Development of a liquid formulation of poorly water-soluble isosteviol sodium using the co-solvent technology

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ABSTRACT
An intravenously injectable liquid formulation of the poorly water-soluble isosteviol sodium (ISVNa) that has a great clinical potential for cardiovascular diseases was developed using the co-solvent technology. The pH and composition of the co-solvent were optimized to obtain a stable liquid formulation (termed as STVNa) based on saline at pH 10.0 containing 25\% (v/v) of ethanol and 20\% (v/v) of propylene glycol. STVNa was physicochemically stable upon storage for more than 3 months under various conditions. In vitro studies showed that STVNa did not induce hemolytic effects up to 9.1\% (v/v) after 3 h of incubation and it was cytocompatible up to 50 \( \mu \)g/mL in H2C9 cells. Furthermore, STVNa showed acceptable safety and pharmacokinetic parameters comparable with those of ISVNa in saline (dissolved at 60 \( ^\circ \)C) upon i.v. injection in Wistar rats. Overall, the results demonstrated that STVNa is a promising formulation of ISVNa for clinical translation.

Introduction
Atherosclerosis and associated coronary artery diseases are the leading causes of death worldwide. Symptoms such as paralysis, impaired speech, or loss of vision can be experienced by atherosclerosis patients due to vascular thrombosis\textsuperscript{1,2}. According to the statistical data, about 795 000 American residents experience a new or relapse stroke annually\textsuperscript{3}. In Russia and China, the estimated death rates are 5–10 times higher than in the United States\textsuperscript{1}.

Anti-thrombotic medicines, blood pressure-lowering medicines, beta-blockers, calcium-channel blockers, renin–angiotensin system agents, lipid-modified medicines, nitrates, and anti-arrhythmic medicines dominated the cardiovascular diseases medicine market according to the prescription rates as reported by Australian Institute of Health and Welfare\textsuperscript{4}. However, various side-effects are associated with these types of drugs. For example, statins are widely used for inhibiting HMG-CoA reductase; however, they may induce safety issues such as serious and uncommon musculoskeletal reactions, for example, rhabdomyolysis. Niacins significantly increase high-density lipoprotein (HDL), but they are poorly tolerable which reduces the patient compliance. Therefore, therapeutic agents for reversing or slowing down the progress of cardiovascular diseases with decreased side-effects are highly demanded\textsuperscript{5}.

Kaurene compounds (belong to the terpene glycosides family) are potential therapeutics for cardiovascular diseases\textsuperscript{5}. For example, stevioside (Figure 1(a)), a non-caloric sweetener, has anti-hypertensive, anti-inflammatory, anti-tumor, and immunomodulatory effects\textsuperscript{6}. Extensive studies showed that steviol (Figure 1(b)) may be the pharmacologically active compound which is metabolized from stevioside (a) in vivo. However, steviol (b) has been reported to be mutagenic when used on a daily basis. Isosteviol (ISV, Figure 1(c)) with a similar chemical structure as steviol (b) is non-mutagenic and exhibits activities for the treatment of coronary diseases, stroke, cerebral ischemia, arrhythmia, etc\textsuperscript{7–10}. Vasodilation induced by ISV (c) is caused by the opening of SKCa and KATP channels\textsuperscript{11}. Moreover, ISV has been applied in the treatment of type 2 diabetes and the cognitive impairment functions\textsuperscript{8,12–14}.

