Determination of related substances in ketoprofen injection by RP-HPLC method

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Abstract: The paper aims to establish a RP-HPLC method for the simultaneous determination of six related substances in ketoprofen injection. The separation was performed on a VP-ODS C_{18} column (4.6mm×250mm, 5μm) with the mobile phase of 6.8% phosphate buffer solution (adjusted to pH3.5 with 85% phosphoric acid)-acetonitrile-water (2:43:55,v/v/v) at a flow rate of 1.2mL·min^{-1}. The detection wavelength and the injection volume were set at 233nm and 20μL, respectively. Impurity A and C were calculated by external standard method. Main component self-compare method with calibration factor was used to calculate impurity B, D, E, F and main component self-compare method without calibration factor was used to calculate unspecified impurity. Related substances and degraded substances were completely separated from ketoprofen. For impurity A and C, the linear range of determination were separately 0.06 μg·mL^{-1} ~ 3.6μg·mL^{-1} and 0.036μg·mL^{-1} ~ 2.4μg·mL^{-1} with the correlation coefficient of 0.9999. The average recoveries (n=9) were 98.13% (RSD=0.35%) and 96.32% (RSD=0.43%). The precision and repeatability for method were good. With reference to ketoprofen (retention time =10.06 min), the relative retention time of impurity B, D, E, F were 0.71, 1.46, 0.59, 2.13, respectively, and the relative correction factors were 0.962, 0.938, 0.957, 0.960, respectively. Finally, determined that the contents of impurity A could not be more than 0.3%, any of the contents of impurity B, C, D, E, F and unspecified impurities could not be more than 0.2%, sum of the contents of impurities other than A and C couldn't be more than 0.5%. The method was proved to be simple, rapid, accurate, sensitive and suitable for the simultaneous determination of six related substances in ketoprofen injection.

Keywords: RP-HPLC, ketoprofen, injection, related substances.

INTRODUCTION

Ketoprofen, 2-(3-benzoylphenyl)-propionic acid, is one of the most widely used non-steroidal anti-inflammatory drugs (NSAIDs) with analgesic, antipyretic and anti-inflammatory effects (Zhang *et al*., 2001; Choi *et al*., 2013), which is mainly used clinically in the treatment of acute renal colic (Baz *et al*., 1995), preoperative and postoperative pain, rheumatism and rheumatoid arthritis(Wollheim *et al*., 1981;Mola *et al*., 1995), as well as myelitis and gout (Imad *et al*., 2012). At present, the most commonly used dosage forms of ketoprofen are conventional capsule, enteric-coated capsules, sustained-release capsules, dispersible tablet, gel, lotion, etc (Chen *et al*., 2001). However, ketoprofen has great irritation to the gastrointestinal tract (Mozaffari *et al*., 2012), so the suitable crowd of oral preparation of ketoprofen are small. Developing ketoprofen injection can not only enhance the bioavailability, but also can reduce side effects, which brought the new choice to the patient who can not be administered orally and has wonderful practical value and clinical application prospect.

In the current version of Chinese Pharmacopoeia, titration method and thin layer chromatography (TLC) are adopted respectively to determine the content and related substances in ketoprofen raw material, and there are no determination method of related substances for the preparation of ketoprofen, namely enteric capsule and liniment (ChP, 2010). For developed ketoprofen injection, the accuracy is not high to use TLC method to determine the related substances, and the operation will be complicated. Furthermore, TLC method is not suitable for the determination of low concentration of ketoprofen solution.

There are 12 impurities of ketoprofen raw materials recorded in the current version of British Pharmacopoeia (BP), among which impurity A-F are included in the quality standard and the impurities A and C are quantitative determined (BP, 2012). In the current version of BP, TLC method is adopted to determine the related substances in ketoprofen gel, HPLC method is used to determine the related substances in ketoprofen capsules and only impurities A and C are determined. In the current version of United States Pharmacopoeia (USP), 2 impurities are determined in ketoprofen raw materials, and no related substances are determined in ketoprofen sustained release capsules (USP, 2012).

Based on the determination method of related substances in ketoprofen raw material in BP (BP, 2012) and USP (USP, 2012), a RP-HPLC method has been developed for the simultaneous determination of six related substances in ketoprofen injection in this paper. The method has proved to be simple, specific, sensitive, accurate, precise and suitable for the quality control of related substances of ketoprofen injection.

