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Short communication

# Liquid chromatography–mass spectrometry method for the determination of thiamphenicol in rabbit tears

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ABSTRACT

A simple and sensitive liquid chromatography–mass spectrometry (LC–MS) method was described for the determination of thiamphenicol in rabbit tears. Chromatographic separation of the analyte was achieved on a C18 column using a mobile phase of acetonitrile and 10 mmol/l ammonium acetate solution (60:40, v/v). Selected ion monitoring (SIM) in negative mode was used for analyte quantification at $m/z$ 354.4 for thiamphenicol and at $m/z$ 137.1 for salylic acid. The run time was less than 6 min. Linearity over the concentration range of 0.032–32.0 ng/ml for thiamphenicol was obtained and the lower limit of quantification was 0.032 ng/ml. For each level of QC samples, inter- and intra-day precisions (R.S.D.) were ≤5.2% and 8.3%, respectively, and the accuracy (RE) was ±2.8%. The present LC–MS method was successfully applied to the pharmacokinetic studies of thiamphenicol in situ forming gel in rabbit tears.

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## 1. Introduction

Thiamphenicol (TAP) [D-threo-2,2-dichloro-*N*-hydroxya-(hydroxymethyl)-*p*-(methyl-sulphonyl)-phenethyl acetamide], is an analogue of chloramphenicol, in which the *p*-nitro group on the benzene ring is replaced by a methylsulphonyl group. The structure of thiamphenicol is shown Fig. 1. It is a broad-spectrum bacteriostatic antibiotic, active against both Gram-positive and Gram-negative pathogens and especially effective against anaerobic organisms. At a sub-cellular level, TAP inhibits the protein synthesis, joining the ribosomes and thus preventing the binding of the amino acid with peptidyl transferase. In contrast to chloramphenicol, TAP has not been associated with fatal aplastic anemia because the nitro group responsible for induced hematological side effect is absent in TAP [1,2]. TAP has a more weakly basic functionality ($pK_a = 7.2$) than chloramphenicol. It is only slightly bound to plasma proteins (approximately 10%) and is not inactivated in the body by metabolic processes [3,4]. TAP is a chiral drug and its L-form is used in humans [5]. A few results have previously been reported concerning the concentration of TAP in serum and cerebrospinal fluid [6], bovine plasma [7], muscles of chicken and beef [8] and in human plasma by gas chromatography (GC) after intravenous injection [9], but there are no reports on the determination of TAP in rabbit tears. Van de Riet et al. applied a chromatographic method to aquatic species with a limit of quantification of 0.3 ng/g for TAP [10], which was not sensitive enough for the pharmacokinetic studies of thiamphenicol in situ forming gel in rabbit tears because levels of the analyte in tears in the initial hours were anticipated to be below or near the limit of quantification of the method. And this method lacks the sensitivity required to reach the minimum required performance limit for TAP (10 g) and did not have a suitable concentration range (between 0.015 and 0.425 ng injected) for pharmacokinetic studies. Moreover, these types of methods require time-consuming sample preparation steps (such as liquid–liquid extraction and skim) and are generally a very expensive means of performing routine analysis. Therefore, an analytical method was required to be sensitive enough to determine the low levels of analyte in tears in the later hours, when it provided key information in choosing the optimal formulation. The optimal formulation displayed a 4–6 h sustained-release profile after administration with lower levels of analyte in the later 0.5 h and had less individual variations. Therefore, a more sensitive and selective analytical method was needed and liquid chromatography–mass spectrometry (LC–MS) was the method of choice. Compared to UV detection, the combination of LC–MS provides enhanced sensitivity and selectivity for the analytes in biological samples and has already been employed in the field of drug development and testing. This paper describes a liquid chromatography–single quadrupole mass spectrometric (LC–MS) method in negative selected ion monitoring (SIM) mode for the determination of thiamphenicol in rabbit tears. The present method offers a simple sample preparation method and

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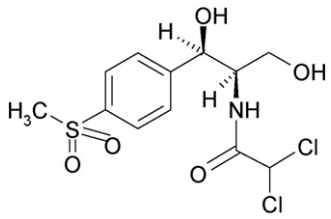

**Fig. 1.** Chemical structure of thiamphenicol.

higher sensitivity with a lower limit of quantification (LLOQ) of 0.032 ng/ml. The described method was validated in terms of selectivity, linearity, LLOQ, accuracy, precision, freeze–thaw cycles and stability of analyte at ambient temperature, and had been successfully applied in the pharmacokinetic studies of thiamphenicol in situ forming gel in rabbit tears.