Intravenous (i.v.) administration of drugs induces quick therapeutic effects and is considered to be the most suitable administration method for unconscious patients. However, i.v. administration of terpene glycosides is highly limited by their low water solubility\textsuperscript{15}. To tackle this problem, various methods have been developed to increase the water solubility of hydrophobic terpene glycosides. For example, ISV (c) is lipophilic\textsuperscript{16}, but its water solubility can be enhanced by converting it to its salt form (i.e. ISVNa)\textsuperscript{5}. However, the solubility of ISVNa (Figure 1(d)) (0.5 mg/mL shown below) is still too low for i.v. administration. ISV (c) has also been incorporated in cyclodextrin (CD) inclusion complexes; however, a solution formulation of ISV (c) incorporated in CD with sufficient stability and acceptable safety for i.v. administration has not been reported yet due to the toxicity, complex preparation process, and high cost of the formulation\textsuperscript{15,17–19}. Herein, we report the development of a liquid formulation of ISVNa (d) for i.v. administration which has acceptable pH value, compatibility with dilute, sufficient physicochemical stabilities upon storage, and good safety for i.v. administration.

Investigations and results

\textbf{Solubility of ISVNa in different solvents}

The solubility of ISVNa was determined to assist the design of the liquid formulation, and the results in water and organic solvents

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acceptable for i.v. injection at 25 °C are provided in Table 1. Apart from PEG 400, the compound exhibited low solubilities in these solvents, especially in water (0.5 mg/mL), which can be explained by the lipophilic hydrocarbon skeleton structure of ISVNa. Furthermore, it was observed that crystals of ISVNa were formed in the mother liquid used in the solubility study several hours after filtration, which confirms the ease of crystallization of ISVNa in water.

**Optimization of the pH of ISVNa solution**

Saline, instead of water, was used to prepare the STVNa injection formulation to ensure a proper osmotic pressure for i.v. injection. Solubilization of ISVNa in saline at different pH values was detected by the turbidity of the systems, which was performed using 96-well microplates and a Multimode Reader. The turbidity of the samples in the pH group was obviously lower than those in the other two groups (Figure 2), which can be explained by the weak acidic nature of ISVNa. The ISV molecules are uncharged at lower pH, whereas they are negatively charged at higher pH. The ionized molecules possess higher water solubility than the neutral species; therefore, increasing pH is effective to enhance the water solubility of ISVNa. At the highest concentration, the solubility of ISVNa in sterilized water was greater than that in saline, which can be ascribed by the common ion effect. To ensure a good compatibility of the liquid formulation with the body, saline with modified pH was chosen for the following studies.

Turbidity of ISVNa solution in saline at pH between 10.2 and 12.0 was much lower than that at pH between 12.9 and 13.3 (Figure 3), which means the optimal pH value for ISVNa solution is between 10.2 and 12.0. For i.v. injection of the ISVNa formulation, a relatively low pH value (10.2) was chosen. ISVNa could be dissolved up to 5 mg/mL in saline at pH 10.2; however, crystallization of ISVNa in the solution occurred 7 days after storage at 4 °C.

**Optimization of the co-solvent system for ISVNa in saline**

Since the maximal solubility of ISVNa in saline at pH 10.2 is still lower than that required for i.v. administration, the solubilization of ISVNa was further improved using several water mixable

![Figure 1. Chemical structures of stevioside (a), steviol (b), ISV (c), and ISVNa (d).](image)

![Figure 2. Turbidity of samples in different solvents with various concentrations (mean ± SD, n = 3).](image)

![Figure 3. Turbidity of samples in different pH values saline with various concentrations (mean ± SD, n = 3).](image)
organic solvents (namely PEG 400, glycerol, propylene glycol, and ethanol) acceptable for i.v. administration by FDA. It was found that ISVNa at 5 mg/mL was soluble in saline with above 40% of ethanol, but insoluble in saline with 11.25% PEG 400, 12.62% glycerol, or 50% propylene glycol (Table 2).

Further studies were performed to use a minimal amount of organic solvents to prepare the liquid formulation of ISVNa using co-solvents. The results (Table 3) show that ISVNa was soluble at 5 mg/mL in different combinations of the organic solvents apart from that of 12.62% glycerol and 11.25% PEG 400. Different combinations of organic solvents (Table 4) were tested, and the co-solvent system containing 25% ethanol (v/v), 20% propylene glycol (v/v), and pH modified to 10 saline (F1-g) was selected as the solvent system for ISVNa due to the low amount of organic solvents used. Formulation F1-g was selected to prepare the ISVNa formulation instead of F1-f that has a lower amount of ethanol because the amount of total excipients (45%) of Formulation F1-g is lower than that of F1-f (55%), and this is meaningful for decreasing the production cost of the final formulation. The optimized formulation of STVNa was prepared by dissolving 20 mg/mL of ISVNa in the co-solvent system, and a clear and transparent solution was obtained.

