Design and content determination of nimesulide injectable formulation

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Abstract: The aim of the present study was to formulate Nimesulide inject able solution, establish a method for content determination, and accumulate data for registration of a new Nimesulide formulation. The optimal Nimesulide inject able formulation was determined based on the results of single factor test and orthogonal test. Moreover, clarity, stability, pH, content and related substances of Nimesulide were used as the main study indicators. The content of Nimesulide in inject able solution was determined by the high performance liquid chromatography (HPLC) method. The mobile phase consisted of V (methanol): V (potassium dihydrogen phosphate, pH 4.2)=60:40 at a flow rate of 1.0mL/min. The detection was carried out with UV detector (λ_{max} =254 nm) under a column temperature of 25°C and an injection volume of 20μL. The optimal inject able formulation was 4% Nimesulide, 4% ethanolamine, 0.1% L-cysteine, 0.01% EDTA-2Na, a suitable amount of lactic acid and water for injection. Nimesulide detection limits range from 20 to 80μg/mL with a correlation coefficient of 0.9995 and high average recovery 99.91% (RSD=0.06%). In conclusion, the formulation was suitable for Nimesulide inject able form, and the determination method was simple, sensitive and accurate. Therefore, the Nimesulide inject able formulation can be used for industrial production and effectively controlled.

Keywords: Nimesulide, inject able form, formulation, content.

INTRODUCTION

Nimesulide, a Non-Steroids Anti-Inflammatory drug (NSAID) (Suleyman *et al*., 2008), was first marketed in Italy by Boehringer Biochemia in 1985 (Ottaviani *et al*., 1993). Moreover, it is the first cyclooxygenase (COX) inhibitor being marketed worldwide. COX is an isozyme, mainly present in two isoforms: COX-1 and COX-2. The commonly used antipyretics and analgesics, such as paracetamol, inhibit both COX-1 and COX-2. It has been reported that inhibiting COX-1 produces untoward effects, whereas, inhibiting COX-2 produces positive effects. Therefore, a highly selective COX-2 inhibitor can improve the effectiveness of NSAIDs and reduce adverse effects (Pi *et al*., 2006; Rao *et al*., 2008).

Nimesulide is a strong selective COX-2 inhibitor, widely used for its antipyretic, analgesic, anti-inflammatory and antirheumatic effects (Jin *et al*., 2011).

Presently, marketed forms of Nimesulide in China are tablets, dispersible tablets, sustained-release tablets, capsules, granules for oral suspension, oral syrup, suppositories, transdermal agent, and latex additives (Li *et al*., 2002; Yang *et al*., 2006; Pan *et al*., 2006; Liu *et al*., 2009; Zhu *et al*., 2010; Khan *et al*., 2011; Yuan *et al*., 2011; Qiu *et al*., 2012; Zhang *et al*., 2012).

To our knowledge, there is no injectable preparation on the market. In 1998, the China Medicine Biotechnology Limited company has patented Nimesulide inject able preparation for publication only (CN 1141088C) without marketing authorization (Rajith *et al*., 1998). This inject able preparation contains mainly oily substrate and organic solvent (90%-97.5%) with almost no water for injection. Therefore, it could increase irritation and environmental pollution.

Our group studied the Nimesulide injectable, belonging to the third new drug class. Moreover, the key technology of its preparation was patented in July 2011 (China ZL 201010042066.9) (Luo *et al*., 2010). Our preliminary research has shown that, the antipyretic, analgesic, anti-inflammatory effects and security of Nimesulide injectable are equal to, if not better than, similar drugs presently used in clinics (Jiang *et al*., 2012; Wang *et al*., 2012). In addition, Nimesulide injectable presented many advantages such as, high concentrations of the primary drug and weak irritation. The administration route of this injection can be intravenous or intramuscular.

In the present study, further investigation to optimize the formulation and content determination has been conducted in order to provide scientific basis for clinical experiments and registration of the Nimesulide injectable preparation.

