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Short communication

High performance liquid chromatography–electrospray ionization mass spectrometric determination of isosorbide 5-mononitrate in human plasma

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Abstract

A novel, selective and sensitive high performance liquid chromatography–mass spectrometric (HPLC–MS) method has been developed for the determination of isosorbide 5-mononitrate (5-ISMN) in human plasma. With acetaminophen as internal standard, sample pretreatment involved one-step extraction with diethyl ether of 0.5 mL plasma. Analysis was performed on an ACQUITY UPLC^{TM} BEH C_{18} column (100 mm × 2.1 mm, 1.7 μm) with mobile phase consisting of acetonitrile–water (20:80, v/v). The detection was carried out by means of electrospray ionization mass spectrometry in negative ion mode with selected ion recording (SIR). Standard curves were linear ($r^2 \geq 0.99$) over the concentration range of 1.04–1040 ng/mL. The lower limit of quantification (LLOQ) was 1.04 ng/mL. The intra- and inter-day precisions (RSDs) were less than 8.6% and 13.4%, respectively, and the accuracy (RE) was within ±0.45%. The method herein described was fully validated and successfully applied to the pharmacokinetic study of 5-ISMN in compound extended-release tablets in 18 healthy male volunteers after oral administration.

Keywords: 5-ISMN; HPLC–ESI–MS; Human plasma; Pharmacokinetics

1. Introduction

Isosorbide 5-mononitrate (5-ISMN), an active metabolite of isosorbide dinitrate (ISDN), is a long-acting organic nitrate vasodilator used in the treatment of angina pectoris [1]. It is usually applied for prophylactic purpose. 5-ISMN has several pharmacokinetic advantages over ISDN in terms of a longer elimination half-life, no first-pass metabolism and no active metabolites, which in all lead to a more predictable and permanent clinical effect [2]. Due to the obvious property of preventing the development of tolerance, the extended-release formulation has been widely adopted [3].

The analytical techniques employed in recent years for the quantification of 5-ISMN in human plasma included gas chromatography with electron-capture detection (GC–ECD) [4–10], GC–MS [11], HPLC [12], HPLC–MS [9,13,14] and HPLC–MS/MS [15,16]. The lower limit of quantification (LLOQ) of all those methods was over 2.3 ng/mL [6]. The reported methods required either a lengthy extraction and/or derivatization procedure, or long analysis time, and may not well meet the requirement of desired throughput, speed and sensitivity in biosample analysis.

In this paper, a novel, selective and highly sensitive approach is described using an LC system comprised of a 1.7 μm particles packed column coupled to mass spectrometry to determine 5-ISMN in human plasma. Liquid–liquid extraction was applied to extract the analyte prior to analysis. This fast HPLC–MS method was sensitive enough to be applied to the pharmacokinetic study of 5-ISMN in healthy volunteers after oral administration of compound 5-ISMN extended-release tablets.

2. Experimental

2.1. Materials and reagents

Compound 5-ISMN extended-release tablets (batch No. 20050501), which contained 60 mg of 5-ISMN and 75 mg of aspirin, was supplied by Shanxi A.T. Biopharmaceutical Corporation (Shanxi, PR China). Reference standards of 5-ISMN

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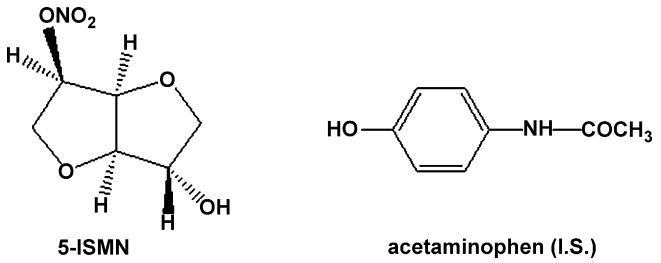


Fig. 1. Structures of isosorbide 5-mononitrate (5-ISMN) and acetaminophen (I.S.).

(99.5% purity), acetaminophen (internal standard, I.S., 99.2% purity) were purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, PR China). The structures of 5-ISMN and I.S. are given in Fig. 1. Acetonitrile was of HPLC grade and was purchased from Dima Company (Richmond Hill, NY, USA). Other chemicals were all of analytical grade. Water was purified by redistillation and filtered through 0.22 μm membrane filter before use.

