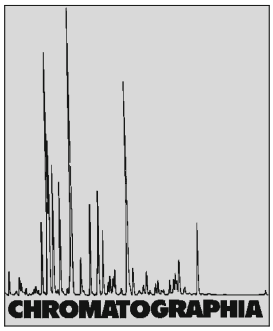
LC Determination of Isosorbide-5-Mononitrate in Human Plasma



Himanshu S. Karmalkar✉, Mohan M. Metku, Milind S. Bagul, Asmita C. Nimkar, Rajen D. Shah

Raptim Research Limited, A-226, TTC Industrial Area, Mahape, Navi Mumbai, Maharashtra 400701, India; E-Mail: hskarmalkar@gmail.com

Received: 3 June 2008 / Revised: 23 November 2008 / Accepted: 15 December 2008

Abstract

A simple, sensitive, selective and cost effective LC–UV method was developed for determination of isosorbide mononitrate in human plasma using guaifenesin as an internal standard. Isosorbide mononitrate in plasma was extracted by a single step liquid extraction using *tert*-butyl methyl ether and chromatographed on a C18 column using water and acetonitrile (80:20 *v/v*) as mobile phase. The method was validated and exhibited a linear range from 51.6 to 2064.4 ng mL^{-1}. The inter- and intra-assay accuracy ranged from 97.2–102.7 to 94.2–105.5%, respectively, with precision less than 10% in both the cases. The LLQ was 51.6 ng mL^{-1}. The validated method was applied to the quantitation of isosorbide mononitrate from plasma samples in a pharmacokinetic study.

Keywords

Column liquid chromatography
Pharmacokinetics
Isosorbide-5-mononitrate in human plasma
Guaifenesin

Introduction

Isosorbide-5-mononitrate [1] (ISMN) which is 8-nitroxy-2, 6-dioxabicyclo [3.3.0] octan-4-ol is an active metabolite of isosorbide dinitrate. ISMN is a long acting organic nitrate vasodilator used in the treatment of angina pectoris. It has several advantages over the parent compound isosorbide dinitrate, such as longer elimination half-life, no first-pass metabolism and no metabolites, leading to more predictable and reproducible clinical effect [2, 3].

The analytical techniques developed in recent years for the quantitation of ISMN from human plasma include LC with a Thermal Energy Analysis detector [4], capillary GC with an electron-capture detector (GC-ECD) [4–13], LC-MS–MS [14–16] and GC–MS [17] However, no LC–UV method has been developed for the determination of ISMN from human plasma.

GC-ECD is the most widely used instrumental method due to its high sensitivity and selectivity. However, it was shown by Pastera et al. [4] that ISMN had a substantial decomposition when the injector temperature was higher than 150 °C. If the injector temperature was low, it promoted the condensation of non-volatile compounds causing contamination of injector system and a rapid decrease in the sensitivity. Morrison et al. [8] used packed GC columns without mononitrate derivatisation.

SPE with ENVI 18 cartridges [5] or Extrelut columns [7] replaced the laborious liquid–liquid extraction in three methods. Gremeau et al. [5] obtained a LLQ of 20 ng mL^{-1}. Pommier et al. [13] reported a method requiring a silylation pretreatment of glassware to prevent adsorption of ISMN to the glass. Jain et al. [14] quantified ISMN via stable acetate adduct formation using LC-ESI-MS–MS to give a LLQ of 10 ng mL^{-1}. The salient features of their method were low sample volume and SPE with direct injection of eluent and short run time.

The method described here was developed with the aim of obtaining a simple, sensitive, selective and cost effective LC–UV analysis with a short

Full Short Communication
DOI: 10.1365/s10337-009-0954-x

Published online: 01 February 2009

run time for the determination of ISMN in human plasma. *tert*-Butyl methyl ether as extraction solvent evaporates very quickly, allowing 100 samples to be processed and analysed in a day. The extraction efficiencies of ISMN using *tert*-butyl methyl ether in polypropylene and glass tubes were found to be similar and consistent, indicating that there was no loss by evaporation of nitrates or sorption to glassware. ISMN is a very weakly UV-absorbing molecule and hence our method is not as sensitive as other reported methods. Although the LLQ value of 51.6 ng mL^{-1} established in our method was higher it is regarded as acceptable. The method was successfully applied to the study of the pharmacokinetics of ISMN after oral administration of 60 mg sustained release (SR) tablets to healthy volunteers.

Experimental

Chemicals and Reagents

All chemicals and reagents used in the study were LC grade. Acetonitrile and *tert*-butyl methyl ether were purchased from Merck (Mumbai, India). Water used in the mobile phase was deionised and purified by a Milli-Q water purification system from Millipore (Bedford, MA, USA). A working standard of ISMN was obtained from Pan Drugs (Gujarat, India), and guaifenesin (GF) which was used as an internal standard was obtained from Unique Pharmaceutical Labs. (Thane, India). Drug free human CPDA (citrate phosphate dextrose adenine) plasma bags (six different lots) were obtained from JVP Blood Bank (Vashi, Navi Mumbai, India).