MATERIALS AND METHODS

Apparatus

Chromatographic analysis was performed using a Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) consisting of LC-20AD pump, an auto sampler (Model SIL-20A) and photodiode array UV-Vis detector

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(Model SPD-M20A); HitachiU-1800 Ultraviolet spectrophotometer (Hitachi, Japan); PHS-3C PH Meter (Shanghai Hong Yi Instrument Co. Ltd., Shanghai, China); YB-2 clarity detector (Tianjin Skylight Optical Instrument Co. Ltd., Tianjin, China).

Reagents

Nimesulide raw material (Lot 20121012) was obtained from Tianjin Institute of Pharmaceutical Research Pharmaceutical Co. Ltd (Tianjin, China). Nimesulide reference standard (Lot 100555-200501) was obtained from National Institute for Food and Drug Control (China). Nimesulide injection was manufactured by our laboratory (Lot 20120205, 20120210, 20120212). Methanol of HPLC grade was purchased from (Tianjin Shield Fine Chemicals Company (Tianjin, China), Potassium dihydrogen was obtained from Kelong Chemical Reagent Company (Chengdu, China), Ethanolamine of medicinal grade (Lot 20111201), Diethanolamine of medicinal grade (Lot 20120211), L-cysteine of medicinal grade (Lot 20111101), Lactic acid of medicinal grade (Lot 20111004), Sodium sulfite of medicinal grade (Lot 20111115), sodium thiosulfate of medicinal grade (Lot 20120207), EDTA-2Na of medicinal grade (Lot 20120101) and EDTA of medicinal grade (Lot 20111121) were obtained from Wuhan Collie Chemical Co. Ltd (Wuhan, China), Shanghai Hui Xing Biochemical Reagent Co. Ltd (Shanghai, China), Guangzhou Su Well Chemical Co. Ltd (Guangzhou, China), and Hunan Huari Pharmaceutical Co. Ltd (Hunan, China), respectively.

Single factor test

Solvents screen

Nimesulide raw material is not soluble in water. Therefore, common alkaline co-solvents such as ethanolamine and diethanolamine were added for a contrast experiment. The amount of the main drug Nimesulide was 4%, while the volume of ethanolamine and diethanolamine were 2.0%, 4.0%, 6.0%, 8.0%, respectively. In order to select the best alkaline co-solvent and its dosage range, eight formulations of Nimesulide injectable were designed. Then, clarity and stability parameters (sediment, crystallize, floccules, discoloration) were observed after 10 days under room temperature and refrigeration conditions. The results are shown in table 1.

Antioxidant screen

Three types of antioxidant including L-cysteine, sodium thiosulfate and sodium sulfite, were screened to increase Nimesulide inject able formulation stability. The amount of the main drug Nimesulide and ethanol amine was 4% each. Three different concentrations were set according to the usual dose of the three antioxidants, respectively.

In order to select the best antioxidant and its dosage range, nine formulations of Nimesulide inject able were prepared, and then tested for clarity and stability parameters as described previously. The results are shown in table 2.

Metal ion chelators screening

EDTA comparing to EDTA-2Na cannot only chelate metal ions in solution but also moderately reduce the injection pH. In the present experiment, six formulations of Nimesulide inject able containing 4% of Nimesulide and 4% of ethanolamine were designed. In order, to select the best metal ion complexing agent and its dosage range, three different concentrations of 0.002%, 0.02% and 0.2% were tested, respectively. Clarity and stability of the obtained formulations were further examined as described above. Results are illustrated in table 3.

Orthogonal test

On the basis of single factor test and pre-experiment results, Nimesulide, ethanolamine, L-cysteine, lactic acid and EDTA-2Na were selected as the main components of the formulation. In order to determine the optimum ratio of each excipient, the orthogonal test was designed (table 4). Furthermore, Nimesulide clarity, stability, pH, content and related substances were used as the main indexes. Results are shown in table 5.

Chromatography conditions

According to the methods reported by Houfei Yan (Hou *et al*., 2011), a series of mobile phases were prepared and then tested to determine Nimesulide content and related substances of the injectable formulation by HPLC.

An Ultimate XB-C_{18} (4.6 mm×250mm, 5μm) column was used. The optimal mobile phase was V (methanol): V (potassium dihydrogen phosphate, pH 4.2) =60: 40 at a flow rate of 1.0mL/min. The detection wavelength was 254nm under a column temperature of 25°C. Recorded chromatograms are shown in fig.1.