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MATERIALS AND METHODS

Apparatus

The HPLC used in this study is composed of a SPD-M20A detector, a LC-20AD pump, and a LC solution liquid chromatography workstation (Shimadzu, Kyoto, Japan). Other apparatuses include MS105DU electronic analytical balance (Mettler Toledo, Shanghai, China) and FE20 pH-meter (Mettler Toledo, Shanghai, China).

Reagents

Ketoprofen reference standard (99.9% purity, Lot 100337-2011104) was obtained from National Institute for Food and Drug Control (China). Ketoprofen impurity A and C were purchased from European Pharmacopoeia Reference Standard (Batch No. 00Q043 and 00Q640, 100% purity). Ketoprofen impurity B, D, E and F were purchased from LGC GmbH (Batch No. 103, 40323, 43281 and 20111, 100% purity). Ketoprofen injection was obtained from Yichang SanXia Pharmaceutical Co., ltd. (Specification of 2ml: 100mg, Lot 20131001, 20131002, 20131003). Potassium Phosphate Monobasic of analytically pure and phosphoric acid of HPLC grade, were purchased from Chengdu Kelong Chemical Reagent Factory (China) and Tianjin Keimiou Chemical Reagent Co., Ltd. (China), respectively.

Chromatographic condition

Shimadzu Shim-pack VP-ODS C_{18} chromatographic column (250mm×4.6mm, 5μm) was adopted, and the mobile phase was composed of 6.8% phosphate buffer (adjusted to pH3.5 with 85% phosphoric acid), acetonitrile and water (2:43:55, v/v/v). The flow rate was 1.2mL/min. The detection wavelength was 233nm. The column temperature was 30^{o}C and the injection sample volume was 20μL.

Preparation of solutions

Preparation of the stock solutions of reference substances

Firstly, 10.0mg of ketoprofen, impurity A, B, C, D, E, F reference substances were precisely weighed respectively and placed into seven 100-mL measuring flask. Then they were dissolved with the mobile phase, diluted to the scale at 100mL, shook, and filtered with a 0.22-μm millipore filter membrane to obtain the stock solution of 100μg/mL ketoprofen, impurity A, B, C, D, E, F reference substance.

Preparation of the solutions of reference substances

Similarly, 10.0mL, 3.0mL, 5.0mL, 2.0mL, 10.0mL, 5.0 mL, 10.0mL stock solution of ketoprofen, impurity A, B, C, D, E, F reference substance were respectively added into seven 100-mL measuring flask to prepare the solution of 2.0μg/mL ketoprofen, 2.0μg/mL impurity A, 5.0μg/mL impurity B, 3.0μg/mL impurity C, 10.0μg/mL impurity D, 5.0μg/mL impurity E, 10.0μg/mL impurity F reference substance.

Preparation of the solutions of testing substances and self-compare solution

Similarly, 2.0mL ketoprofen injection (containing about 100mg of Ketoprofen) was added into a 100-mL measuring flask to prepare the solution of the testing substance. 0.2mL solution of the testing substance was added into a 100-mL measuring flask to prepare self-compare solution.

Preparation of the solution of system suitability

Similarly, 10mL, 3.0mL, 5mL, 2.0mL, 10mL, 5mL, 10mL stock solution of 100μg/mL ketoprofen, impurity A, B, C, D, E, F reference substance were added into a 100-mL measuring flask to prepare the solution of system suitability.

Preparation of the solution of blank excipients

According to the proportion of prescriptions, excipients were added into a 100-mL measuring flask to prepare the solution of blank excipients.

System suitability test

The prepared solutions of ketoprofen, impurity A, B, C, D, E, F reference substance, testing substance, system suitability, and blank excipients were determined under the chromatographic conditions mentioned above. Among of them, impurity A and C were determined for six times for calculating the value of RSD.

Specificity tests

The prepared various solutions of testing substance (2.0 mL) were heated to 105°C for 5h, and subjected to 2-h illumination with the intensity of 4500 lux. Then, at room temperature, it was respectively treated with 2mL of 30 % hydrogen peroxide solution for 1h, 2mL of 0.1M sodium hydroxide solution for 2h, and 2mL of 0.1M hydrochloric acid solution for 2h. A series of testing samples were tested with the injection volume of 20μL under the chromatographic conditions mentioned above.