2.2. Apparatus and operation conditions

2.2.1. Liquid chromatography

Liquid chromatography was performed on ACQUITY^{TM} UPLC system (Waters Corp., Milford, MA, USA) with autosampler and column oven enabling temperature control of analytical column. ACQUITY UPLC^{TM} BEH C_{18} column (100 mm × 2.1 mm, 1.7 μm; Waters Corp., Milford, MA, USA) was employed. The column temperature was maintained at 35 °C. Chromatographic separation was achieved with an isocratic elution using the mobile phase composed of acetonitrile–water (20:80, v/v). The flow rate was set at 0.3 mL/min. The autosampler was conditioned at 4 °C. The injection volume was 5 μL using partial loop mode for sample injection.

2.2.2. Mass spectrometry

Mass spectrometric detection was carried out on a Micromass® Quattro micro^{TM} API mass spectrometer (Waters Corp., Manchester, UK) with an electrospray ionization (ESI) interface. The ESI source was set in negative ionization mode. Selected ion recording (SIR) mode was used for the quantification. The $[M-H]^-$ ions *m/z* 190.0 for 5-ISMN and *m/z* 150.0 for acetaminophen were selected as detecting ions. The optimized ionization conditions were as follows: capillary 2.9 kV, cone 18 kV, source temperature 105 °C and desolvation temperature 300 °C. Nitrogen was used as desolvation and cone gas with the flow rate of 500 and 30 L/h, respectively. Multipliers were set at 650 V and the dwell time was 0.2 s. All data collected in centroid mode were acquired and processed using MassLynx^{TM} NT 4.0 software with QuanLynx^{TM} program (Waters Corp., Milford, MA, USA).

2.3. Calibration standards and quality control samples

Stock solutions of 5-ISMN and acetaminophen (I.S.) were prepared in methanol at concentrations of 0.5 mg/mL and 0.1 mg/mL, respectively, and were stored at 4 °C. The stock solution of acetaminophen was further diluted with water to prepare the working internal standard solution containing 1 μg/mL of acetaminophen. Calibration samples for 5-ISMN were prepared in blank human plasma at concentrations of 1.04, 10.4, 20.8, 52.0, 104, 208, 520 and 1040 ng/mL. The quality control (QC) samples were prepared in blank plasma at low, middle, and high concentrations of 2.08, 104 and 832 ng/mL, respectively. The following assay procedures were the same as described below. In each run, a blank plasma sample (without I.S.) was also analyzed.

2.4. Plasma sample preparation

Aliquots of 0.5 mL of collected plasma sample in 10 mL clean glass tubes were spiked with 50 μL of I.S. working solution (1 μg/mL) and vortexed for 30 s. After adding 3 mL of diethyl ether, the tubes were vortexed for 1 min and centrifuged at 1330 × *g* for 10 min. The upper organic layer was then transferred into another set of clean glass tubes and evaporated to dryness under a gentle stream of nitrogen at 40 °C. The residue was reconstituted in 300 μL of solution consisted of acetonitrile–water (20:80, v/v), followed by vortexing and centrifugation at 12,753 × *g* for 10 min. The supernatant was transferred into 700-μL glass vials, and aliquot of 5 μL was injected into the LC–MS system for analysis.

2.5. Method validation

Validation runs were conducted on three consecutive days. Each validation run consisted of a minimum of one set of calibration standards and six sets of QC plasma samples at three concentrations. The results from QC plasma samples in three runs were used to evaluate the precision and accuracy of the method developed. The peak area ratios of 5-ISMN/I.S. of unknown samples were then interpolated from the calibration curve to give the concentrations of 5-ISMN. During routine analysis, each analytical run included a set of calibration standards, a set of QC plasma samples in duplicate and plasma samples to be determined.

2.5.1. Selectivity

Selectivity was investigated by comparing chromatograms of blank plasma obtained from six subjects with those of corresponding standard plasma sample spiked with 5-ISMN and acetaminophen (1 μg/mL) and plasma sample after an oral dose of compound 5-ISMN extended-release tablets.