Sample preparation

A precise volume of Nimesulide inject able formulation (1mL) was first placed in 50mL volumetric flask and made up to the mark with methanol. Afterward, 1mL of the resulting dilution was placed in 25mL volumetric flask then diluted with methanol. After a gentle agitation, the sample solution of 32μg/mL Nimesulide was obtained.

Blank solution preparation

In view to prepare the blank solution, an injectable formulation Nimesulide free was diluted following the same method as in the sample solution.

Reference solutions

The standard solution was made up as follows: 25mg of Nimesulide standard were accurately weighed and dissolved in 50mL volumetric flask with methanol, then gently shaken to form homogenous Nimesulide solution of 500μg/mL.

Table 1: Effect of ethanolamine and diethanolamine on nimesulide solution

No.	1	2	3	4	5	6	7	8
Nimesulide (g)	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Ethanolamine (mL)	2.0	4.0	6.0	8.0	0.0	0.0	0.0	0.0
Diethanolamine (mL)	0.0	0.0	0.0	0.0	2.0	4.0	6.0	8.0
Constant volume (mL)	100	100	100	100	100	100	100	100
Clarity	a^-	a^+	a^+	a^+	a^-	a^-	a^-	a^-
Stability	b^-	b^+	b^+	b^+	b^-	b^-	b^-	b^-

Table 2: Effect of different types of antioxidants (sodium sulfite, L-cysteine, sodium thiosulfate) and their dosage on clarity and stability of Nimesulide injection

No.	1	2	3	4	5	6	7	8	9
Nimesulide (g)	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Ethanolamine (mL)	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Sodium sulfite (g)	0.20	0.30	0.40	0	0	0	0	0	0
L-cysteine (g)	0	0	0	0.05	0.10	0.15	0	0	0
Sodium thiosulfate (g)	0	0	0	0	0	0	0.05	0.10	0.15
Constant volume (mL)	100	100	100	100	100	100	100	100	100
Clarity	a^-	a^-	a^-	a^-	a^-	a^-	a^-	a^-	a^-
Stability	b^-	b^-	b^-	b^+	b^+	b^+	b^-	b^-	b^-

Table 3: Effect of EDTA and EDTA-2Na on clarity and stability of Nimesulide injection

No.	1	2	3	4	5	6
Nimesulide (g)	4	4	4	4	4	4
Ethanolamine (mL)	4	4	4	4	4	4
EDTA-2Na (g)	0.002	0.002	0.002	0	0	0
EDTA (g)	0	0	0	0.002	0.002	0.002
Constant volume (mL)	100	100	100	100	100	100
Clarity	a^+	a^+	a^+	a^-	a^-	a^-
Stability	b^+	b^+	b^+	b^-	b^-	b^-

. * a^-: Unclarity, a^+: Clarity, b^-: Unstability, b^+: Stability

20µL of sample, blank and reference solution were injected into HPLC, respectively. Then, their chromatograms were recorded.

Linear Relationship

A series of working reference solutions were prepared by diluting the Nimesulide reference solution with methanol at concentrations of 20, 30, 40, 50, 60, 70 and 80µg/mL, respectively. Then, 20µL of each resulting standard solution was injected onto the HPLC column. Thereafter, the mean calibration curve and the correlation coefficient were calculated. Results are illustrated in fig. 2.

Precision

Different operators evaluated the precision of the method by analyzing six samples of Nimesulide standard solution. The precision was expressed as the relative standard deviation (RSD%).

Repeatability

The repeatability of the assay method was evaluated by analyzing six replicates of Nimesulide sample solution by one operator, under the same conditions.

Average recovery

Three separate volumes (1mL) of Nimesulide sample solution were prepared. A precise volume of Nimesulide reference solution 1.0, 1.5 and 2.0mL was placed in three 50mL volumetric flasks, respectively, and made up to the mark with methanol. 1mL of the obtained solutions was added to the prepared sample solutions. Then, 20µL of the resulting mixtures were injected onto HPLC. Thereafter, the recovery was calculated.