## 2. Experimental

### 2.1. Chemicals and materials

Thiamphenicol standard reference (99.5% purity) was purchased from Shandong Zibo Xinhua-Chemferm Industrial Pharmaceuticals Co. Ltd. Salylic acid (99.5% purity) was purchased from Tianjin Bodi Chemicals Co. Ltd. Thiamphenicol pH-triggered in situ gel (2.5 mg/ml, batch no. 060301) and Thiamphenicol eye drops (2.5 mg/ml, batch no. 060302) were from Shenyang Pharmtech Institute of Pharmaceuticals (Shenyang, China). Acetonitrile of HPLC grade was purchased from Tianjin Concord Tech Reagent Company (Tianjin, China). All the other reagents were of analytical grade.

### 2.2. Instrument and LC–MS conditions

HP 1100 series LC/MSD G1946D (Agilent, USA) was used in the present work. Chromatographic separation was performed on a Diamonsil™ C18 column (150 mm × 4.6 mm i.d., 5 μm, Dikma, China) at ambient temperature. The mobile phase consisting of a mixture of acetonitrile and 10 mmol/l ammonium acetate solution (60:40, v/v) was delivered at a flow rate of 0.5 ml/min. The injection volume was 20 μl. The mass spectrometer was operated in the negative electrospray ionization (ESI) mode. The optimized ionization conditions were as follows: nitrogen flow rate, 7.0 ml/min; gas temperature, 300 °C; nitrogen pressure, 30 psig; capillary current, 24 nA; collision induced dissociation (CID), 90 V for thiamphenicol and 90 V for salylic acid. SIM mode was used for the quantification of thiamphenicol at $m/z$ 354.4 and of salylic acid at $m/z$ 137.1. The retention time was 4.5 min for thiamphenicol and 4.8 min for salylic acid.

### 2.3. Preparation of calibration standards and quality control samples

Stock solutions (0.01 mg/ml) of thiamphenicol and salylic acid (internal standard) were individually prepared in methanol. The stock solution of thiamphenicol was further diluted with methanol to give a series of standard solutions with concentrations of 0.032, 0.064, 0.320, 1.6, 16.0, and 32.0 ng/ml. The stock solution of salylic acid was further diluted with methanol to give a concentration of 2 ng/ml. Calibration standards of thiamphenicol (0.032, 0.064, 0.320, 1.6, 16.0, and 32.0 ng/ml) were prepared by spiking appropriate amount of the standard solutions of thiamphenicol into blank artificial tears. Quality control (QC) samples were prepared at concentrations of 0.032, 1.6, 32.0 ng/ml of thiamphenicol using the artificial tears. The spiked samples were then treated following the sample preparation procedure as indicated in Section 2.4.

### 2.4. Sample preparation

Five microlitres of each tear sample were transferred to a 1-ml centrifuge tube. Ten microlitres of salylic acid in methanol (2 ng/ml), 100 μl of acetonitrile were added and shaken well. Twenty microlitres was injected onto the LC column.

