**Possible Role of Electrolytes on the Formation of Precipitates during the Infusion of Nafamostat Mesilate in Hemodialysis**

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Nafamostat mesilate (NFM) is used as an anticoagulant during hemodialysis in patients who have had complications due to hemorrhages. The formation of precipitates, which could lead to the interruption of hemodialysis has been reported when NFM is infused into blood during hemodialysis. We report herein on an examination of possible factors that could cause this. The effects of electrolytes such as phosphates, citrates or succinates on the formation of precipitates were examined by mixing NFM with aqueous solutions or plasma that contained these electrolytes. The formation of precipitates was observed in all electrolyte solutions when higher concentrations of NFM were mixed at around physiological pH. In the case of plasma, precipitates were observed when solutions containing higher concentrations of NFM were mixed with plasma that contained phosphate and citrate. In addition, the formation of precipitates under dynamic conditions where NFM was infused into flowing electrolyte solutions was also evaluated. The data suggested that such precipitates might be formed and disrupt the blood flow and/or an NFM infusion when NFM is infused into blood flowing in the hemodialysis circuit. The findings presented herein suggest the serum levels of anionic electrolytes (e.g., phosphate), the type of excipients present in pharmaceutical products (e.g., succinic acid or citric acid), the concentration of NFM used for the infusion or the rates of NFM infusion and blood flow are all factors that could affect precipitate formation during NFM infusions for hemodialysis.

**Key words** nafamostat mesilate; phosphate; precipitate; hemodialysis

**INTRODUCTION**

Nafamostat mesilate (NFM), a synthetic serine protease inhibitor, is used for the treatment of acute pancreatitis and disseminated intravascular coagulation (DIC). NFM is also used as an anticoagulant during hemodialysis in patients who have had complications related to hemorrhaging, since its short half-life could lower the risk of further hemorrhaging. Several recent studies have reported that NFM is efficacious for the treatment of the coronavirus disease 2019 (COVID-19) patient with severe respiratory failure and/or coagulopathy. In contrast to such a variety of uses, the formation of precipitates to form remains unclear. To address this issue, in present study, effects of the electrolytes such as phosphates, citrates or succinates on the formation of a precipitate were investigated by mixing NFM with aqueous solutions or plasma that included these electrolytes. In addition to such relatively static conditions, the formation of precipitates under more dynamic conditions in which NFM is infused into flowing electrolyte solutions was also evaluated.

**MATERIALS AND METHODS**

**Materials** NFM was generously supplied by the Kobayashi Kako Co., Ltd. (Fukui, Japan), 1-Heptanesulfonic acid sodium salt was obtained from Wako Pure Chemical Corporation (Osaka, Japan). Human plasma was purchased from Interstate Blood Bank, Inc. (Memphis, TN, U.S.A.). All other chemicals were obtained from commercial sources and were of the highest grade available.

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Formation of Precipitate by Mixing Solutions of NFM and Electrolytes To prepare the electrolyte solutions for the experiments, aqueous solutions of electrolytes A and B were mixed at volume ratios of 0:5, 1:4, 2:3, 3:2, 4:1, and 5:0. As combinations of electrolytes A and B, phosphoric acid (A)-sodium dihydrogen phosphate (B), sodium dihydrogen phosphate (A)-disodium hydrogen phosphate (B), citric acid (A)-trisodium citrate (B) and succinic acid (A)-disodium succinate (B) were used. The concentrations of the electrolytes (10, 50, and 100 mM) which are higher than those typically used in clinical situations were initially used to characterize precipitate formation. To prepare 10, 50, and 100 mM electrolyte solutions for precipitate experiments, 10, 50, and 100 mM electrolyte A solutions were mixed with same concentrations of electrolyte B solutions, respectively. An aqueous solution of NFM (10 µL) was mixed with these prepared electrolyte solutions (290 µL) on a 96-well plate to give NFM concentrations of 0.2 or 2 mg/mL. Since nafamostat does not show an absorption at 540 nm, the enhancement in absorbance at 540 nm (ΔAbs 540) when NFM is mixed with electrolytes is likely due to a turbidity increase as shown in Fig. 1A. Thus, the level of precipitate formation was evaluated by monitoring the enhancement in absorbance at 540 nm (ΔAbs 540) with a microplate reader (Infinite M200 PRO, Tecan Japan Co., Ltd., Kanagawa, Japan). The pH of the suspension or solution after mixing A and B were separately measured with a compact pH meter (LAQUAtwin pH-22, HORIBA, Ltd., Kyoto, Japan).