Determination

Three batches of Nimesulide inject able solution were tested, each batch containing three samples. Sample solutions were prepared as described above. Then, 20µL of each sample was injected into HPLC and the content of Nimesulide was determined referring to regression equation.

Related Substances

A self-control method to determine the related substances of Nimesulide inject able solution was established under the same chromatographic conditions previously described.

Table 4: Orthogonal test of Nimesulide injection design

Levels	Factors		
	A (%)	B (%)	C (%)
1	3	0.08	0.005
2	4	0.10	0.010
3	5	0.12	0.015

A: Ethanolamine, B: L-cysteine, C: EDTA-2Na

A precise amount of Nimesulide equivalent to 40mg was first weighed to prepare a sample solution at a concentration of 50µg/L. A volume of the resulting solution was then diluted with the mobile phase to obtain a 1% self-control solution. Each solution was subjected to HPLC analysis. The detector's sensitivity was adjusted to detect the principal component (Nimesulide) at accurate integration. The total peak area of impurities was subsequently compared with the control solution peak area.

RESULTS

Single factor testing

Nimesulide dissolved well in ethanol amine solution of 4% and 8%, but not in diethanol amine solution (table 1). Used L-cysteine of 0.01% and 0.20% as antioxidants provided clarity and stability to the inject able solution (table 2). Moreover, EDTA comparing to EDTA-2Na crystallized the injection (table 3). Therefore, ethanolamine, L-cysteine and EDTA-2Na were selected as the main excipients of the injectable formulation.

Orthogonal testing

According to range analysis results, the order of various factors influencing the injection was: ethanolamine amount (A) >L-cysteine amount (B) >EDTA-2Na amount (C). According to the optimal level of each factor, the optimal prescription of the injection was A2B2C2. This suggested that the best inject able formulation is ethanolamine 4%, L-cysteine 0.10%, EDTA-2Na 0.01%, suitable amount of lactic acid and water for injection (table 5).

Formulation and technology

On the basis of orthogonal test results, the optimal Nimesulide inject able formulation preparation process was as follows: an amount of water for injection equivalent to 40% of the total volume was placed into a suitable container. 4% ethanolamine was first added and mixed. Then, 4% Nimesulide was slowly added, and submitted to a vigorous stirring. Afterward, 0.10% L-cysteine and 0.01% EDTA-2Na were sequentially added, gently stirred until complete dissolution, and then diluted with water for injection to 90% of the total volume. The pH was further adjusted to 10-10.5 with lactic acid. Finally, the volume was completed to 100% with water for injection. After reaching equilibrium, the injectable solution was passed through a 0.45µm and 0.22µm micro porous membrane filter, respectively. The obtained filtrate, under nitrogen condition was filled into 5mL ampoules, then submitted to 100□ steam sterilization circulation for 30min (Specification: 5mL: 0.2g). According to this preparation process, 3 batches of Nimesulide inject able (20120605, 20120610 and 20120612) were manufactured, each batch containing 10,000 units.

Chromatogram of Nimesulide

Nimesulide reference and sample exhibited a characteristic peak appearing approximately at the same time. Furthermore, solvent and excipients had no chromatographic interference with Nimesulide (fig. 1).

Linearity

The method displayed a good linearity within the ranges of 20-80µg/mL (fig. 2). The regression equation was A=25505C-25997 with a correlation coefficient R2 = 0.9995 (n=7); where A represents the peak area and C represents the concentration.