Limit of detection (LOD) and Quantitation (LOQ)

The prepared solutions of reference substances of ketoprofen, impurity A and C were gradually diluted with the mobile phase until the peak height was 3 times of the baseline noise to obtain the minimum detection limit. In addition, the prepared solutions were gradually diluted with the mobile phase until the peak height was 10 times of the baseline noise to obtain the minimum quantitation limit.

Linear correlation

The calibration curve was plotted on the basis of the peak areas and the experimental solution concentrations. A series of standard solutions of ketoprofen (0.125, 0.5, 1.0, 2.0, 2.4μg/mL), impurity A (0.06, 0.75, 1.5, 3.0, 3.6 μg/mL) and impurity C (0.036, 0.5, 1.0, 2.0, 2.4μg/mL)

were respectively prepared by dissolving the corresponding amounts of standard substances of ketoprofen, impurity A and C with the mobile phase. Then 20μL of each solution was injected into HPLC. A linear curve was plotted with the concentrations of ketoprofen, impurity A and C against the peak areas.

Solution stability test

The same prepared solutions of testing substance were tested with the injection volume of 20μL under the chromatographic conditions mentioned above at 0, 2, 4, 6, 8, 12, 16, 20 and 24h, respectively. Determine the peak areas and calculate the values of RSD.

Precision tests

The solutions of ketoprofen, impurity A and C reference substances (20μL) were injected into HPLC for six times to determine their peak areas, which were used to calculate the value of RSD.

Repeatability tests

The repeatability of the determination method was evaluated with six samples selected from the same batch. According to the method above, peak areas of impurity A and C, single largest unspecified impurities, the sum of impurities other than impurity A and C were determined to calculate the values of RSD under the chromatographic condition mentioned above.

Recovery rate tests

Nine samples of the solution of blank excipients were used to determine recovery rate tests. Various volumes of stock solutions of ketoprofen, impurity A and C (table 1), were respectively added into nine samples in nine 50 mL measuring flasks. Then after dilution, shaking, and filtration with a 0.22-μm millipore filter, the obtained solutions were determined to calculate the recovery rates of ketoprofen, impurity A and C.

Determination methods

The prepared solutions (20μL) of testing substances, various reference substances and self-compare solution were injected into HPLC. We recorded the chromatogram and computed the concentrations of impurity A and C with the peak areas by the external standard method, computed the concentrations of impurity B, D, E, F with the peak areas by main component self-compare method with calibration factor, computed the concentrations of single largest unspecified impurity and the sum of impurities other than impurity A and C with the peak areas by main component self-compare method without calibration factor.

The relative retention time and calibration factor of impurity B, D, E, F

A series of standard solutions of ketoprofen , impurity B, D, E, F(0.05, 0.1, 0.2, 0.4, 0.8μg/mL) were respectively prepared by dissolving the corresponding amounts of standard substances of ketoprofen, impurity B, D, E, F with the mobile phase. Then 20μL of each solution was injected into HPLC. A linear curve was plotted with the concentrations of ketoprofen, impurity B, D, E, F against the peak areas. Calculated the relative retention time (RRT) of impurity B, D, E, F relative to ketoprofen with the mean absolute retention time (T_R) of series concentration of each impurity, respectively. Calculated the relative correction factors (*f*) of impurity B, D, E, F relative to ketoprofen with the ratio of slope of linear curve of each impurity and that of ketoprofen, respectively.

RESULTS

Results of system suitability test

The main components could be efficiently separated from the impurities, as shown in fig. 1, the RSD value of peak area of impurity A and C were less than 2.0%, and the theoretical plate number were over 2000, which all can meet system suitability requirements.

Results of specificity tests

Ketoprofen injection was stable under the conditions of high temperature, strong alkali and strong acidity. Although the degradation products were increased significantly under the conditions of oxidation and illumination, the main components could be efficiently separated from the impurities, as shown in fig. 2.

Limit of detection (LOD) and quantitation (LOQ)

The minimum detection limits of ketoprofen, impurity A and impurity C were respectively 40ng (S/N≥3), 18ng (S/N≥3) and 10ng (S/N≥3). The minimum quantitation limits of ketoprofen, impurity A and impurity C were respectively 125ng (S/N≥10) , 60ng (S/N≥10) and 36ng (S/N≥10).