HPLC Analysis of Precipitates Precipitates formed by mixing solutions of NFM and electrolytes were dissolved in the following mobile phase and analyzed by HPLC. HPLC analyses were carried out as described in a previous report with minor modifications. The HPLC system consisted of a Hitachi model D-2000 Elite HPLC system (Hitachi Co., Tokyo, Japan). Inertsil ODS-2 (5 µm particle size, 250 × 4.6 mm I.D., GL Sciences Inc., Tokyo, Japan) was used as the stationary phase and was maintained at 40°C. Nafamostat was detected using a mobile phase consisting of acetonitrile and 0.1 M acetic acid containing 0.03 mM 1-heptanesulfonic acid sodium electrolyte (30:70 (v/v)) at a flow rate of 1.0 mL/min in the isocratic mode. The detection wavelength was fixed at 260 nm and monitored during 15 min for each sample. The retention time of nafamostat was 7.8 min.

Formation of Precipitate by Mixing NFM with Human Plasma Concentration of inorganic phosphate in human plasma was initially determined to be 0.885 mM by using Malachite Green Phosphate Detection Kit (R&D Systems, Inc., Minneapolis, MN, U.S.A). Aqueous solutions of electrolytes (5 µL) were mixed with human plasma (285 µL) to give phosphate, citrate and succinate concentrations of 0, 0.2, 0.5, 1.0, and 2.0 mM or sodium chloride concentration of 0, 2, 5, 10, 50, 100, and 200 mM. An aqueous solution of NFM (10 µL) was mixed with human plasma containing electrolytes (290 µL) on a 96-well plate to give NFM concentrations of 0.1 or 1 mg/mL. Precipitate formation (Fig. 1B) was evaluated by measuring the enhancement in absorbance at 540 nm (ΔAbs 540) with a microplate reader.

Formation of a Precipitate by Infusing NFM into the Flow of Electrolyte Solution The formation of a precipitate when NFM was mixed with several electrolytes under dynamic flow conditions was investigated by using a flow system with an infusion line as shown in Fig. 2. Disodium hydrogen phosphate was used as the electrolyte. Constant flows of 0, 1, 2, or 4 mM electrolyte solution (12 or 24 mL/h) in the polytetrafluoroethylene (PTFE) tube (0.25 mm I.D. 1.5 mm O.D., Eyela Co., Ltd., Tokyo, Japan) were generated by an HPLC pump (Model L-2130, Hitachi Co.). These flow rates which are lower than those typically used in clinical situations were used to characterize precipitate formation. A solution of NFM (1,
2, and 4 mg/mL) was infused into the flowing electrolyte solution through a three-way tube connector at constant rates (6 or 12 mL/h) by a syringe pump (110 VAC, KD Scientific Inc., Holliston, MA, U.S.A.) during 10 min. Precipitate formation and the accompanying disturbance in the flow system during the infusion of NFM were indirectly detected by monitoring the increase in pressure displayed in the HPLC pump.

**Statistical Analysis** All data are expressed as the mean ± standard deviation. The data were analyzed statistically using one-way ANOVA, followed by Tukey multiple comparisons *post hoc* test to evaluate differences between the means. A probability value of $p < 0.05$ was considered to be significant.

**RESULTS**

**Formation of Precipitates on Mixing Solutions of NFM and Electrolytes** The formation of precipitates when solutions of NFM and several electrolytes were mixed was investigated. Increases in the absorbance at 540 nm (ΔAbs540) were observed for all of the electrolyte solutions investigated when an NFM solution was added to concentrations of 2 mg/mL (Fig. 3), indicating that a precipitate was formed. The increase in absorbance was more obvious at higher pH than at lower pH. For phosphoric acid-sodium dihydrogen phosphate, the increase tended to be dependent on the electrolyte concentration.

At lower NFM concentrations (0.2 mg/mL), a significant
increase in absorbance was observed at around pH 7 in the case if the sodium dihydrogen phosphate–disodium hydrogen phosphate system (Fig. 4). In addition, smaller increases were also found around pH 7 in the case of the citric acid–trisodium citrate system. The changes in absorbance for sodium dihydrogen phosphate–disodium hydrogen phosphate and citric acid-trisodium citrate system were much smaller at lower NFM concentration (0.2 mg/mL; Fig. 4) than at higher NFM concentrations (2 mg/mL; Fig. 3).

HPLC Analysis of Precipitates Formed by Mixing Solutions of NFM and Electrolyte  The precipitates formed by mixing solutions of NFM and electrolytes (NFM; 2 mg/mL, electrolytes; 10 mM) were analyzed by HPLC. HPLC chromatograms of precipitates when using sodium dihydrogen phosphate, disodium hydrogen phosphate and trisodium citrate or disodium succinate were nearly the same as that of an aqueous NFM solution (Supplementary Fig. 1).