**Sterilization of STVNa**

Autoclaving sterilization is an easy and preferable method to ensure sterility of liquid formulations, which can be applied if the formulation is compatible with heat and moisture. Stability of STVNa was studied after autoclaving sterilization. The clarity of STVNa was not changed after the sterilization operation, that is, no color change and precipitation were observed. The pH of the STVNa formulation was not affected by the sterilization. STVNa was chemically stable during this process without formation of impurities as detected by HPLC (High-Performance Liquid Chromatography), and the content of ISVNa was within the acceptable range 95–105% (Supplementary Table S1).

**Dilution compatibility of STVNa**

For i.v. administration, dilutions of STVNa with different clinically used solvents are necessary, and therefore the compatibility of STVNa with the dilution operations was assessed. Table 5 shows that the pH of the formulation after dilution with the solvents was declined to between 8.4 and 7.3, which was in the acceptable range. The turbidity of STVNa diluted in saline was nearly 0, whereas in GS and GNS, the value increased to between 0.002 and 0.006; therefore, saline is considered as the most suitable solvent for dilution of the formulation for further (pre-)clinical studies.

**Physicochemical stability of STVNa**

**Low temperature stability study**

Since STVNa injection is an aqueous formulation that would be stored at low temperatures (4 and –20 °C, respectively) and warmed up to room temperature before administration, it is necessary to monitor the content and possible impurities of the formulation during the cooling–heating cycles. The characteristics (physical appearance, pH, content, and impurities) of STVNa after recovery from low temperatures are presented in Supplementary Table S2. The physical appearance (color and clarity) and pH of STVNa were not changed after 3 cycles of storage at 4 and –20 °C, respectively, with storage at each temperature for 2 days. Content of ISVNa in the recovered STVNa was within the acceptable range of 95–105%, and impurities in STVNa were not detected by HPLC. The overall results indicate a good stability of STVNa after storage at low temperatures.

**Influential factors study**

The pH, clarity, impurities, and content of STVNa were monitored after storage at high temperature (60 °C, 10 days) and under high light irradiation (4500 ± 500 Lux, 10 days), respectively. Supplementary Table S3 shows that the pH, clarity, and content of STVNa after storage at the afore-mentioned conditions were

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Table 1. Solubility of ISVNa in different solvents at 25 °C.

| Solvents | Ethanol | Propylene glycol | Water | PEG 400 | Glycerin |
|----------|---------|------------------|-------|---------|---------|
| Solubility (mg/mL) | 11.7 | 3.7 | 0.5 | 33.6 | 23.3 |

Table 2. Formulations table containing single solvent and drug.

| Ingredients | Batch code | ISVNa (mg) | Ethanol (v/v) | Propylene glycol (v/v) | PEG 400 (v/v) | Total volume* (mL) | Clarity |
|-------------|------------|------------|---------------|-----------------------|---------------|-------------------|--------|
| F-I         | 50         | 49%        | –             | –                     | –             | 10                | +      |
| F-II        | 50         | 40%        | –             | –                     | –             | 10                | +      |
| F-III       | 50         | 35%        | –             | –                     | –             | 10                | –      |
| F-IV        | 50         | –          | 50%           | –                     | –             | 10                | –      |
| F-V         | 50         | –          | –             | 12.62%                | –             | 10                | –      |
| F-VI        | 50         | –          | –             | 11.25%                | –             | 10                | –      |

*Saline at pH 10 was used as the diluent. +: qualified; –: unqualified.