### 2.5. Method validation

Validation runs were conducted on three separate days. Each validation run consisted of a set of calibration standards at seven concentrations over the concentration range (each in triplicate) and QC samples at three concentrations ($n = 6$ at each concentration). The results from QC samples in three runs were used to evaluate the accuracy and precision of the method developed. The concentrations of analytes in artificial tear samples were determined by back-calculation of the observed peak area ratios of the analytes and internal standards from the best-fit calibration curves. During routine analysis, each analytical run included a set of calibration standards, a set of QC samples in duplicate and tear samples to be determined. Selectivity of the method was investigated by comparing chromatograms of blank tears, standard artificial tear samples spiked with thiamphenicol (1.6 ng/ml) and salylic acid (2 ng/ml) and the tear samples after administration of 30 μl thiamphenicol in situ forming gel (2.5 mg/ml). An additional test was performed to demonstrate if there was any interference from the tear matrix. The test was conducted as follows: standard solutions of thiamphenicol of 0.032 and 32.0 ng/ml (in triplicate for each concentration) were added into blank tears after extraction and determined by the present LC–MS method. Standard solutions of thiamphenicol at 0.032 and 32.0 ng/ml were directly determined without extraction. Based on the percentage of peak area ratio of the peak area of the analyte added into tears after extraction to that of the analyte added without extraction, RE (%) was calculated to evaluate the accuracy of the determination without interferences from the matrix. The linearity of each calibration curve was determined by plotting the peak area ratios ($y$) of the analyte to the internal standard versus the nominal concentrations ($x$) of the analyte. The extraction recoveries of thiamphenicol were determined at low, medium and high concentrations (0.032, 1.6 and 32.0 ng/ml) by comparing the responses from tear samples spiked before extraction with those of the corresponding standard solutions without extraction. Freeze/thaw stability of thiamphenicol in tears was determined at the levels of 0.032, 1.6 and 32.0 ng/ml to measure the accuracy and precision of samples that had underwent three freeze–thaw cycles. QC samples at the indicated levels were stored at −20 °C for 24 h and thawed unassistedly at room temperature. When completely thawed, the samples were refrozen. These freeze–thaw samples were analyzed to see if there was any variation due to thawing of the samples. Sample stability was determined by analyzing QC samples containing thiamphenicol of 0.032, 1.6 and 32.0 ng/ml after sample preparation and exposure to the ambient temperature over a time period of 24 h.

### 2.6. Application of the LC–MS method

The LC–MS method was successfully applied in the pharmacokinetic studies of thiamphenicol in situ forming gel in six rabbits. The in vivo pre-corneal drainage of each formulation was determined after an instillation of 30 μl solution onto the left cornea. A small plastic vial containing an aliquot of 100 μl solution to be tested was placed near the eye of the rabbit. After

instillation, the eyelids were kept closed for 5 s to prevent the loss of the instilled solution. Each formulation was tested on three rabbits. Food and water intake were free during the study. Tear samples (5 μl) were obtained immediately before dosing and at 10, 20, 30, 60, 90, 120, 180, 240 and 360 min after dosing. They were collected in tubes and kept frozen at −20 °C until analysis. Thiamphenicol-containing tears were determined by the present LC–MS method.

## 3. Results and discussion

### 3.1. Method development

Owing to the complex matrices such as fluids, tissues and organs, sample preparation is usually required for the determination of pharmaceuticals in biological samples in order to remove the possible interfering matrix components and increase selectivity and sensitivity. Because of the scarcity of tear sample, it is very difficult to use liquid–liquid extraction for the sample preparation. Organic solvent precipitation was used for the sample preparation. This simple procedure produced a clean chromatogram for the blank tear sample and yielded satisfactory recoveries for the analytes from the tear fluid. In this work, 100 μl of acetonitrile was added in the process of sample preparation. A Diamonsil™ C18 column (150 mm × 4.6 mm i.d., 5 μm) was used. Other chromatographic conditions, especially the composition of mobile phase, were tested to achieve good resolution and symmetric peak shapes of analytes as well as a short run time. Internal standard plays an important role in biopharmaceutical analysis and is often required to have similar physical and chemical properties to analyte in terms of solubility and acid–base properties ($pK_a$). On the basis of the above requirements, salylic acid was found to be suitable for the present work and finally used as the internal standard. In order to obtain a higher sensitivity, the optimum conditions of ESI were discussed systematically. The mass spectrometer was calibrated in the negative ion mode. Voltages across the capillary and the octapole lenses were tuned by an automated procedure to maximize the signal for the ion of interest. All optimal voltage conditions for each target compounds were accomplished by introducing analytes into mass spectrometer. The SIM mode was used in all experiments to monitor the protonated molecular species of thiamphenicol and salylic acid. The optimal parameters of the MS detector were evaluated by the sensitivity (amount of base ion current). We discussed the optimal conditions of spray voltage, sheath gas flow rate, auxiliary gas flow rate and capillary temperature in negative ESI mode and vaporizer temperature, discharge current, sheath gas flow rate, auxiliary gas flow rate, capillary temperature in negative ion mode. It was found that acetonitrile and 10 mmol/l ammonium acetate solution (60:40, v/v) could achieve our purpose and were finally adopted as the mobile phase for chromatographic separation. The retention time was 4.5 min for thiamphenicol and 4.8 min for salylic acid. The run time was less than 6 min. Negative ESI source was used. MS parameters involving capillary temperature, vaporizer temperature and flow rate were tested to obtain an optimum ionization yield of the analytes. Daughter ion scanning discovered that the simultaneous addition of ammonium acetate into the mobile phase further enhanced the sensitivity (see Fig. 2) because the acetate ion promoted the ionization of analytes. Thiamphenicol is easily fragmented into many fragments with discrete abundance. This is also the reason that the detection sensitivity is low. The fragmentor energy was tested to achieve maximum response of the fragment ion peaks, i.e. 90 V for thiamphenicol and 90 V for the internal standard. SIM in negative mode was used for the quantification of