The result of linear correlation and regression equations

The method both displayed good linearity correlation for ketoprofen, impurity A and impurity C within various ranges (0.125-2.4μg/mL for ketoprofen, 0.06-3.6 μg/mL for impurity A and 0.036–2.4μg/mL for impurity C). The regression equation of ketoprofen was obtained as: $Y=2.6\times10^5 X-248.14$ (r=0.9999); The regression equation of impurity A was obtained as: $Y_A=1.1\times10^5 X+955.88$ (r=0.9999); The regression equation of impurity C was obtained as: $Y_C=4.1\times10^5 X+885.95$ (r=0.9999); where Y is the peak areas of the analytes and X is the concentration of the analytes (μg/mL).

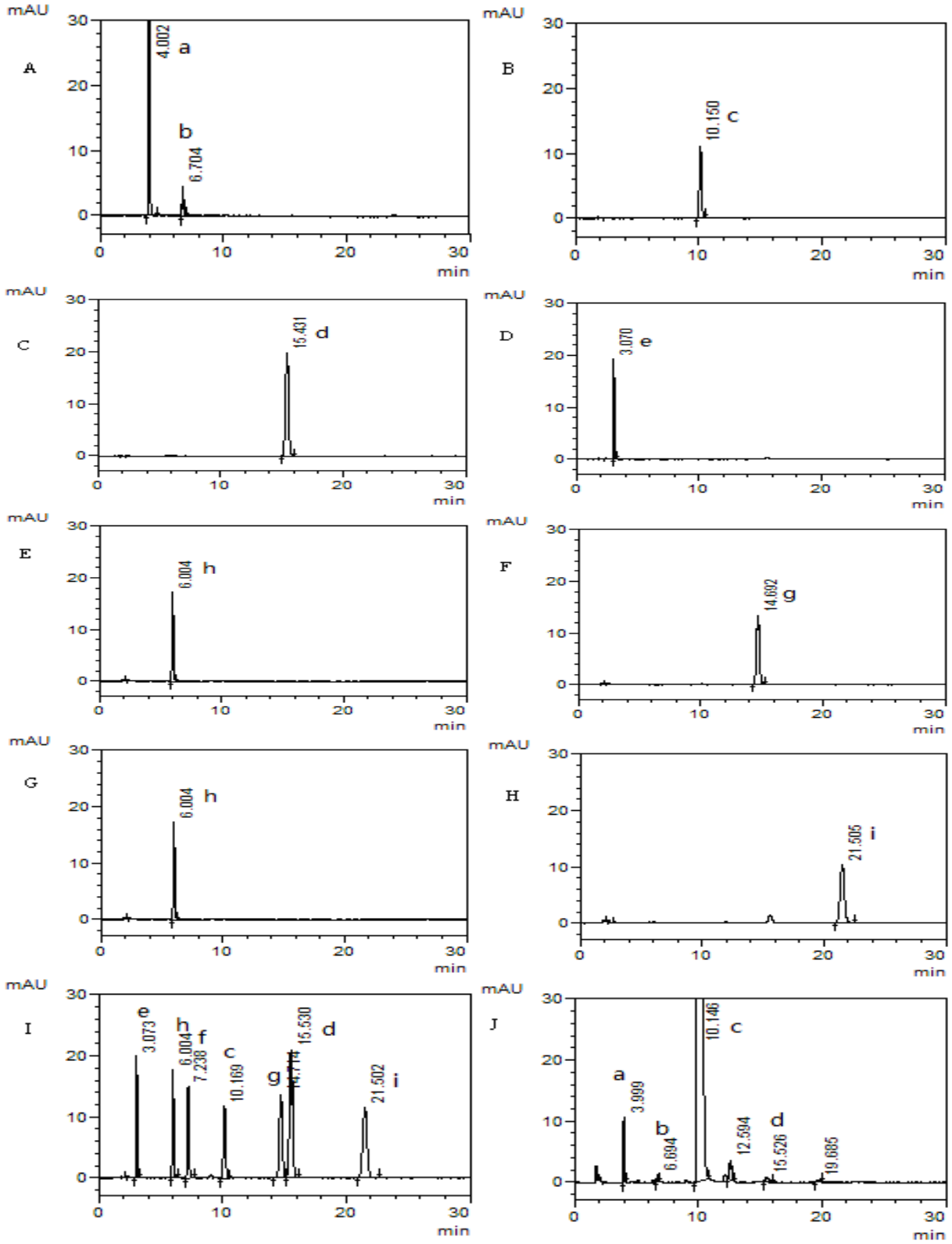


Fig. 1: The chromatograms of system suitability test: (A) blank excipients solution, (B) ketoprofen reference substance solution, (C) impurity A reference substance solution, (D) impurity C reference substance solution, (E) impurity B reference substance solution, (F) impurity D reference substance solution, (G) impurity E reference substance solution, (H) impurity F reference substance solution, (I) system suitability solution, (J) testing substance solution. a: excipients1; b: excipients2; c:ketoprofen; d:impurity A; e:impurity C; f: impurity B; g: impurity D; h: impurity E; i: impurity F.