Table 3. Formulations table containing co-solvent and drug to screen excipients species.

| Ingredients | Batch code | ISVNa (mg) | Ethanol (v/v) | Propylene glycol (v/v) | PEG 400 (v/v) | Total volume* (mL) | Clarity |
|-------------|------------|------------|---------------|-----------------------|---------------|-------------------|--------|
| F1          | 50         | 29%        | 50%           | –                     | –             | 10                | +      |
| F2          | 50         | 49%        | –             | 12.62%                | –             | 10                | +      |
| F3          | 50         | 49%        | –             | –                     | 11.25%        | 10                | +      |
| F4          | 50         | –          | 50%           | 12.62%                | –             | 10                | +      |
| F5          | 50         | –          | 50%           | –                     | 11.25%        | 10                | +      |
| F6          | 50         | –          | –             | 12.62%                | 11.25%        | 10                | –      |

*Saline at pH 10 was used as the diluent. +: qualified; –: unqualified.
within the acceptable ranges, and impurities were not detected by HPLC.

**Acceleration stability study**

In the acceleration stability study (40 ± 2°C, 75 ± 5% relative humidity, 3 months), the pH, clarity, and content of STVNa after 3 months were consistent. Two degradants of 0.032 and 0.036% compared to ISVNa with retention times of 10.12 and 15.55 min were detected by HPLC at the 3-month time point (Supplementary Table S4), which are in the acceptable range according to ICH guidelines.

**Long-term stability study**

In the long-term stability study (25 ± 2°C, 60 ± 10% relative humidity, 3 months), the pH, clarity, and content of STVNa were not affected, and there were two degradants generated with the same retention times as those in the acceleration stability study (0.037 and 0.037%, respectively, compared to ISVNa) at the 3-month time point (Supplementary Table S5). These results indicate that the physicochemical stabilities of STVNa were in the acceptable range for at least 3 months.

**Hemolysis study of STVNa**

Hemolytic activity is a critical issue for injectable formulations containing organic solvents. To assess the hemolytic activity, the release of hemoglobin from red blood cells (RBCs) is detected as an indication of the membrane-damaging. The release of hemoglobin from RBCs incubated with STVNa of 5.7% and 9.1% (v/v) were compared with that of saline, and was significantly lower than that of water (Figure 4). The results indicate that STVNa has a good hemolytic compatibility which is favorable for i.v. administration.

**Cytocompatibility of STVNa**

Cytotoxicity is another concern for injectable formulation containing organic solvents. The cytotoxicity effect of STVNa and its corresponding blank solvent in H2C9 cells were determined by standard MTT assay. STVNa did not affect the viability of the cells with the concentration of ISVNa up to 50 μg/mL for 24 h, and the blank solvent at corresponding concentrations was also cytocompatible as shown in Figure 5. Moreover, it can be roughly calculated that after in vivo injection, the maximal concentration of ISVNa in the blood is around 20 μg/mL which is far below the

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Table 4. Formulations table containing co-solvent and drug to optimize excipients amounts.