Formation of Precipitates by Mixing NFM with Human Plasma in the Presence of Electrolytes  The formation of precipitates on mixing NFM and human plasma were investigated. An increase in absorbance at 540 nm (ΔAbs540) was observed when an NFM solution was added to plasma that contained a high concentration of phosphoric acid, sodium dihydrogen phosphate, disodium hydrogen phosphate, citric acid or trisodium citrate to give NFM concentrations of 1 mg/mL (Fig. 5A), indicating that a precipitate had formed. In contrast, no obvious change was observed in the cases of succinic acid or disodium succinate. At a lower NFM concentration (0.1 mg/mL), no change was detected in any of the plasma including those with added electrolytes (Fig. 5B). In addition to these electrolytes, the effects of sodium chloride on precipitate formation between NFM and human plasma was also examined. No significant change in ΔAbs540 was observed even at higher NFM and sodium chloride concentrations (Fig. 6).

Formation of Precipitates When NFM Is Infused into a Flowing Electrolyte Solution  An NFM solution was infused into a flowing electrolyte solution in order to investigate whether sufficient precipitate formation could occur to have an effect on the flow. Disodium hydrogen phosphate was used as an electrolyte. The disturbance of flow which was monitored
DISCUSSION

NFM, a synthetic serine protease inhibitor, has been used for the treatments of acute pancreatitis and DIC.\(^\text{1,2}\) NFM has also been used as an anticoagulant in hemodialysis patients with several hemorrhage complications.\(^\text{3,4}\) In contrast to these various uses, precipitation formation occurs which requires treatment to be interrupted, especially during hemodialysis.\(^\text{9,10}\) Therefore, we examined the possible factors that could contribute to this by mixing various electrolytes with NFM.

The addition of NFM at concentration of 2 mg/mL to solutions of electrolytes caused the formation of white precipitates as evidenced by high ΔAbs\(_{540}\) values (Fig. 3). Precipitate formation was more pronounced at higher pH. Since nafamostat is hydrolyzed to p-guanidinobenzoic acid (PGBA) and 6-amidino-2-naphthol (AN) at higher pH (over pH 7) (Supplementary Fig. 2), it is possible that such degradation products might contribute to the precipitate formation. However, the HPLC chromatograms of precipitates indicated that the NFM was largely intact (Supplementary Fig. 1), suggesting that the precipitate contains nafamostat and not a hydrolysis product. It is well known that the mesilate component of NFM can be exchanged with the other anionic salts, and that this can cause precipitation to occur. Among the electrolytes investigated, phosphate, succinate or citrate that could cause precipitation by salt exchange, especially when high concentrations of NFM are used and when these electrolytes are present at around physiological pH (Figs. 3, 4).

The concentration of phosphate (inorganic phosphorus) is normally 0.80–1.60 mmol/L in serum.\(^\text{15}\) However, in some diseases such as kidney failure or hypoparathyroidism,\(^\text{15–17}\) the phosphate concentration can exceed 1.60 mmol/L, a condition that is referred to as hyperphosphatemia. Under such a high serum phosphate level, a precipitate was observed in cases of higher concentrations of NFM (1 mg/mL) regardless of the type of electrolytes (i.e., phosphoric acid, sodium dihydrogen, and phosphate-disodium hydrogen phosphate) (Fig. 5), suggesting that insoluble salts were formed due to salt exchange between the mesilate moiety in NFM and phosphate at plasma pH (pH 7.4). Thus, in a hyperphosphatemic state, the risk of precipitate formation would likely be increased when a high concentration of infused NFM is used. Chloride ion is also a major electrolyte in serum. The serum chloride concentration is normally 95–105 mmol/L\(^\text{15}\) and exceeds 105 mmol/L in cases of hyperchloremia which is very common in critically ill patients.\(^\text{5,18,19}\) Precipitation was not observed even when NFM was mixed with much higher concentrations of sodium chloride solutions (as increased chloride concentration: approx. 200 mmol/L) (Fig. 6). Hence, precipitate formation would be expected to be unlikely when mixed with NFM in plasma compared to phosphate. In addition to phosphate and chloride, several other anions (e.g., bicarbonate, sulfate, nitrate or lactate) in plasma could exchange with the mesilate of NFM, although further studies will be needed to confirm this possibility.

In some pharmaceutical products that contain NFM, citric acid or succinic acid are sometimes included to control the pH of the prepared solution.\(^\text{10}\) Such additives may become anionic electrolytes and exchange with the mesilate of NFM. Hirashima et al. and Honda and Saito suggested that the type of excipients or impurities in products can affect the frequency of precipitate formation during hemodialysis.\(^\text{10,13}\) Interestingly, a precipitate in serum was observed only under conditions of higher serum concentrations of NFM (1 mg/mL) and citrate (2 mM) (Fig. 5). Meanwhile, precipitates were formed both in citric acid-trisodium citrate and succinic acid–disodium succinate solutions at around pH 7.4 when a high concentration of NFM was added (Fig. 3). Taking such differences between the data in serum and aqueous solutions into consideration, the formation of precipitates in serum appears to be accelerated in the presence of citrate and can be prevented when succinate is present. Hence, pharmaceutical products that are composed of both NFM and succinic acid might minimize the precipitate formation in serum compared to those that contain citric acid.