In order to evaluate the matrix effect on the ionization of analytes, i.e. the potential ion suppression or enhancement due to co-eluting matrix components, three concentration levels of 5-ISMN (2.08, 104 and 832 ng/mL), each in triplicate, were added to the extracts of 0.5 mL of blank plasma sample, dried and reconstituted with 300 μL of mobile phase, the corresponding peak areas (A) were compared with those of the 5-ISMN standard solutions dried directly and reconstituted with the same volume of mobile phase (B). The ratio (A/B × 100)% was used to evaluated the matrix effect. The matrix effect of internal standard was evaluated using the same procedure.

2.5.2. Linearity and LLOQ

Calibration curves were prepared by assaying standard plasma samples at eight concentrations of 5-ISMN ranged 1.04–1040 ng/mL. The linearity of each calibration curve was determined by plotting the peak area ratio (y) of 5-ISMN/I.S. versus the nominal concentration (x) of the analyte. The calibration curves were constructed by weighted ($1/x^2$) least squares linear regression [17].

The lower limit of quantification (LLOQ) is defined as the lowest concentration on the calibration curve at which an acceptable accuracy (RE) within ±20% and a precision (RSD) below 20% were obtained.

2.5.3. Precision and accuracy

The precision and accuracy of assay were determined from QC plasma samples at three concentration levels of 5-ISMN (2.08, 104 and 832 ng/mL). Precision was expressed as relative standard deviation (RSD %) and accuracy as (mean found concentration − added concentration)/(added concentration) × 100%. Intra-day precision and accuracy were determined by repeated analysis of a set of standards on 1 day ($n = 6$), while inter-day precision and accuracy by repeated analysis on 3 consecutive days ($n = 6$ series per day), using standard curve prepared on the same day.

2.5.4. Extraction recovery

The extraction recovery of 5-ISMN was determined by comparing the peak areas obtained from plasma samples spiked with analyte before extraction with those from samples, to which analyte was added after extraction. This procedure was repeated for samples at three concentrations of 2.08, 104 and 832 ng/mL.

2.5.5. Stability

The stability of 5-ISMN and I.S. stock solutions was evaluated after storage at room temperature for 6 h and at 4 °C for 30 days. The stability of 5-ISMN and I.S. working solutions was investigated at room temperature for 5 h.

QC plasma samples of three concentration levels were subjected to the conditions below. Short-term stability was assessed by analyzing QC plasma samples kept at room temperature for 4 h that exceeded the routine preparation time of samples. Long-term stability was determined by assaying QC plasma samples after storage at −20 °C for 30 days. Freeze–thaw stability was investigated after three freeze (−20 °C)–thaw (room temperature) cycles. Post-preparative stability was assessed by analyzing the extracted QC plasma samples kept in the autosampler at 4 °C for 4 h.

2.6. Application of the assay

In order to demonstrate the reliability of this method in the pharmacokinetic study of 5-ISMN, it was used to determine plasma concentrations of 5-ISMN after single as well as multiple dose administration of compound 5-ISMN extended-release tablets to 18 healthy volunteers. The pharmacokinetic study was approved by local Ethics Committee and all volunteers signed informed consent to participate in the study according to the principles of the Declaration of Helsinki.

Single dose study: subjects fasted overnight before dosing (one tablet each) until 2 h after dosing. Blood samples were collected before dosing (time 0) and at 0.5, 1.0, 1.5, 2.5, 4.0, 5.0, 6.0, 8.0, 12.0, 16.0, 24.0, 36.0 h after dosing. The blood samples were immediately centrifuged at 1330 × g for 10 min, the plasma was removed and stored at −20 °C until analysis.

Multiple dose study: the compound 5-ISMN extended-release tablet was administered to the subjects once daily for 7 consecutive days (at 0, 24, 48, 72, 96, 120 and 144 h). Venous blood samples were obtained at 16 time points (at 72, 96, 120, 144, 144.5, 145, 145.5, 146.5, 148, 149, 150, 152, 156, 160, 168, 180 h). The blood samples were immediately centrifuged at 1330 × g for 10 min, the plasma was removed and stored at −20 °C until analysis.