| Ingredients                                                                 | 100 mL          | 250 mL          |
|-----------------------------------------------------------------------------|-----------------|-----------------|
| Batch code ISVNa (mg) Ethanol (v/v) Propylene glycol (v/v) Glycerol (v/v) PEG 400 (v/v) Total volume (mL) Clarity |                 |                 |
| F1-a 50 5% 20% - - 10 -                                                      |                 |                 |
| F1-b 50 5% 30% - - 10 -                                                     |                 |                 |
| F1-c 50 5% 40% - - 10 -                                                     |                 |                 |
| F1-d 50 15% 20% - - 10 -                                                    |                 |                 |
| F1-e 50 15% 30% - - 10 -                                                    |                 |                 |
| F1-f 50 15% 40% - - 10 -                                                    |                 |                 |
| F1-g 50 25% 20% - - 10 -                                                    |                 |                 |
| F1-h 50 25% 30% - - 10 +                                                   |                 |                 |
| F1-i 50 25% 40% - - 10 +                                                   |                 |                 |
| F2-a 50 20% - 10% - 10 -                                                   |                 |                 |
| F2-b 50 30% - 10% - 10 -                                                   |                 |                 |
| F2-c 50 40% - 10% - 10 -                                                   |                 |                 |
| F3-a 50 20% - - 10% - 10 -                                                 |                 |                 |
| F3-b 50 30% - - 10% - 10 -                                                 |                 |                 |
| F3-c 50 40% - - 10% - 10 +                                                 |                 |                 |
| F4-a 50 - 20% 10% - 10 -                                                   |                 |                 |
| F4-b 50 - 30% 10% - 10 -                                                   |                 |                 |
| F4-c 50 - 40% 10% - 10 -                                                   |                 |                 |
| F5-a 50 - 20% 10% - 10 -                                                   |                 |                 |
| F5-b 50 - 30% 10% - 10 -                                                   |                 |                 |
| F5-c 50 - 40% 10% - 10 +                                                   |                 |                 |

Saline at pH 10 was used as the diluent. +: qualified; -: unqualified.

Table 5. Compatibility of STVNa in different dilutions.

| pH Turbidity within 12 h | Impurity (%) | Content (%) | pH Turbidity within 12 h | Impurity (%) | Content (%) |
|--------------------------|--------------|-------------|--------------------------|--------------|-------------|
| Saline                   | 8.0 ± 0.0    | 0.001 ± 0   | N.D.                     | 7.3 ± 0.0    | 0 ± 0.001   |
| GS                       | 8.4 ± 0.2    | 0.002 ± 0.001 | N.D.                     | 7.7 ± 0.1    | 0.002 ± 0.002 | N.D. | 1.9 |
| GNS                      | 8.2 ± 0.0    | 0.006 ± 0.001 | N.D.                     | 7.6 ± 0.1    | 0.002 ± 0   | N.D. | 1.9 |

Data displayed as mean ± SD (n = 3). N.D.: not detectable.

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![Figure 4. Hemoglobin release of rabbit RBCs incubated with different samples for 3 h at 37°C (mean ± SD, n = 3).](image-url)

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highest drug concentration in the present study (50 μg/mL); therefore, it suggests that the STVNa may have a good safety profile.

Pharmacokinetics study of STVNa

The ISV concentration in plasma versus time is presented in Figure 6. It is important to note that for the formulation of ISVNa in saline, dissolution of ISVNa was achieved with the assistance of heating at 60°C and the freshly prepared solution was injected to avoid precipitation of the compound, which is practically challenging for further clinical use of ISVNa. The pharmacokinetics profiles of ISVNa in STVNa and directly dissolved in saline are rather comparable, which suggests that the excipients used in STVNa hardly influenced the pharmacokinetics of ISVNa.

Discussion

ISVNa and related compounds are poorly water soluble due to the hydrophobic hydrocarbon skeleton in their chemical structures. Therefore, for i.v. administrations of this type of compounds, it is highly needed to develop liquid formulations that meet the requirement for i.v. route and are physicochemically stable during storage at various conditions. There are varieties of solubilization methods for hydrophobic compounds reported in the literature, including using surfactants, using co-solvent technique, liquisolid technique, incorporation of hydrophobic compounds in nanoparticulate systems (e.g. liposomes, micelles, and microemulsions), and host–guest inclusion complexation. However, surfactants are strictly limited for i.v. administration due to their obvious toxicity and nanoparticulate systems are known to be challenging for industrial and clinical translations. In the present study, a liquid formulation of ISVNa for i.v. administration was developed by adjusting the pH value and using co-solvents that are well accepted for pharmaceutical industry and clinics.