Instrument and Chromatographic Conditions

The LC instrument was an Agilent 1200 series LC (Waldbronn, Germany) equipped with an isocratic pump (G1310A), autosampler (G1329A), Peltier cooler (G1330B), thermostatted column compartment (G1316A) and a variable wavelength detector (G1314B). EZChrome Elite software (version-3.2.1) was used for data handling. Analysis was performed on a 5 μm Merck Purosphere RP18e column (Merck, Darmstadt, Germany) (150 cm × 4.6 mm i.d.) with water:acetonitrile (80:20 *v*/*v*) at 1 mL min^{-1}. The column was maintained at 20 °C. Detection was carried out at 220 nm.

Preparation of Stock and Working Solutions

Primary stock solutions of ISMN and GF were separately prepared by dissolving an appropriate amount in methanol to yield a concentration of 1 mg mL^{-1} of each. The standard working solutions were obtained by further dilution of the stock solutions with mobile phase. The GF working solution had a concentration of 25 μg mL^{-1}. All solutions were stored at 2–8 °C and equilibrated to room temperature before use.

Preparation of Calibration Standards and Quality Control Samples

Calibration standards were prepared at 8 different concentrations of 51.6, 103.3, 206.6, 413.1, 826.2, 1238.6, 1651.5 and 2064.4 ng mL^{-1} of ISMN in plasma. Quality control (QC) samples were prepared at three different concentrations of low QC (LQC-151.7), medium QC (MQC-908.3) and high QC (HQC-1816.7) ng mL^{-1}. All of these samples were prepared by spiking appropriate amounts of standard stock solutions into the drug free plasma samples. They were stored at −20 °C.

Method Development

Dichloromethane, diethyl ether, *n*-hexane, *tert*-butyl methyl ether and their mixtures with each other were tried for the extraction of ISMN and GF. With dichloromethane the extraction efficiency of the analyte was 12%. With a mixture of dichloromethane and *n*-hexane (1:1 *v*/*v*) the recovery of the analyte was 22%. Interference from plasma at the retention time of ISMN was observed in samples extracted with diethyl ether. *tert*-Butyl methyl ether was the solvent which showed no interference at the retention time of ISMN and GF and yielded an extraction efficiency of better than 50%. An appropriate internal standard can control extraction and LC injection variability. Although Guaifenesin is not chemically and structurally related to the analyte, it was chosen as an internal standard for its stability in prepared solutions and a good response at the detection wavelength. Its selection was also based on its good chromatographic and extraction behavior.

Sample Preparation

Calibration standard samples and quality control samples were extracted by using liquid–liquid extraction. To each polypropylene vial containing 0.5 mL sample, 50 μL of IS was added at a concentration of 25 μg mL^{-1} and also 3.0 mL of *tert*-butyl methyl ether, which were then vigorously vortex-mixed for 3 min followed by centrifuging at 4,500*g* at 4 °C for 5 min. The organic phase was transferred to a new polypropylene vial and evaporated to dryness in a water bath at 40 °C under a stream of nitrogen gas. The dried residue was reconstituted with 100 μL of mobile phase and vortex-mixed briefly. A 50 μL aliquot was injected into the LC system.

Method Validation

The method validation was carried out according to the US Food and Drug Administration bioanalytical method validation guidance [18].

The specificity of the method was tested by screening six different batches of drug free human plasma. Each blank sample was tested for interferences at the retention time of ISMN and GF using the proposed extraction procedure. Frequently used over-the-counter medicines such as acetaminophen, ibuprofen, diclofenac and cetirizine were also injected to check for interference at the retention time of the analyte and GF. For sensi-

tivity determination, six samples at LLQ concentration (51.6 ng mL^{-1}) of ISMN were investigated and the reproducibility and precision were determined.

Linearity was tested for the concentration range of 51.6–2064.4 ng mL^{-1}. For the determination of linearity, a blank sample, a zero sample (blank + IS) and eight calibration standards were used. Samples were quantified using the ratio of peak area of analyte to that of the IS. A weighted linear regression (1/concentration) was performed with nominal concentrations of calibration levels. Peak area ratio was plotted against plasma concentrations and standard curves were calculated.

The intra-day precision and accuracy of the assay were measured by analyzing six spiked samples of ISMN at each QC level (151.7, 908.3 and 1816.7 ng mL^{-1}). The inter-day precision and accuracy was determined over 3 days by analyzing 30 QC samples ($n = 6$ for each concentration level) each day.