3. Results and discussion

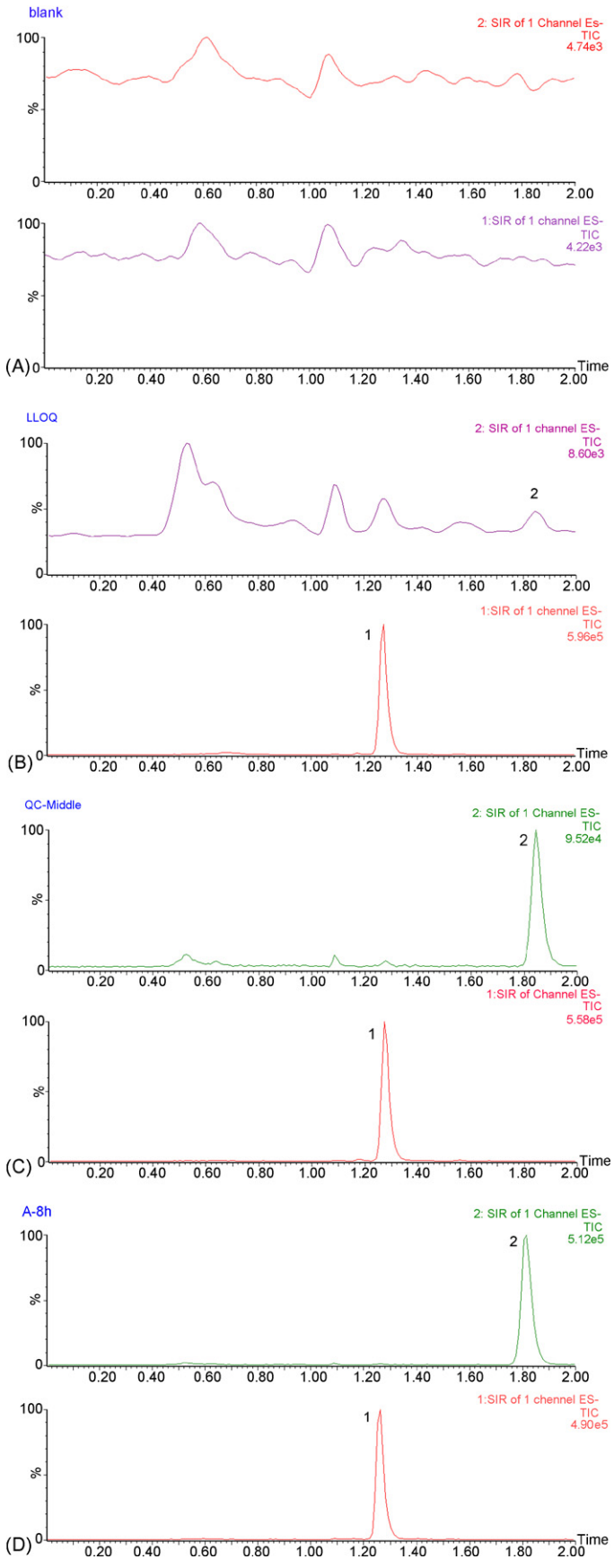
3.1. LC–MS analysis

Under the optimized conditions described above (Section 2.2), the electrospray ionization of 5-ISMN and I.S. produced abundant $[M\text{-}H]^-$ at mass-to-charge ratio (m/z) of 190.0 and 150.0, respectively. The analytes were identified and determined by using selected ion recording (SIR) with the corresponding $[M\text{-}H]^-$ ions from 5-ISMN and I.S. Two channels were used for recording, channel 1 for I.S. with the retention time of 1.27 min, and channel 2 for 5-ISMN with the retention time of 1.85 min. Both 5-ISMN and I.S. were rapidly eluted with retention times less than 2 min, and the total run time was just 2.2 min per sample, which greatly met the requirement for high sample throughput in bioanalysis. Typical SIR chromatograms of a blank plasma sample spiked with 5-ISMN of 104 ng/mL and I.S. (1 μg/mL) are depicted in Fig. 2C. They were detected with excellent separation as well as peak shapes, and very narrow chromatographic peaks with peak width about 6 s.