The solubility of ISVNa was shown to be sensitive to the pH value. Therefore, the first strategy was to adjust the pH value of saline that is used as the solvent. Theoretically, as the pH increases, the turbidity should decrease due to the ionized form of ISVNa at basic pH values. ISVNa can be ionized by increasing the medium pH value, which can explain the better solubility of ISVNa and lower turbidity of the solution when increasing the pH from 10.0 to 12.0. However, it should be pointed out that while elevating the pH value by adding NaOH, the Na+ concentration of the solution became higher, which can cause precipitation of ISVNa due to the common ion effect and can explain the higher turbidity of the solution when further increasing the pH value to 13.3. Since the pH value of ISVNa in normal saline (5 mg/mL) was nearly 9, pH values lower than 9 were not studied in the present study, and therefore, pH of 10.2 was selected in the final formulation. Although the solubility of ISVNa in saline was enhanced to 5 mg/mL at pH 10.0, it was found that crystallization of the compound occurred after 7 days of storage at 4°C. The enhanced solubility of ISVNa (5 mg/mL) was insufficient to achieve certain doses for i.v. administration and the stability of the solution was apparently too low for transportation and storage of the formulation. Therefore, four organic solvents which are acceptable by FDA for i.v. administration (below certain concentration limits) were incorporated to further increase the solubility of ISVNa and its stability during storage. After screening the individual solvents at the maximal concentrations allowed and different combinations of them, the optimized solvent system for ISVNa composed of saline at pH 10.0, 25% of ethanol and 20% of propylene glycol was obtained. Similar solvents containing ethanol and propylene glycol for hydrophobic drugs such as phenobarbital, pentobarbital sodium, phenytoin sodium, digoxin, and diazepam have been applied in clinics. The liquid formulation of ISVNa in the optimized co-solvent at 20 mg/mL (2%, w/w) (i.e. STVNa) was prepared for the in vitro and in vivo studies. In a previous publication, the water solubility of terpene glycoside was increased to 0.1–7% (w/w) by incorporation in CD inclusion complex. For example, Reb C (which belongs to the terpene glycoside family) was solubilized at 2 and 4% (w/w) in γ-CD and the solutions were clear for 30 and 4 days. In the present study, ISVNa was solubilized up to 20 mg/mL in the optimized solvent system and the solutions were physicochemically stable for at least 90 days. Compared to other solubilization techniques that have been applied in the pharmaceutical field, the current strategy is preferred because that 1) the production of STVNa is simple and cost-effective, and is therefore more industrially applicable; 2) the used organic solvents and their concentrations in STVNa are well accepted in clinics.

Sterilization of STVNa by autoclaving was conducted to ensure the safety of the formulation for i.v. administration, and STVNa was compatible with the sterilization process. The formulation was diluted to 1 and 0.4 mg/mL. According to the previous pharmacological studies performed by Tan and Hu et al., the effective dose of ISVNa in rat was between 0.2 and 10 mg/kg, which is equal to the clinical dose of the STVNa at 0.4 and 20 mg/mL when the injection volumes were 5 mL. Stabilities of STVNa under different conditions that may apply to its production, transportation, and storage were systemically assessed. STVNa was shown to be
stable during storage at low temperatures, and negligible amounts of impurities were generated during the acceleration and long-term studies with harsh conditions involved, and both impurities and contents were in the acceptable range according to ICH guidelines\(^\text{24}\). The good stability of STVNa can be explained by the composition of the solvent, in which excipients prone to degradations such as lipids or polymers are not used.

To preliminarily study the safety of STVNa for i.v. administration, the hemolytic effect and cytocompatibility of STVNa were examined, and the results showed that STVNa has an acceptable safety for i.v. injection, which was also confirmed that no significant acute toxicities were observed in Wistar rats that received STVNa intravenously. Pharmacokinetics parameters of ISVNa in STVNa and that in saline dissolved at 60 °C were comparable to each other. However, solubilization of ISVNa in saline was achieved with heating at 60 °C prior to injection, which is poorly applicable in clinics.

