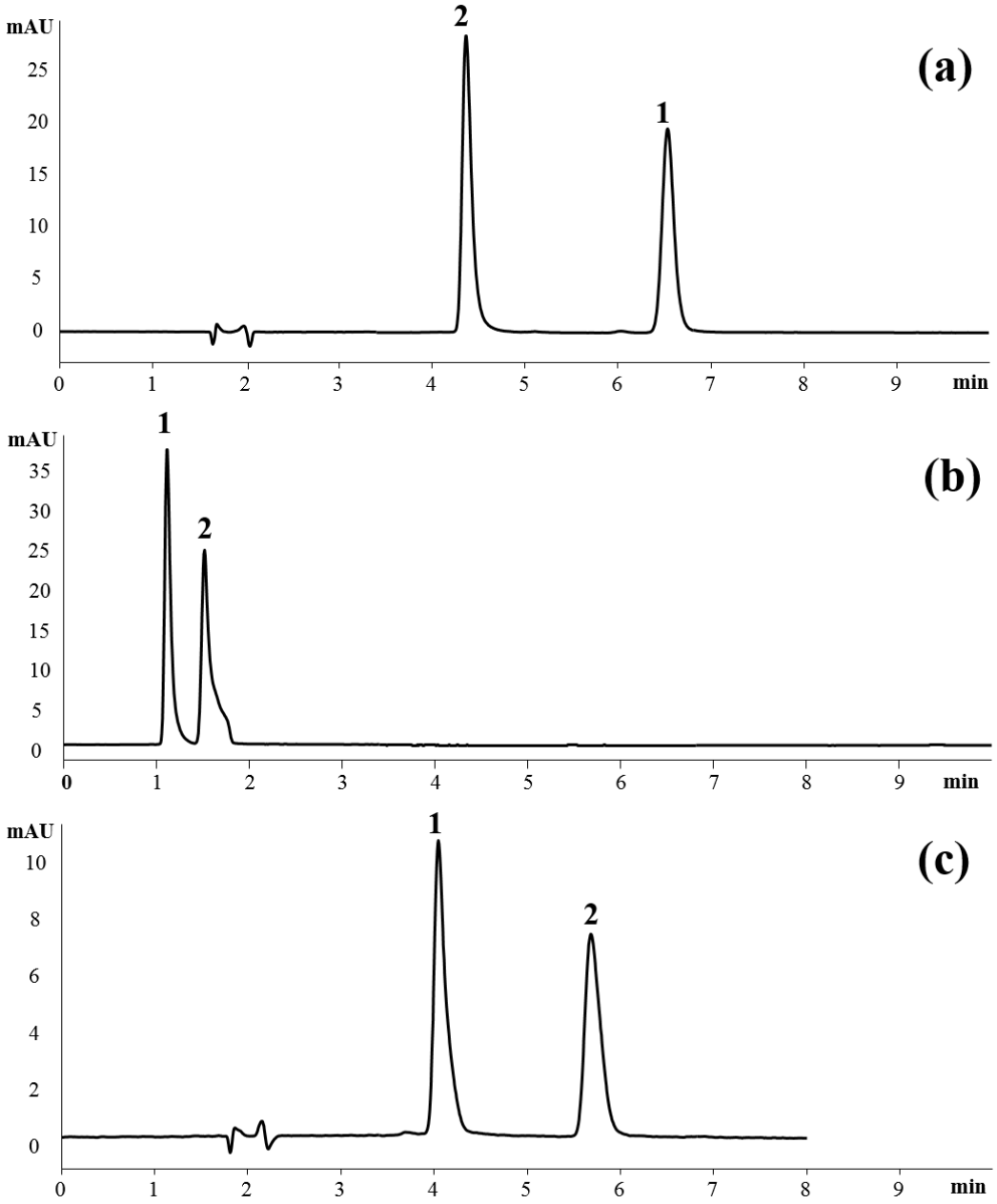

Figure 4. Chromatogram of a standard mixture with each analytical method. (**a**) USP, (**b**) KP, and (**c**) proposed method. Peaks: (1) pralidoxime chloride and (2) pyridine-2-aldoxime (the concentration is 30 μg/mL each).