The fast speed and narrow chromatographic peaks were due to the 1.7 μm particles featured LC system. According to the Van Deemter equation, decrease in the size of the particle packed into modern HPLC columns significantly increases the number of height equivalent theoretical plates (HETP) for a given length column. The use of 1.7 μm particles allows the LC system to push the limits of both analysis speed and peak capacity without compromising resolution.

3.2. Selection of internal standard

The ideal I.S. is the compound that has structural similarity to the analyte, but sometimes it is difficult to get such reference standard. We investigated several substances such as theophylline, carbamazepine, tramadol hydrochloride and acetaminophen. Finally, acetaminophen was chosen as the I.S. by virtue of its similarity in chromatographic behavior, mass spectrometric response and extraction recovery to 5-ISMN. Acetaminophen may be used in selfmedication, which would impair the accuracy of the method. However, in pharmacokinet-



ics or bioequivalence study all subjects should sign the informed consent clearly declaring not taking any drugs 2 weeks before the study until it is finished, therefore the interference with I.S. should not be present. In our study, blood samples from every subject before dosing (time 0) were analyzed and no peak was observed in the retention time region of I.S. which indicated the practical applicability of the method.

3.3. Sample preparation

Liquid–liquid extraction was chosen as the method for sample pretreatment because this technique could produce not only purified but also concentrated samples. Ethyl acetate and diethyl ether were attempted as the extraction solvent and the latter was finally adopted by virtue of its high extraction efficiency and less interference. Besides, extraction procedure was investigated with and without adding 1 M HCl (50 μL), and the latter was found to give a cleaner supernatant and a higher recovery. It was reported by Pennings and de Haas [5] and Ali et al. [18] that 5-ISMN adheres to plastic, hence, we carried out a preliminary experiment to compare the recovery of using plastic tubes with that of using glass tubes with QC samples of 104 ng/mL, each in triplicate, the recovery was 41.3 ± 10.8% and 65.2 ± 2.8%, respectively, indicating the recovery was indeed irreproducibly decreased when plastic tubes were used. Therefore, the sample extraction was performed with diethyl ether and glassware used throughout.

3.4. Method validation

3.4.1. Selectivity

Fig. 2 represents SIR chromatograms of 5-ISMN and acetaminophen in a blank plasma sample (A); a blank plasma sample spiked with 5-ISMN at 104 ng/mL and I.S. (1 μg/mL) (C) and a plasma sample from a volunteer 8.0 h after oral administration of 5-ISMN (D). No interferences were observed at the retention times of the analytes. The retention times for 5-ISMN and I.S. were 1.85 and 1.27 min, respectively.

For all six different blanks, the retention time regions of 5-ISMN and I.S. were free from endogenous interfering peaks. Because selected ion recording (SIR) mode was used for quantification, the determination of 5-ISMN in human plasma was free from interferences of aspirin, another component of the tablets, and its metabolites. To evaluate the matrix effect, the peak areas of blank plasma extracts spiked with analyte post-extraction (A) were compared with those of the standard solutions dried directly and reconstituted with mobile phase (B). All the ratios (A/B × 100)% were between 85% and 115%, which means no matrix effect for 5-ISMN and acetaminophen in this method.

Fig. 2. Representive SIR chromatograms of 5-ISMN (peak 2, channel 2) and acetaminophen (peak 1, channel 1) in human plasma samples. (A) A blank plasma sample; (B) a blank plasma sample spiked with 5-ISMN at the LLOQ of 1.04 ng/mL and I.S. (1 μg/mL); (C) a blank plasma sample spiked with 5-ISMN at 104 ng/mL and I.S. (1 μg/mL); (D) a plasma sample from a volunteer 8.0 h after oral administration of 5-ISMN. The retention times for 5-ISMN and I.S. were 1.85 and 1.27 min, respectively.