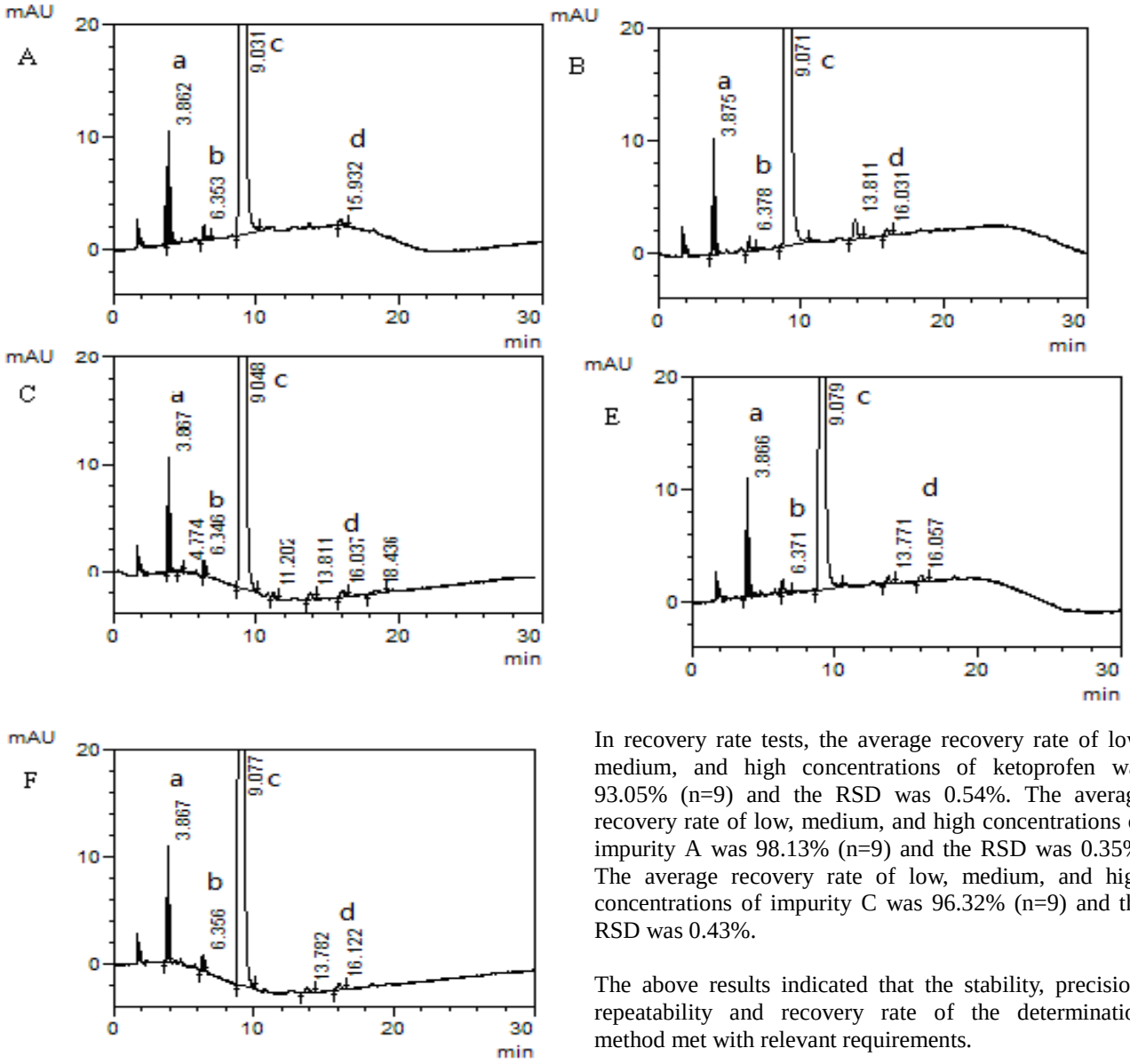


Fig. 2: The Chromatograms of specificity experiments: (A) Undestroyed, (B) Destroyed by heat at about 105°C, (C) Decomposed by strong light irradiation, (D) Decomposed by 30%

Results of stability, precision, repeatability and recovery rate tests

In stability tests, the RSD value of impurity A, single largest unspecified impurities, the sum of impurities other than impurity A were 2.82%, 1.43%, 1.69%, respectively, which suggested the solution of testing substances was stable within 24h under this system condition. In precision tests, RSDs obtained by the peak areas of ketoprofen, impurity A and impurities C were respectively 0.32%, 0.43%, 0.29%. In repeatability tests, RSDs obtained by the peak areas of impurity A, single largest unspecified impurities, the sum of impurities other than impurity A were respectively 1.37%, 2.19% and 1.72%. In recovery rate tests, the average recovery rate of low, medium, and high concentrations of ketoprofen was 93.05% (n=9) and the RSD was 0.54%. The average recovery rate of low, medium, and high concentrations of impurity A was 98.13% (n=9) and the RSD was 0.35%. The average recovery rate of low, medium, and high concentrations of impurity C was 96.32% (n=9) and the RSD was 0.43%.

The above results indicated that the stability, precision, repeatability and recovery rate of the determination method met with relevant requirements.

Results of the relative retention time and calibration factor of impurity B, D, E, F