Even at the same concentration, differences in peak response may occur among the analytical methods depending on the retention time and peak sharpness. For example, in the KP method, due to the very short retention time, the peaks were sharp, and the signals were strong. However, although the patterns were similar in the USP and our proposed method, there was a difference in response, which is believed to be due to differences in the analytical sensitivity depending on the mobile phase.

Several analytical methods for pralidoxime, including the USP, using a mobile phase containing ion-pairing reagents have been developed [11,13,16,32,40–42] and are summarized in Table 2. The use of ion-pairing reagents increases the capacity factor. However, ion-pairing reagents can cause irreversible damage to the column in terms of performance, such as changes in selectivity and extended equilibration times, owing to the adsorption of counterions onto the stationary phase [43].

Table 2. Established ion-pairing method for the determination of pralidoxime chloride.

Analyte	Column	Mobile Phase	Wavelength	Ref.
Pralidoxime chloride (USP)	L1 (150 mm × 4.6 mm, 3–3.5 µm)	A: 2 mM tetraethylammonium chloride and 8 mM sodium octanesulonate monohydrate in water (pH 4.3) B: Acetonitrile A/B (83:17, *v*/*v*)	270 nm	[40]
Atropine, pralidoxime chloride, obidoxime chloride, and HI-6	Agilent Zorbax Rx-C_{18} (250 mm × 4.6 mm, 5 µm)	A: 50 mM sodium dihydrogenorthophosphate, 1–5 mM trimethylamine, and 0.5–1 mM l-octanesulfonic acid sodium salt in water (pH 3.5) B: Acetonitrile	203 nm	[11]
Pralidoxime mesylate and its degradation products	Thermo Hypersil^{TM} ODS (125 mm × 4.6 mm)	A: 0.1 M trimethylamine phosphate and 10 mM sodium lauryl sulfate in water (pH 3.0) B: Methanol A/B (90:10, *v*/*v*)	262 nm	[13]
Pyridine aldoximes	Agilent Zorbax Rx-C_{18} (250 mm × 4.6 mm, 5 µm)	A: Sodium phosphate dibasic dehydrate, citric acid, and 1-octane sulfonic acid in water (pH 3.7) B: Acetonitrile	276 nm	[16]
Pralidoxime chloride	Agilent Zorbax SB-C_{18} (150 mm × 4.6 mm, 5 µm)	A: 0.01% trifluoroacetic acid in water B: 0.01% trifluoroacetic acid in methanol	- (LC/MSD)	[41]
Eserine and pralidoxime chloride	CHOMASIL C_{18} (250 mm × 4.6 mm, 5 µm)	A: 10 mM potassium dihydrogen phosphate and 10 mM heptane-1-sulfonic acid in water (pH 3.0) B: Acetonitrile A/B (70:30, *v*/*v*)	238 nm	[42]

3.4. Method Validation

The proposed HPLC method with a chaotropic salt was validated for the determination and quantification of pralidoxime chloride according to the Ministry of Food and Drug Safety (MFDS) guidelines on the validation of analytical procedures to prove the linearity, precision, accuracy, and recovery.

3.4.1. Linearity

A calibration curve was prepared in the concentration range of 10–50 µg/mL of the pralidoxime chloride standard. A strong linear relationship was observed between the corresponding peak area and concentration ($y = 7.64267x + 1.30$, $r^2 > 0.9999$).

3.4.2. Intra- and Inter-Day Accuracy and Precision

Intra- and inter-day accuracy and precision were assessed by performing three repeated measurements daily for three days within the concentration range of 20–50 µg/mL. Accuracy is expressed as the observed concentration of the nominal concentration, and precision was calculated using the relative standard deviation (RSD). The obtained accuracy and precision values are listed in Table 3. The intra- and inter-day accuracy ranged between 97.44% and 101.01%, and the intra- and inter-day precision values ranged between 0.12 and 2.56%.

Table 3. Precision and accuracy of pralidoxime chloride.