**Conclusion**

In this study, a liquid formulation of ISVNa (i.e. STVNa) for i.v. injection was developed. ISVNa was dissolved at 20 mg/mL in the formulation which showed sufficient physicochemical stability upon storage for at least three months under various conditions. The formulation did not induce hemolytic effects up to 9.1% (v/v) after 3 h of incubation and it showed good cytocompatibility up to 50 μg/mL in H2C9 cells. Finally, STVNa exhibited high safety after i.v. injection and similar pharmacokinetic parameters as ISVNa in saline dissolved at 60 °C. Further studies to assess the therapeutic efficacy of the liquid formulation are currently ongoing.

**Experimental**

**Materials**

ISVNa was provided by Key-Pharma Biomedical Inc. with a purity greater than 99.9% (Dongguan, China). All formulation components met USP requirements for pharmaceutical excipients. Ampoules were friendly donated by JingGu Packing Material co., Ltd. (Chengdu, China). HPLC grade solvents were purchased from Fisher Scientific (Shanghai, China), Water was purified by a Milli-Q Ultrapure Water Purifier (Darmstadt, Germany).

**Methods**

**Solubility of ISVNa in different solvents**

The equilibrium solubility method was used for the solubility study\(^\text{11}\). An excess amount of ISVNa was placed in contact with 10 mL solvents in sealed tubes. Samples were maintained with gentle agitation for 12 h at 25 °C using a constant temperature vibrator. The saturated solution was centrifuged (12,000 RPM, 3 min) and the supernatant was filtered through a 0.45 μm micro-porous membrane. The concentration of ISVNa in the supernatant was determined using HPLC (Shimadzu Corporation, Kyoto, Japan).

**Optimization of pH for ISVNa solution in saline**

Three groups of samples were prepared by adding the required weight of ISVNa to saline at pH 10.2 (pH adjusted using sodium hydroxide solution), saline, and sterilized water at room temperature. Four concentrations (0.5, 1, 2.5, and 5 mg/mL) of ISVNa were prepared for each group. The samples were shaken for 30 s for 6 times with a 5-min interval between two operations by a vortex mixer. The turbidity of the samples was determined using a photometric method with a detection wavelength at 550 nm. In the next experiment, the pH value of ISVNa in saline was optimized at four ISVNa concentrations (2.5, 5, 8, and 10 mg/mL) using the same preparation and detection methods.

**Optimization of co-solvent for ISVNa solution in saline**

To increase the solubility of ISVNa in saline at pH 10.2, 4 organic solvents (PEG 400, glycerol, propylene glycol, and ethanol) permitted by FDA for i.v. administration were used. First, each of the 4 solvents with the maximum amounts (11.25%, 12.62%, 50%, and 49%, respectively) allowed by FDA for i.v. administration were tested with ISVNa at 5 mg/mL\(^\text{22}\). Second, two of these organic solvents were used together at the maximal allowable amounts described above. Finally, the amounts of co-organic solvents were decreased to the minimal level while completely dissolving ISVNa in saline at pH 10.2. All samples were stored at 4 °C and visually monitored to observe the precipitation of ISVNa for 10 days. An optimized formulation of ISVNa (referred to as STVNa) at 20 mg/mL was used for the following studies.

**Sterilization method of STVNa**

STVNa was performed by autoclaving. STVNa was placed upright in an autoclave sterilizer (TOMY, SX-500) with a condition of 15 psi and 121 °C for 15 min. pH, clarity, impurities, and content were studied before and after sterilization. pH was measured by a pH meter (Satorio, Germany). Clarity was visually assessed. Content and impurities were determined by HPLC.

**Dilution compatibility of STVNa**

To evaluate the compatibility of STVNa with dilution solvents which are used in clinics for i.v. administration, at 25 °C, 5 mL of STVNa was diluted with 100 and 250 mL saline, 100 and 250 mL 5% glucose solution (GS), and 100 and 250 mL 5% glucose solution and saline (GNS), respectively. Three different solvents (saline, GS, and GNS) at pH between 7.3 and 8.4, which are in the acceptable range for i.v. injection, were used in the diluent compatibility study. pH and turbidity of the formulation after dilution were measured using methods described above.