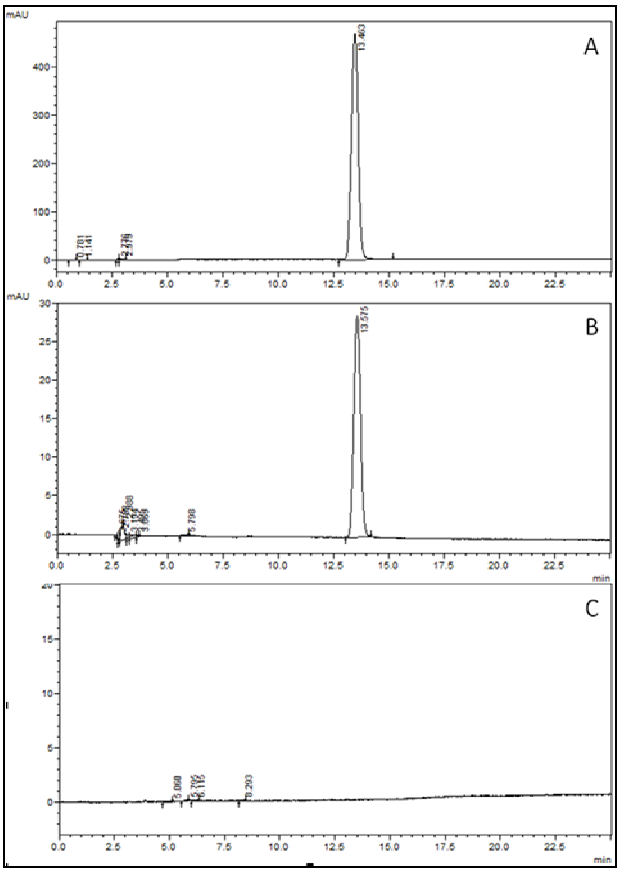

Fig. 1: Chromatogram of Nimesulide injectable content.

A: reference solution B: sample solution C: blank solution

Table 5: Nimesulide injection orthogonal test results (mean±SD, n=3).

No.	Factor			Clarity	Stability	pH	Nimesulide (%)	Related substances(%)
	A	B	C					
1	1	1	1	a^+	b^-	10.19±0.04	91.2±0.09	1.02±0.06
2	1	2	2	a^+	b^-	10.18±0.05	92.3±0.05	0.87±0.05
3	1	3	3	a^+	b^-	10.19±0.03	91.7±0.06	0.92±0.04
4	2	1	2	a^+	b^+	10.21±0.02	98.2±0.02	0.82±0.02
5	2	2	3	a^+	b^+	10.19±0.01	97.6±0.01	0.76±0.03
6	2	3	1	a^+	b^+	10.17±0.02	96.9±0.02	0.87±0.02
7	3	1	3	a^+	b^+	10.22±0.03	94.7±0.04	0.69±0.04
8	3	2	1	a^+	b^+	10.21±0.01	97.6±0.03	0.73±0.02
9	3	3	2	a^+	b^+	10.23±0.02	96.2±0.05	0.84±0.01
K_1	275.2	284.1	285.7					
K_2	292.7	287.5	286.7					
K_3	288.5	284.8	284					
k_1	91.73	94.7	95.23					
k_2	97.57	95.83	95.57					

Precision

Precision was investigated using prepared Nimesulide sample solution. The method exhibited a good precision with RSD of 0.28%.

Repeatability

RSD of the content determination was 0.38%. Therefore, the method reproducibility was satisfactory.

Average recovery

The recoveries of Nimesulide in low, medium, and high concentrations were 99.88% (RSD=0.08%), 99.91% (RSD=0.06%) and 99.95% (RSD=0.04%), respectively. The average recovery was 99.91% and the RSD was 0.04%.

Content and Related Substances

Nimesulide content was determined by calculating the peak area with the external standard method. Furthermore, the main self-compare component without calibration factor was used to calculate Nimesulide related substances content and was found to be less than 1 % (table 6).

DISCUSSION

Selection of solvent

Nimesulide was included in the 2010 edition of Chinese Pharmacopoeia. Nimesulide is slightly soluble in methanol, ethanol and ether, almost insoluble in water, however, dissolves in acetone, dimethyl form amide and chloroform. Moreover, an organic solvent can improve the solubility of Nimesulide. Yet, it will not only increase the cost of the injection but also cause an irritation and lead to environmental pollution when produced in industry. It has been reported in literature that Nimesulide dissolves easily in alkaline solution (Alexanian *et al*., 2008). Therefore, in order to increase Nimesulide solubility and minimize the amount of organic solvent, we considered the use of alkaline solution in the present study. After repeated tests, water was selected as the solvent and ethanolamine as an organic weak base to adjust the pH, thus increasing the solubility of Nimesulide. Furthermore, a proper amount of lactic acid was added to lower the final pH, reducing irritation at the injection site.

Table 6: Nimesulide and related substances content of three batches Nimesulide injectable (mean±SD, n = 3).