Analyte	Concentration (μg/mL)	Precision (RSD, %)		Accuracy (%)	
		Intra-Day	Inter-Day	Intra-Day	Inter-Day
Pralidoxime chloride	10	0.66	8.79	99.18	94.51
	20	0.20	2.56	100.18	97.44
	30	0.13	0.65	100.52	101.01
	40	0.44	0.77	99.77	100.59
	50	0.47	0.12	99.96	100.09

3.4.3. Recovery

The recovery was calculated according to a standard addition method procedure at low, medium, and high concentrations (2, 5, and 10 μg/mL) of pralidoxime chloride. The recovery was determined at low, medium, and high concentrations within the linearity range by repeating the test five times. The mean recovery was 99.02–99.55% with an RSD of 0.36–0.62% (Table 4).

Table 4. Recovery of pralidoxime chloride (n = 5).

Analyte	Base (μg/mL)	Spiked (μg/mL)	Found (μg/mL)	Recovery	
				Mean, %	RSD, %
Pralidoxime chloride	26.17	2	28.04	99.55	0.36
		5	30.88	99.06	0.53
		10	35.89	99.22	0.62

3.4.4. Repeatability

The repeatability of the proposed method was evaluated by performing six repeated measurements (n = 6) involving pralidoxime chloride under the same operating conditions. The RSD was calculated to determine the repeatability. The RSD of six repeated injections was 0.31%. The repeatability was considered satisfactory because an RSD value of less than 1.0% was obtained.

4. Conclusions

We introduced an efficient, rapid, and innovative RP-HPLC technique for the analysis and quantification of pralidoxime chloride in antidote autoinjectors using sodium hexafluorophosphate ($NaPF_6$) as a chaotropic salt in the mobile phase. This approach demonstrated an enhanced retention of polar compounds when a chaotropic salt was employed as a mobile-phase additive. Experimental observations indicated a sequential increase in retention time with different chaotropic salts, following the order of $PF_6^- > ClO_4^- > BF_4^-$, and this effect was particularly notable when compared to the effective ionic liquid $[EMIM]PF_6$. Consequently, a method for analyzing pralidoxime was established by incorporating 30 mM of $NaPF_6$ into the mobile phase. The method underwent rigorous validation, demonstrating high linearity, precision, accuracy, and recovery, ensuring its reliability and effectiveness for practical applications. These validation parameters affirm the method's suitability for the intended analytical purposes, providing a robust foundation for its application in the quality control and regulatory compliance of pralidoxime chloride. Distinct from the methods recommended by the USP and KP, which suggest the use of ion-pairing agents that are potentially harmful to the column, our method demonstrated an improved separation of pralidoxime and the internal standard using safer chaotropic salts. Additionally, this approach is both cost-effective and environmentally friendly, characterized by a brief analysis duration of only 8 min and the minimal use of organic solvents, thus reducing potential harm to humans and the environment. This newly developed method extends beyond the quantitative analysis of pralidoxime chloride, presenting a versatile alternative for examining amine compounds. Its applicability across a range of substances signifies a breakthrough, offering broader analytical capabilities.

Author Contributions: Conceptualization, S.B.H. and B.S.; methodology, B.S. and J.K.; validation, B.S., H.-s.K., J.-Y.L., S.S. and C.H.J.; data curation, B.S., E.B., D.-K.L. and S.B.H.; writing—original draft preparation, B.S., D.-K.L. and S.B.H.; writing—review and editing, D.L., H.L. and D.-K.L. and S.B.H.; funding acquisition, D.-K.L. and S.B.H. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by a grant from the Ministry of National Defense in 2021. This research was supported by a Chung-Ang University research grant for 2021.