Results of the relative retention time and calibration factor of impurity B, D, E, F were showed in table 2.

Results of determination of related substances of ketoprofen injection

Results of determination of related substances of three batch ketoprofen injection were showed in table 3.

DISCUSSION

Quality control of six related substances

Impurity A-F reference substances were too expensive, for which it is costly and complicated to calculate all the impurities with external standard method. Impurity A and C were scanned at full wavelength with diode array

Table 1: Sample preparation for recovery rate test

ketoprofen (mL)	1.0	1.0	1.0	2.0	2.0	2.0	4.0	4.0	4.0
Impurity A (mL)	1.5	1.5	1.5	3.0	3.0	3.0	6.0	6.0	6.0
Impurity C (mL)	1.0	1.0	1.0	2.0	2.0	2.0	4.0	4.0	4.0
Add solutions above into nine 50 mL measuring flasks and followed with dilution, shaking, and filtration.									
1mL solution from each measuring flasks was mixed with 1mL blank excipients solution.									

Table 2: The relative retention time and calibration factor of impurity B, D, E, F

Compound	t_R/min	RRT	Linear equation	Correlation coefficient	Correction factors (*f*)
Ketoprofen	10.17	-	*Y*=28410 *X*-612.92	0.9996	-
Impurity B	7.24	0.71	*Y*=27194*X*-817.17	0.9996	0.957
Impurity D	14.71	1.45	*Y*=26148*X*-96.417	0.9999	0.920
Impurity E	6.00	0.59	*Y*=26447*X*-200.71	0.9997	0.931
Impurity F	21.50	2.11	*Y*=27265*X*+400.00	0.9998	0.963

Table 3: Results of determination of related substances of three batch ketoprofen injection (%, n=6).