It was suggested that such precipitates can be formed and disturb blood flow and/or NFM infusion (Fig. 7), although a disodium hydrogen phosphate solution was used instead of blood in our experiments. In the present study, the flow was disturbed when higher concentrations of NFM (4 mg/mL) were infused at an infusion rate of 6 mL/hr, as evidenced by a significant change in pressure and the observation of precipitates in the flow tube. Pharmaceutical products that include NFM...
at levels of 10 and 50 mg are dissolved in 1 and 5 mL of 5% glucose (NFM 10 mg/mL), respectively, and then infused at an infusion rate of 20–50 mg/hr (2–5 mL/h) in the hemodialysis circuit. The general blood flow rate for hemodialysis (200 mL/min = 6 L/h) was significantly higher than the flow rates used in this study (12–24 mL/h). Since an increase in the rates of flow and infusion minimized the accumulation of a precipitate (Figs. 7D–F) possibly due to the clearance of precipitates just after their formation, such precipitates might be difficult to observe in actual hemodialysis with higher blood flow. In addition, longer inner diameters of flow (3.3–4.7 mm) and infusion (2.4 mm) tubing for hemodialysis compared to those in this study (0.25 mm) could also affect the flow and infusion and thus reduce the extent of precipitation. Since size and shape of primary precipitates and their growth rates could be factors that affect the accumulation or clearance of precipitates, they also should be investigated in a future study.

However, several reports have indicated that precipitates are actually formed during hemodialysis even though the preparation and infusion of NFM were conducted appropriately. Flow could be hindered in the region where blood flow and the NFM infusion mingle from different directions, thus leading to the formation, growth and the accumulation of a precipitate. Indeed, precipitates were observed in the flow tube connected to the drain side of the three-way connector (Fig. 8). Although Arishima et al. reported on precipitate formation in the circuit of blood flow, Hirashima et al. detected precipitates in the infusion tube. In hemodialysis, the infusion rate of NFM (2–5 mL/h) is much slower than blood flow (6 L/h). Such differences in rates of infusion and blood flow may cause backflow from the region of faster flow (blood flow) to the region of slower flow (NFM infusion), resulting the accumulation of un-cleared precipitates in the infusion tube. Thus, precipitation formation could occur at region where the infusion and blood flow merge. In addition, it should be noted that the formation and accumulation of a precipitate could be accelerated by an elevated concentration of anionic electrolytes such as phosphate and/or high concentrations of NFM.

NFM concentrations of 0.1–4 mg/mL were used in this study, since the solution of pharmaceutical product (10 mg/mL) that is used is diluted when it is clinically infused into the hemodialysis circuit. Although the dilution ratio is different between conditions of hemodialysis (e.g., infusion and blood flow rates), our data obtained in which such a wide range of NFM concentrations were used would permit more relevant assessments of precipitate formation during the infusion of NFM in hemodialysis conditions.

Several studies recently reported on the efficacy of an NFM infusion for the treatment of COVID-19 patients with severe respiratory failure and/or coagulopathy. Therefore, in addition to the use of NFM for hemodialysis, acute pancreatitis and DIC, extending its clinical use can be anticipated in the near future. The formation of precipitates by the infusion of NFM into blood could lead to the interruption of NFM treatment for hemodialysis, acute pancreatitis, DIC and COVID-19. The use of succinic acid in pharmaceutical products as a pH adjuster could contribute to the reduction of this risk. A faster infusion flow rate of a lower concentration of NFM may also become an alternative option to prevent precipitate formation although its therapeutic effects and side effects would need to be confirmed in future experiments. In addition, serum levels of anionic electrolytes, especially phosphate, should be carefully monitored before an NFM treatment since they could become risk factors for precipitate formation.

CONCLUSION

The findings presented herein provide fundamental and valuable information in terms of our understanding of the mechanism responsible for precipitate formation that can occur as the result of an NFM infusion. In addition, these findings are also valuable in terms of developing new formulations and/or dosage regimens for the administration of NFM during hemodialysis or treatments of acute pancreatitis, DIC and COVID-19.

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Conflict of Interest The authors have the following interests. The nafamostat mesilate used in this study was a gift from the Kobayashi Kako Co., Ltd. There are no further patents, products under development or marketed products to declare. This does not alter the authors’ adherence to journal policies on sharing data and materials, as detailed in the guide for authors.

Supplementary Materials The online version of this article contains supplementary materials.

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