Data Availability Statement: Data are contained within the article.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Pulkrabkova, L.; Svobodova, B.; Konecny, J.; Kobrlova, T.; Muckova, L.; Janousek, J.; Pejchal, J.; Korabecny, J.; Soukup, O. Neurotoxicity evoked by organophosphates and available countermeasures. *Arch. Toxicol.* **2023**, *97*, 39–72. [CrossRef]
2. Tang, X.; Wang, R.; Xie, H.; Hu, J.; Zhao, W. Repeated pulse intramuscular injection of pralidoxime chloride in severe acute organophosphorus pesticide poisoning. *Am. J. Emerg. Med.* **2013**, *31*, 946–949. [CrossRef] [PubMed]
3. Pirollo, K.F.; Moghe, M.; Guan, M.; Rait, A.S.; Wang, A.; Kim, S.-S.; Chang, E.H.; Harford, J.B. A Pralidoxime Nanocomplex Formulation Targeting Transferrin Receptors for Reactivation of Brain Acetylcholinesterase After Exposure of Mice to an Anticholinesterase Organophosphate. *Int. J. Nanomed.* **2024**, *19*, 307–326. [CrossRef] [PubMed]
4. John, H.; Blum, M.-M. Review of UV spectroscopic, chromatographic, and electrophoretic methods for the cholinesterase reactivating antidote pralidoxime (2-PAM). *Drug Test. Anal.* **2012**, *4*, 179–193. [CrossRef] [PubMed]
5. Antoniello, A.A.; Pauls, P.; Awad, N.I.; Sobolewski, K.; Fernández, D.; Bridgeman, P. Optimization of antidote stocking, availability, and administration practices for a large multihospital organization. *Am. J. Health-Syst. Pharm.* **2022**, *80*, S1–S10. [CrossRef] [PubMed]
6. Rawas-Qalaji, M.; Thu, H.E.; Hussain, Z. Chapter 23—Potential alternative treatments and routes of administrations: Nerve agents poisoning. In *Sensing of Deadly Toxic Chemical Warfare Agents, Nerve Agent Simulants, and their Toxicological Aspects*; Das, S., Thomas, S., Das, P.P., Eds.; Elsevier: Amsterdam, The Netherlands, 2023; pp. 539–568.
7. Chung, S.P.; Roh, H.-K. Antidote for organophosphate insecticide poisoning: Atropine and pralidoxime. *Korean Med. Assoc.* **2013**, *56*, 1057–1066. [CrossRef]
8. Vijayaraghavan, R. Autoinjector device for rapid administration of drugs and antidotes in emergency situations and in mass casualty management. *J. Int. Med. Res.* **2020**, *48*, 0300060520926019. [CrossRef] [PubMed]
9. Menke, B.A.; Ryu, C.; Justin, G.A.; Chundury, R.V.; Hayek, B.R.; Debiec, M.R.; Yeh, S. Ophthalmic manifestations and management considerations for emerging chemical threats. *Front. Toxicol.* **2023**, *5*, 1281041. [CrossRef] [PubMed]
10. Schroeder, A.C.; Digiovanni, J.H.; Von Bredow, J.; Heiffer, M.H. Pralidoxime chloride stability-indicating assay and analysis of solution samples stored at room temperature for ten years. *J. Pharm. Sci.* **1989**, *78*, 132–136. [CrossRef]
11. Paddle, B.M.; Dowling, M.H. Simple high-performance liquid chromatographic method for assessing the deterioration of atropine-oxime mixtures employed as antidotes in the treatment of nerve agent poisoning. *J. Chromatogr.* **1993**, *648*, 373–380. [CrossRef]
12. Bariakar, U.V.; Patel, U.N. Pralidoxime Chloride. In *Analytical Profiles of Drug Substances*; Academic Press: Cambridge, MA, USA, 1988; pp. 533–569.
13. Utley, D. Determination of 2-hydroxyiminomethyl-1-methyl-pyridinium methanesulphonate (pralidoxime mesylate, P2S) and its degradation products in solution by liquid chromatography. *J. Chromatogr. A* **1983**, *265*, 311–322. [CrossRef]
14. Prue, D.G.; Johnson, R.N.; Kho, B.T. High-performance liquid chromatographic determination of pralidoxime chloride and its major decomposition products in injectable solutions. *J. Pharm. Sci.* **1983**, *72*, 751–756. [CrossRef] [PubMed]
15. Ellin, R.I. Stability of concentrated aqueous solutions of pralidoxime chloride. *J. Pharm. Sci.* **1982**, *71*, 1057–1059. [CrossRef] [PubMed]
16. Szegi, P.; Kalász, H.; Laufer, R.; Kuca, K.; Tekes, K. Pyridinium aldoxime analysis by HPLC: The method for studies on pharmacokinetics and stability. *Anal. Bioanal. Chem.* **2010**, *397*, 579–586. [CrossRef]
17. Žuvela, P.; Skoczylas, M.; Jay Liu, J.; Bączek, T.; Kaliszan, R.; Wong, M.W.; Buszewski, B. Column Characterization and Selection Systems in Reversed-Phase High-Performance Liquid Chromatography. *Chem. Rev.* **2019**, *119*, 3674–3729. [CrossRef]
18. Suh, J.H.; Kim, J.; Jung, J.; Kim, K.; Lee, S.G.; Cho, H.-D.; Jung, Y.; Han, S.B. Determination of thiamine in pharmaceutical preparations by reverse phase liquid chromatography without use of organic solvent. *Bull. Korean Chem. Soc.* **2013**, *34*, 1745–1750. [CrossRef]
19. Zhao, S.; Li, L. Chemical derivatization in LC-MS-based metabolomics study. *TrAC Trends Anal. Chem.* **2020**, *131*, 115988. [CrossRef]
20. Iturrospe, E.; Da Silva, K.M.; Talavera Andújar, B.; Cuykx, M.; Boeckmans, J.; Vanhaecke, T.; Covaci, A.; van Nuijs, A.L.N. An exploratory approach for an oriented development of an untargeted hydrophilic interaction liquid chromatography-mass spectrometry platform for polar metabolites in biological matrices. *J. Chromatogr. A* **2021**, *1637*, 461807. [CrossRef]