Batch No.	Impurity A ($\bar{x}$ ± SD)	Impurity C	Impurity B,D,E,F	Single unspecified impurity ($\bar{x}$ ± SD)	Total unspecified impurity ($\bar{x}$ ± SD)
20131001	0.019±0.001	N.D	N.D	0.111±0.001	0.209±0.001
20131002	0.020±0.001	N.D	N.D	0.110±0.002	0.210±0.001
20131003	0.019±0.001	N.D	N.D	0.102±0.001	0.202±0.002

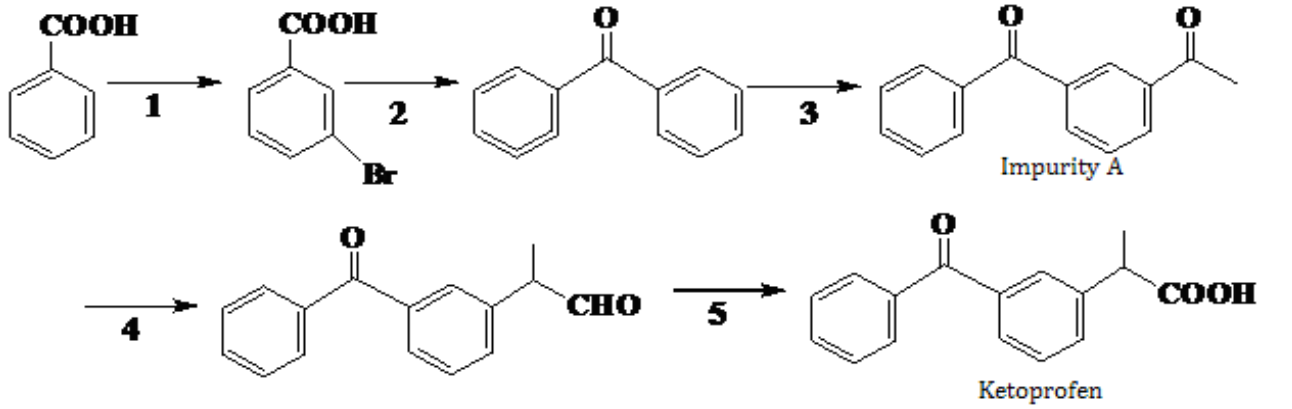
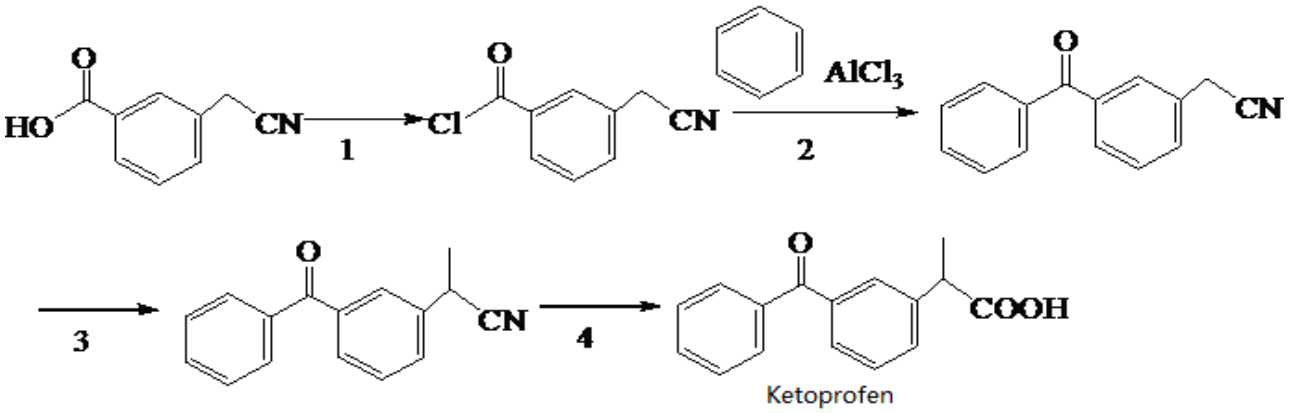


Fig. 3: A synthetic route of ketoprofen.



detector and the maximum absorption wavelength were found to be significantly different from ketoprofen (ketoprofen at 255nm, impurity A and C at 233nm), that is to say, the response value of impurity A and C were significantly different from ketoprofen at a certain wavelength. Therefore, it was inaccurate to calculate the