Table 1
Precision and accuracy for the determination of 5-ISMN in human plasma (intra-day: $n = 6$; inter-day: $n = 6$ series per day, 3 days)

Added C (ng/mL)	Found C (ng/mL)	Intra-run RSD (%)	Inter-run RSD (%)	Accuracy RE (%)
1.04 (LLOQ)	1.04 ± 0.11	9.1	14.7	−0.37
2.08 (Low)	2.07 ± 0.19	7.8	13.4	−0.43
104 (Middle)	103.5 ± 8.5	7.8	7.6	−0.45
832 (High)	834.5 ± 75.8	8.6	8.8	0.30

3.4.2. Linearity and LLOQ

Calibration curve was linear for 5-ISMN in the concentration range of 1.04–1040 ng/mL ($r^2 \geq 0.99$) by using weighted least square linear regression analysis with a weight factor of $1/x^2$. Representative regression equation for the calibration curve was $y = 1.025 \times 10^{-2}x + 9.885 \times 10^{-4}$ ($r^2 = 0.9973$).

The lower limit of quantification (LLOQ) for 5-ISMN was 1.04 ng/mL with precision and accuracy presented in Table 1, and a corresponding chromatogram is given in Fig. 2B. Compared with the previous methods [4–16] regarding the determination of 5-ISMN in human plasma, our method demonstrated a higher sensitivity with an LLOQ of 1.04 ng/mL. The high sensitivity obtained was attributed to the efficient chromatographic separation afforded by the fast LC system with use of 1.7 μm particles packed column, which leads to a reduction in the number of co-eluting species and it seems to be particularly advantageous when coupled to ESI–MS, providing a higher spectral purity of peaks and an apparent reduction in ion suppression hence lower limits of detection. The sensitivity of the current method could be further improved by reducing sample reconstitution volume.

3.4.3. Precision and accuracy

The data of intra- and inter-day precision and accuracy for 5-ISMN quantification are shown in Table 1. For each QC level of 5-ISMN, the intra- and inter-day precisions (RSDs) were less than 8.6% and 13.4%, and accuracy (RE) was within ±0.45%, indicating acceptable precision and accuracy of the present method.

3.4.4. Extraction recovery and stability

The mean extraction recoveries of 5-ISMN from human plasma were 69.2 ± 3.6%, 65.2 ± 2.8%, and 63.2 ± 3.7% at concentration levels of 2.08, 104 and 832 ng/mL, respectively.

The stock solutions of 5-ISMN and I.S. were found to be stable at room temperature for 6 h and at 4 °C for 30 days. Both working solutions were stable at room temperature for 5 h. Table 2 summarizes the results of short-term stability, long-term stability, Freeze–thaw stability of 5-ISMN in plasma and post-preparative stability. All the results well met the criterion for stability measurements.

Table 2
Stability of 5-ISMN in human plasma at three QC levels ($n = 5$)

Stability	Accuracy (mean ± SD) (%)		
	2.08 (ng/mL)	104 (ng/mL)	832 (ng/mL)
Short-term stability	109.3 ± 2.8	109.6 ± 3.0	106.6 ± 9.4
Long-term stability	106.7 ± 7.5	100.3 ± 1.8	89.8 ± 3.6
Freeze–thaw stability	106.2 ± 5.9	105.2 ± 9.4	104.9 ± 2.4
Post-preparative stability	100.0 ± 7.6	97.6 ± 9.6	102.0 ± 4.4

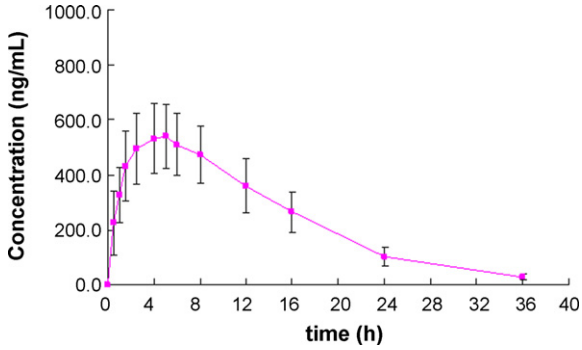


Fig. 3. Mean plasma concentration–time curve of 5-ISMN in 18 volunteers after a single oral dose of 5-ISMN.

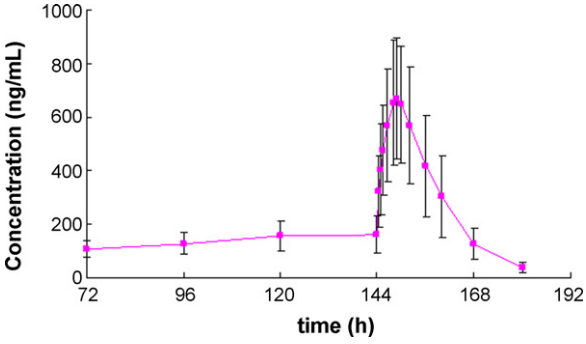


Fig. 4. Mean plasma concentration–time curve of 5-ISMN in 18 volunteers after a multiple oral dose of 5-ISMN.