Lot No	Nimesulide (%)	Related substances (%)
20120605	99.28±0.11	0.64±0.0058
20120610	100.00±0.18	0.77±0.0058
20120612	98.33±0.36	0.72±0.01

Selection of antioxidant

Sodium sulfite, sodium metabisulfite, butylated phenol, sodium bisulfite, L-cysteine, sodium thiosulfate, and tert-butyl-hydroxyanisole ether are antioxidants commonly used in inject able preparations. We observed that, sodium metabisulfite and sodium bisulfite were suitable antioxidants for acid injection, whereas, sodium thiosulfate, sodium sulfite and L-cysteine are appropriate for alkaline injection. In addition, butylated phenol and

tert-butyl hydroxy anisole are an oil-soluble and a fat-soluble antioxidant, respectively. Those are unstable under the influence of light or in the presence of metal ions. Moreover, their cost is relatively high.

In the single-factor test, we conducted a screening of alkaline injection antioxidants, wherein, sodium sulfite, sodium thiosulfate and L-cysteine were tested. The results showed that both sodium sulfite and sodium thiosulfate in contact with Nimesulide immediately led to precipitation. In contrast, after adding L-cysteine, Nimesulide solution was clear and more stable. Furthermore, L-cysteine presents a strong reduction property. In inject able solution, it oxidizes into cysteine then consumes itself, preventing or retarding drug oxidation, thereby maintaining the drug stability (Zhang *et al*., 2009). Therefore, L-cysteine was chosen as the antioxidant for our injectable formulation.

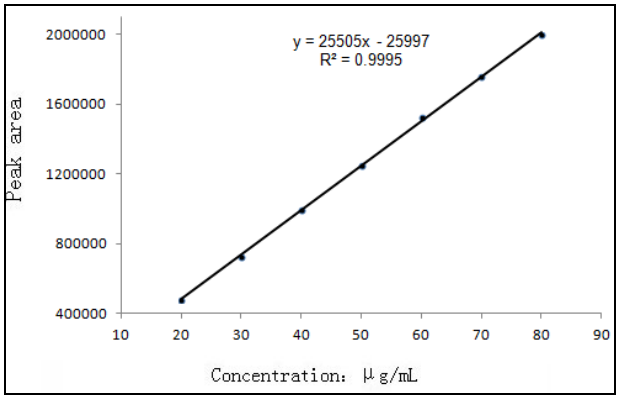

Fig. 2: Nimesulide calibration curve.

Mobile phase selection

In the British Pharmacopoeia 2010 edition, acetonitrile with 0.5mol/L potassium dihydrogen phosphate is described as the appropriate mobile phase to determine Nimesulide content in raw materials and solid preparation (Ruela *et al*., 2009). On the other hand, M.J. Yu used Na_2HPO_4 (pH=7.0) with methanol as mobile phase in order to test Nimesuide raw materials and solid dosage forms (Yu *et al*., 2002). However, Y.H. Li, *et al*., reported that the main drug and impurity peaks cannot be completely separated showing high tailing factor (Li *et al*., 2006).

According to previous test results (Feng *et al*., 2010; Mokry *et al*., 2010; Zhao *et al*., 2010), we optimized and adjusted the types and proportions of the mobile phase. Hence, the best mobile phase was a 40:60 (v/v) mixture of methanol and 0.004mol/L potassium dihydrogen phosphate (pH 4.2). The content and related substances of Nimesulide injectable were accurately determined. Furthermore, the main drug and impurity peaks were distinctly separated.

In summary, on the basis of single factor experiment and orthogonal test results, our research group developed and optimized Nimesulide injectable formulation. Moreover, we established an effective HPLC method for injection content determination that showed high resolution, accuracy, precision, high recovery and good reproducibility. This method was applied to all three prepared batches of Nimesulide inject able, wherein, the measured levels were within a range of 90.0% -110.0%. Hence, the method demonstrated to be suitable for testing Nimesulide injectable content.

CONCLUSION

The developed formulation was appropriate for Nimesulide inject able. Moreover, the HPLC established method was simple, sensitive and accurate, and therefore, can find use in industrial production and quality control work.

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