21. Jandera, P.; Hájek, T. Dual-mode hydrophilic interaction normal phase and reversed phase liquid chromatography of polar compounds on a single column. *J. Sep. Sci.* **2020**, *43*, 70–86. [CrossRef]
22. Axente, R.E.; Stan, M.; Chitescu, C.L.; Nitescu, V.G.; Vlasceanu, A.-M.; Baconi, D.L. Application of Ionic Liquids as Mobile Phase Additives for Simultaneous Analysis of Nicotine and Its Metabolite Cotinine in Human Plasma by HPLC–DAD. *Molecules* **2023**, *28*, 1563. [CrossRef] [PubMed]
23. Goyon, A.; Blevins, M.S.; Napolitano, J.G.; Nguyen, D.; Goel, M.; Scott, B.; Wang, J.; Koenig, S.G.; Chen, T.; Zhang, K. Characterization of antisense oligonucleotide and guide ribonucleic acid diastereomers by hydrophilic interaction liquid chromatography coupled to mass spectrometry. *J. Chromatogr. A* **2023**, *1708*, 464327. [CrossRef] [PubMed]
24. Violi, J.P.; Bishop, D.P.; Padula, M.P.; Steele, J.R.; Rodgers, K.J. Considerations for amino acid analysis by liquid chromatography-tandem mass spectrometry: A tutorial review. *TrAC Trends Anal. Chem.* **2020**, *131*, 116018. [CrossRef]
25. Long, Z.; Guo, Z.; Xue, X.; Zhang, X.; Nordahl, L.; Liang, X. Selective separation and purification of highly polar basic compounds using a silica-based strong cation exchange stationary phase. *Anal. Chim. Acta* **2013**, *804*, 304–312. [CrossRef] [PubMed]
26. Abdulhussain, N.; Nawada, S.; Schoenmakers, P. Latest Trends on the Future of Three-Dimensional Separations in Chromatography. *Chem. Rev.* **2021**, *121*, 12016–12034. [CrossRef]
27. Lu, J.; Xiong, X.; Ma, M.; Chen, B.; Chen, Y.; Schmitz, O.J. Enhancing the compatibility of normal-phase chromatography x reversed−phase chromatography by combination of low−temperature sensitive aqueous-phase compatible normal-phase chromatography and at−column dilution modulation. *J. Chromatogr. A* **2023**, *1691*, 463821. [CrossRef] [PubMed]
28. Kartsova, L.A.; Makeeva, D.V.; Kravchenko, A.V.; Moskvichev, D.O.; Polikarpova, D.A. Capillary electrophoresis as a powerful tool for the analyses of bacterial samples. *TrAC Trends Anal. Chem.* **2021**, *134*, 116110. [CrossRef]
29. Kumagai, M.; Kato, S.; Arakawa, N.; Otsuka, M.; Hamano, T.; Kashiwagi, N.; Yabuki, A.; Yamato, O. Quantification of Histidine-Containing Dipeptides in Dolphin Serum Using a Reversed-Phase Ion-Pair High-Performance Liquid Chromatography Method. *Separations* **2021**, *8*, 128. [CrossRef]
30. Voicu, V.; Albu, F.; Tache, F.; Musilek, K.; Kuca, K.; Medvedovici, A. LC–MS/MS approaches for the assay of bis-quaternary pyridinium oximes used as AChE reactivators in biological matrices. *Bioanalysis* **2013**, *5*, 793–809. [CrossRef]
31. Voicu, V.; Medvedovici, A.; Radulescu, M.; Iorgulescu, E.-E.; David, V. Unusual Retention Behavior of Some Cationic-Type Aldoximes Used as AChE Reactivators Under Ion-Pairing Liquid Chromatographic Mechanism. *Anal. Lett.* **2010**, *43*, 1267–1276. [CrossRef]