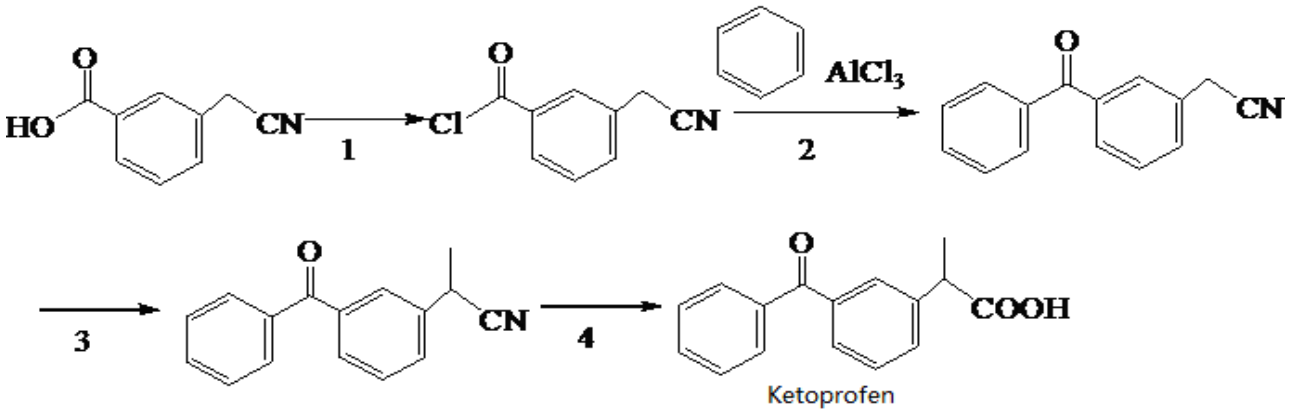


Fig. 4: Another synthetic route of ketoprofen.

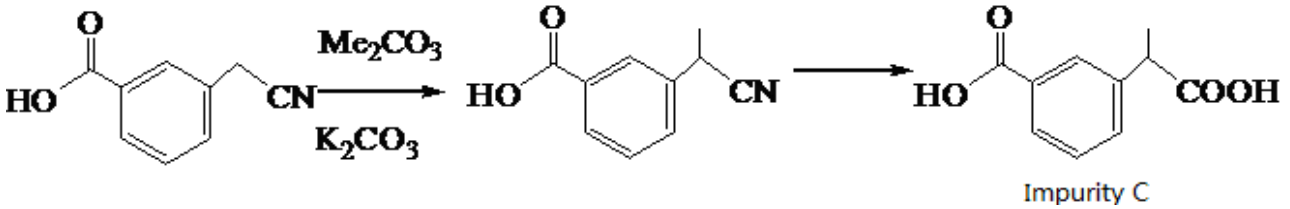


Fig. 5: Sources of impurities C.

contents of impurity A and C with main component self-compare method. So, external standard method was obtained to determine impurity A and C. Many tests were performed to show that no impurity B, D, E, F were detected in our products. Taking the feasibility of productive practice into consideration, main component self-compare method with calibration factor was used to calculate impurity B, D, E, F. For other unspecified impurities, the reference substances were not available and the contents were usually less, so main component self-compare method without calibration factor was used to calculate unspecified impurity.

The origin of impurity A and C

Referring to the pharmacopoeia of each country and relevant quality standards, determined to take impurity A and C as the main controlled object with regard to related substances of ketoprofen injection. A common synthetic route of ketoprofen (Lv *et al*., 2000) was shown in fig. 3. Taking benzoic acid as starting material, through bromination, Friedel-crafts reaction, Grignard reaction, 3-acetyl-diphenylketone (impurity A) was formed. Then, through Darzens reaction, ketoprofen was produced.

Another common synthetic route of ketoprofen (Liao *et al*., 1997) was shown in fig. 4. In this route, if the starting material 3-(α-cyanomethyl) benzoic acid, didn't involve into reaction at the first and second step, and involve into reaction at the third step, thus, the impurity C was produced, as was shown in fig. 5.

The literature showed that impurity A and C were significantly increased with ketoprofen in long-term storage (Dvořák *et al*., 2004). In the specificity tests, the degradation products were increased significantly under the conditions of oxidation and illumination, especially impurity A. Therefore, in the progress of production and storage, attention should be paid to protect the injection from illumination and oxidation. Consequently, both intermediate in synthetic progress and degradation products in storage progress can be the origin of impurity A and C.

CONCLUSION

The method was proved to be simple, rapid, accurate, sensitive and suitable for the simultaneous determination of six related substances in ketoprofen injection.

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