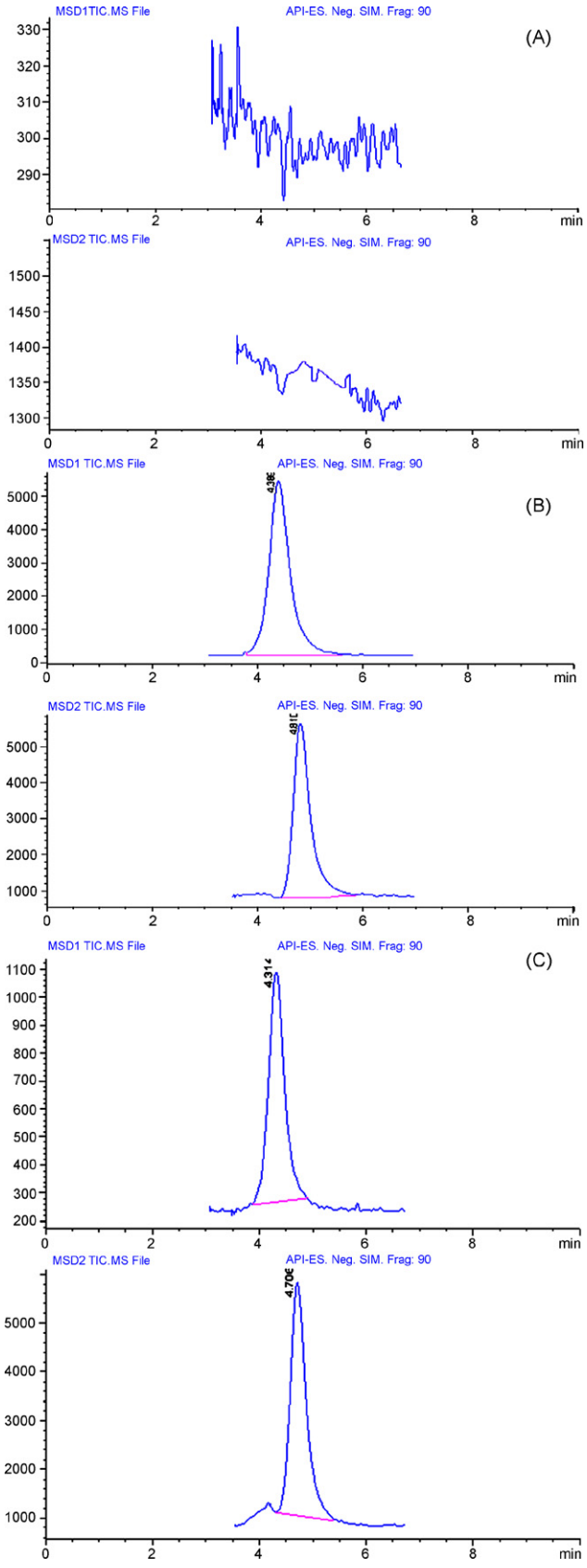


**Fig. 2.** Representative SIM chromatograms of (A) blank rabbit tear fluid sample; (B) blank tear fluid sample spiked with thiamphenicol (1.6 ng/ml) and salylic acid (2 ng/ml); (C) tear fluid sample at 60 min after administration of thiamphenicol in situ forming gel (0.25%, w/v) to rabbit with the measured concentration of analyte at about 1.2 ng/ml. Two channels were used for the quantification, i.e. MSD1 for thiamphenicol ($t_R$ = 4.5 min) and MSD2 for salylic acid ($t_R$ = 4.8 min).