32. USP monographs. *Pralidoxime Chloride for Injection*; USP-NF; Unites States Pharmacopoeia: Rockville, MD, USA, 2023.
33. *The Korean Pharmacopoeia*, 12th ed.; The KFDA Notification: Cheongju, Republic of Korea, 2020.
34. Protti, M.; Carotti, A.; Mercolini, L.; Sardella, R. Chapter 12—Co-solvents and mobile phase additives in HPLC. In *Liquid Chromatography*, 3rd ed.; Fanali, S., Chankvetadze, B., Haddad, P.R., Poole, C.F., Riekkola, M.-L., Eds.; Elsevier: Amsterdam, The Netherlands, 2023; Volume 1, pp. 283–321.
35. Dullinger, P.; Horinek, D. Solvation of Nanoions in Aqueous Solutions. *J. Am. Chem. Soc.* **2023**, *145*, 24922–24930. [CrossRef] [PubMed]
36. Djajić, N.; Krmar, J.; Rmandić, M.; Rašević, M.; Otašević, B.; Zečević, M.; Malenović, A.; Protić, A. Modified aqueous mobile phases: A way to improve retention behavior of active pharmaceutical compounds and their impurities in liquid chromatography. *J. Chromatogr. Open* **2022**, *2*, 100023. [CrossRef]
37. Aliyeva, M.; Brandão, P.; Coutinho, J.A.P.; Ferreira, O.; Pinho, S.P. Solubilities of Amino Acids in the Presence of Chaotropic Anions. *J. Solut. Chem.* **2023**, 1–11. [CrossRef]
38. Soares, B.P.; Abranches, D.O.; Sintra, T.E.; Leal-Duaso, A.; García, J.I.; Pires, E.; Shimizu, S.; Pinho, S.P.; Coutinho, J.A.P. Glycerol Ethers as Hydrotropes and Their Use to Enhance the Solubility of Phenolic Acids in Water. *ACS Sustain. Chem. Eng.* **2020**, *8*, 5742–5749. [CrossRef]
39. Lazarevska Todevska, E.; Piponski, M.; Stefova, M. Development and validation of an analytical method for the determination of related substances in bisoprolol fumarate in dosage forms by HPLC-UV-DAD. *Maced. J. Chem. Chem. Eng.* **2021**, *40*, 263–276. [CrossRef]
40. *USP43-NF38-3637*; Pralidoxime Chloride for Injection. USP-NF: Frederick, MD, USA, 2020.
41. Hoffman, R.S.; Mercurio-Zappala, M.; Bouchard, N.; Ravikumar, P.; Goldfrank, L. Preparing for chemical terrorism: A study of the stability of expired pralidoxime (2-PAM). *Disaster Med. Public Health Prep.* **2011**, *6*, 20–25. [CrossRef] [PubMed]
42. Banerjee, S.; Chattopadhyay, P.; Ghosh, A.; Kaity, S.; Veer, V. Development and validation of a reverse phase liquid chromatography method for the simultaneous quantification of eserine and pralidoxime chloride in drugs-in-adhesive matrix type transdermal patches. *Drug Res.* **2013**, *63*, 450–456. [CrossRef] [PubMed]
43. Seo, N.; Lee, Y.R.; Park, H.S.; Truong, Q.-K.; Lee, J.-Y.; Chung, H.K.; Choi, Y.; Kim, B.; Han, S.B.; Kim, K.H. Determination of urazamide in pharmaceutical preparation with room temperature ionic liquid. *Arch. Pharmacal Res.* **2017**, *40*, 364–372. [CrossRef] [PubMed]