4. Application to a pharmacokinetic study

The present method was successfully applied to the pharmacokinetic study of 5-ISMN in compound extended-release tablets after oral administration in healthy male volunteers.

Mean plasma concentration–time curves of 5-ISMN in single and multiple dose study are shown in Figs. 3 and 4, respectively.

5. Conclusion

A sensitive, selective and rapid HPLC–ESI–MS method for the determination of 5-ISMN in human plasma is described. Compared with the methods published, the present method is featured by a simple sample preparation procedure, higher sensitivity with an LLOQ of 1.04 ng/mL, satisfactory selectivity and short run time of 2.2 min. The method was successfully applied to the pharmacokinetic study of 5-ISMN in compound extended-release tablets in healthy volunteers.

References

[1] P. Needleman, P.B. Corr, E.M. Johnson, in: A.G. Gilman, T.W. Rall, A.S. NIes, P. Taylor (Eds.), Goodman and Gilman's: The Pharmacological Basis of Therapeutics, eighth ed., Pergamon Press, New York, 1990.

[2] J.P. Kampmann, Drugs 33 (Suppl 4) (1987) 5.
[3] U.E. Jonsson, Eur. J. Clin. Pharmacol. 38 (Suppl 1) (1990) S15.
[4] T. Kosoglou, J.E. Patrick, A. Cohen, E. Radwanski, D. Christopher, M.B. Affrime, Clin. Ther. 17 (1995) 241.
[5] Ed J.M. Pennings, Ed J.M. de Haas, J. Chromatogr. B 675 (1996) 332.
[6] F. Pommier, N. Gauduchear, V. Pineau, A. Sioufi, J. Godbillon, J. Chromatogr. B 678 (1996) 354.
[7] Q. Zhang, X.F. Huang, P. Ji, L.H. Fu, B.X. Yan, S.L. Wei, Acta Pharma. Sin. 33 (1998) 373.
[8] W.X. Luo, Y.D. Zhang, J.P. Shen, Y. Ding, D.K. Huang, J.P. Luo, J. Qiu, Z.H. Jiang, Acta Pharma. Sin. 20 (1999) 951.
[9] A. Stockis, S.D. Bruyn, X. Deroubaix, B. Jeanbaptiste, E.L. Jr, F. Nollevaux, G. Poli, D. Acerbi, Eur. J. Pharm. Biopharm. 53 (2002) 49.
[10] J. Pastera, L. Vysloužil, J. Květina, J. Chromatogr. B 800 (2004) 271.
[11] C.L. Marty, C.L. Mattei, J.L. Chabard, E. Beyssac, J.M. Aiache, M. Madesclaire, J. Chromatogr. B 663 (1995) 153.
[12] J.X. Wang, L. He, T.L. Li, Y.P. Qin, Y.G. Zou, T. Li, Chin. New Drugs J. 8 (1999) 524.
[13] L. Liu, K.X. Li, Y. Li, G.W. He, C.H. Sun, Chin. J. Clin. Pharmacol. 17 (2001) 215.
[14] Y.Q. Hu, H.Y. Xue, Y.N. Hou, H.C. Liu, Chin. Pharm. J. 39 (2004) 931.
[15] T.B. Vree, E. Dammers, R. Valducci, Int. J. Clin. Pharmacol. Ther. 42 (2004) 463–472.
[16] L.C. Silva, L.S.O.B. Oliveira, G.D. Mendes, G. Garcia, A.S. Pereira, G.D. Nucci, J. Chromatogr. B 832 (2006) 302–306.
[17] A.M. Almeida, M.M. Castel-Branco, A.C. Falcão, J. Chromatogr. B 774 (2002) 215–222.
[18] S.L. Ali, H. Blume, Z. Fresenius, Anal. Chem. 321 (1985) 257.