**Table 1**
Statistical analysis of calibration graph in the determination of TAP (see text for details)

| | Results |
|---|---|
| Intercept ($10^{-3}$) | 7.30 |
| Slope ($10^{-2}$) | 9.55 |
| Linear dynamic range (ng/ml) | 0.032–32.0 |
| Limit of quantification (ng/ml) | 0.032 |
| Standard deviation of the intercept ($10^{-3}$) | 1.12 |
| Standard deviation of the slope ($10^{-2}$) | 0.78 |
| Correlation coefficient | 0.9987 |

For linearity study, sample are 0.032, 0.064, 0.320, 1.6, 16.0 and 32.0 ng/ml, respectively; $N = 3$ for each volume.

thiamphenicol and the internal standard at $m/z$ 354.4 and $m/z$ 137.1, respectively. Two-channel mode was used, i.e. channel 1 (MSD1) for thiamphenicol and channel 2 (MSD2) for the internal standard.

### 3.2. Selectivity

The results for selectivity are shown in Fig. 2, indicating a clean chromatogram from blank tear sample after sample preparation by organic solvent precipitation. The results demonstrated the absence of endogenous interferences from the tear matrix and the satisfactory selectivity of the present method for the determination of thiamphenicol in rabbit tears.

### 3.3. Linearity

To evaluate the linearity of the LC–MS method, calibration curves of tear fluids were determined in triplicate on three separate days. As shown in Table 1, representative regression equation for the calibration curve was $y = 9.55 \times 10^{-2}x + 7.30 \times 10^{-3}$ ($r = 0.9987$, $n = 6$) for thiamphenicol. Good linearity was observed over the concentration range of 0.032–32.0 ng/ml.

### 3.4. Lower limit of quantification

The LLOQ is defined as the lowest concentration analyzed with an accuracy of less than 20% and a precision of less than 20%. LLOQ for thiamphenicol was found to be 0.032 ng/ml. A typical SIM chromatogram of tear sample spiked with analyte at the LLOQ level is shown in Fig. 3. At LLOQ level, the inter- and intra-day precision (R.S.D.) were 3.0 and 8.3%, and the accuracy (RE) was 3.0%.

### 3.5. Accuracy and precision

The accuracy and precision of the method were evaluated based on the data from QC tear samples at three concentrations (0.032, 1.6 and 32.0 ng/ml) in three validation runs. The accuracy was determined by calculating the percentage of deviation observed in the analysis of QC samples and expressed in the relative error (RE). The intra- and inter-day precision was expressed as the relative standard deviation (R.S.D.). As shown in Table 2, for each QC level of thiamphenicol, the inter- and intra-day precisions (R.S.D.) were less than 5.2 and 8.3%, and the accuracy (RE) was ±2.8%, indicating

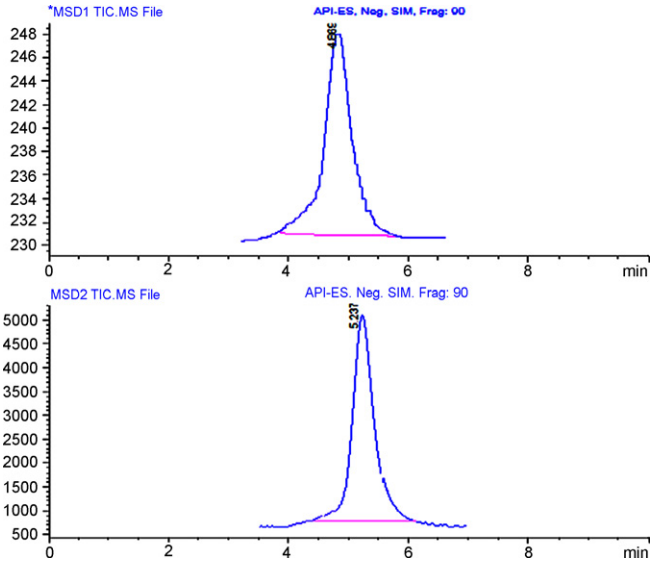


**Fig. 3.** Representative SIM chromatograms of blank tear sample spiked with thiamphenicol (0.032 ng/ml) and salylic acid (2 ng/ml). Two channels were used for quantification, i.e. MSD1 for thiamphenicol ($t_R = 4.5$ min) and MSD2 for salylic acid ($t_R = 4.8$ min).

acceptable accuracy and precision of the present LC–MS method for the determination of thiamphenicol in rabbit tears.