**Physicochemical stabilities of STVNa**

The stability assessment was composed of 4 separated studies including low temperature stability study (3 cycles of storage at 4 and −20 °C in glass ampoules sealed for 2 days and warming up to room temperature), influential factors study (60 °C and 4500 ± 500 Lux stored in glass ampoules sealed for 10 days, respectively), acceleration stability study (40 ± 2 °C, 75 ± 5% relative humidity, 3 months), and long-term stability study (25 ± 2 °C, 60 ± 10% relative humidity, 3 months) according to Chinese Pharmacopoeia\(^\text{32}\). For acceleration and long-term stability study, the formulations were stored in glass ampoules sealed using a constant temperature and humidity chamber. These samples were analyzed with respect to clarity, pH, impurities, and content as described above.

**Hemolysis study of STVNa**

Blood was collected gently from New Zealand rabbits from veins into a clean glass beaker and fibrinogen was rapidly removed by stirring the blood with cotton swabs. The residual RBCs were washed with an appropriate amount of saline and centrifuged at 1300 rpm for 3 min, and resuspended in saline after removing the supernatant. The operation was repeated for two more times. Finally, RBCs suspension was diluted to 2% (v/v) in saline, and the...
suspension was incubated with saline (negative control group), distilled water (positive control group), and STVNa (5.7% and 9.1% (v/v)), respectively. The mixtures were incubated at 37°C for 3 h in a water bath and samples were withdrawn at 0.5, 1, 2, and 3 h. The RBCs in the mixture were centrifuged to the bottom and the release of hemoglobin was measured by a Multimode Reader (PerkinElmer, Waltham, MA) of the supernatant at 540 nm.

**Cytocompatibility of STVNa in H2C9 cells**

H2C9 cells (generation 8) were plated in a 25 cm² culture flask in DMEM contained 10% FBS and 1% penicillin and streptomycin. The cells were sub-cultured in a 96-well plate at a density of 5 × 10³ cells/well in 100 μL of DMEM (supplemented with 10% FBS and 1% penicillin and streptomycin) for 18 h. Subsequently, the medium was replaced and the cells were washed with PBS. Afterwards, the STVNa with ISVNa concentration ranging from 0.5 μg/mL to 50 μg/mL and the blank solvent from 0.5 μg/mL to 500 μg/mL in DMEM with 2% FBS were added to the cells. After 24 h, the medium was removed and fresh DMEM and 20 μL MTT solution (5 mg/mL in DMEM) were added to each well. After 4 h of incubation, the medium was removed and 150 μL of DMSO was added to each well to solubilize any formazan crystals. The absorbance was measured at 492 nm using a Multimode Reader after shaking for 10 min.

Cells incubated with blank cell culture medium were used as the control group. The cell viability was calculated as Cell viability = (AT/AC) × 100

(1)

where AT is the absorbance of cells incubated with STVNa or blank solvent and AC is the absorbance of cells of the control group.

**Pharmacokinetics study of STVNa**

The animal experiment and the protocol were approved by the Ethics Committee of South China University of Technology. Pharmacokinetics of STVNa was evaluated by analysis of the plasma ISV concentration versus time and AUC_{inf} in Wistar rats. Rats were divided into two groups (n = 4) and fasted overnight. The pharmacokinetics was assessed following i.v. injection of STVNa diluted 2 times in saline at a dose of 10 mg/mL. ISVNa dissolved in saline at 10 mg/mL after incubation at 60°C was used as control. Blood samples were collected before and after injection at various time points. Plasma was obtained by centrifuging the blood samples at 4000 rpm for 10 min at 4°C and stored at −20°C before analysis.

**Statistical analysis**

All experimental data were presented as the mean value of at least three replicates ± SD; p < 0.05 was considered statistically significant.

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**Disclosure statement**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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