### 3.6. Extraction recovery

The extraction recovery of thiamphenicol from rabbit tears was determined by comparing peak areas from tear samples spiked before extraction with those of the corresponding standard solutions without extraction. The results showed that the extraction recovery from rabbit tear fluid were 95.8 ± 0.03, 99.2 ± 0.08 and 89.4 ± 0.7% at the thiamphenicol concentrations of 0.032, 1.6 and 32.0 ng/ml, respectively.

### 3.7. Freeze–thaw cycles

The results of three freeze–thaw cycles showed that the analyte was stable in rabbit tears through three freeze–thaw cycles. For the three levels of the analyte in tear fluid, the intra- and inter-day precisions (R.S.D.) ranged from 3.1 to 6.5% and from 8.4 to 10.3%, respectively. The accuracy (RE) ranged from 2.7 to 6.2%.

### 3.8. Stability

The stability of thiamphenicol in the supernatant was determined. The analyte was found to be stable for at least 24 h at ambient temperature after sample preparation with an accuracy (RE) ranging from 6.4 to 9.5% at three levels of QC samples.

### 3.9. Application of the developed LC–MS method

The present LC–MS method achieved satisfactory results for the determination of thiamphenicol in rabbit tears and was

**Table 2**
Accuracy and precision for the determination of thiamphenicol in rabbit tears (3 days, six replicates each day)

| Calculated $C$ (ng/ml) | Found $C$ (ng/ml) | Intra-day precision R.S.D. (%) | Inter-day precision R.S.D. (%) | Accuracy RE (%) |
|---|---|---|---|---|
| 0.032 | 0.03 | 8.3 | 3.0 | 0.2 |
| 1.6 | 1.59 | 5.1 | 5.2 | 0.08 |
| 32.0 | 28.6 | 4.8 | 3.2 | 2.8 |

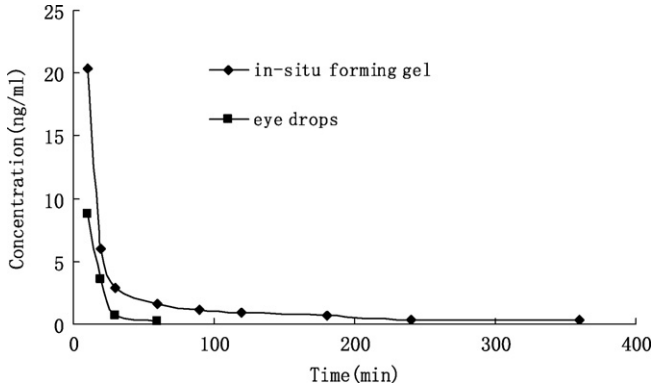


**Fig. 4.** Mean tear fluid concentration–time profiles of thiamphenicol after administration of thiamphenicol in situ forming gel and its eye drops to rabbits.

successfully applied in the pharmacokinetic study of thiamphenicol in situ forming gel and its eye drops following administration to rabbit. Fig. 4 shows the thiamphenicol concentrations in the tear fluid as a function of time. The concentrations of thiamphenicol in situ forming gel were higher than those of its eye drops almost at each time point. This indicates that at the initial time period, the formulation experienced a smaller pre-corneal elimination owing to its in situ forming gel property.

## 4. Conclusions

A sensitive and selective LC–MS method for the determination of thiamphenicol in rabbit tears has been established. Compared with the methods published, the present LC–MS method featured a simple procedure for sample preparation, higher sensitivity with a LLOQ of 0.032 ng/ml, satisfactory selectivity and short run time (less than 6 min). It was successfully applied to the pharmacokinetic studies of thiamphenicol in situ forming gel in rabbit.

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