The validation was carried out using human plasma which contained CPDA as anticoagulant. Normally EDTA is used as an anticoagulant and it was therefore necessary to assess the anticoagulant effect and understand its impact on the quantitation of ISMN from a plasma matrix. A significant anticoagulant effect would prevent using EDTA as an anticoagulant. Anticoagulant effect was performed by comparing LQC and HQC samples ($n = 3$) prepared in EDTA anticoagulant-based human plasma and compared against calibration curve prepared in CPDA anticoagulant-based plasma.

Evaluation of stability of samples was based on the comparison of stored samples against freshly prepared samples of the same concentration. The percentage difference between the back calculated concentrations obtained in the stored samples and freshly prepared samples was evaluated. Four aliquots each of LQC and HQC concentrations were used for the stability study. For assessment of long term stability, the samples were kept in deep freezer at -20 ± 5 °C for 30 days and thereafter analyzed. Bench top stability was studied on samples kept at ambient temperature (20–30 °C) for 8 h. The processed samples were kept in the autosampler at 7 °C for 24 h and then injected to determine the autosampler stability The freeze–thaw stability (samples stored at -20 ± 5 °C), was studied after subjecting the samples to three freeze–thaw cycles. For dry extract stability the dried extracts were kept at 2–8 °C for 24 h after which they were brought to ambient temperature, reconstituted and then analyzed. In order to determine the stability of ISMN in solution, the working solution was kept at 2–8 °C for 6 days. Thereafter, the mean areas of ISMN from six replicate chromatographic runs were compared to that of mean area of a freshly prepared solution of the same concentration.

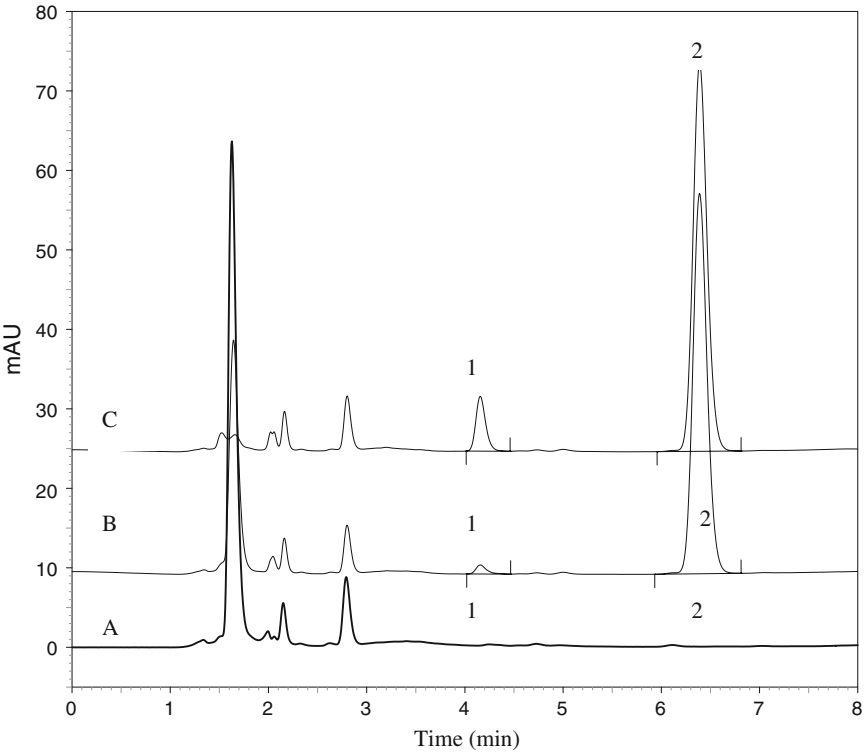


Fig. 1. Representative chromatograms of ISMN (**1**) and guaifenesin (**2**) in human plasma. A Blank human plasma; B blank plasma spiked with ISMN and IS at LQC level; C plasma sample from a subject 4 h after oral administration of 60 mg sustained release (SR) isosorbide-5-mononitrate formulation

Results and Discussion

Using the chromatographic conditions described above, the peaks of ISMN and GF eluted at about 3 and 6 min, respectively. The total analysis time was 8 min.

No interfering peaks were observed at the retention times of either the analyte or IS in six different batches of drug-free human plasma samples. A representative LC chromatogram of extracted blank plasma is shown in Fig. 1. The precision for the six plasma samples spiked with ISMN at LLQ concentration was 5.9% with accuracy ranging from 98.6 to 102.7%.

The method exhibited a good linear response over the range of concentration from 51.6 to 2064.4 ng mL^{-1}. The mean correlation coefficient was 0.998. The mean accuracy values of each calibration standard were close to each other.

The intra-day accuracy ranged between 94.9 and 105.5% with a precision of 6.9–9.2%. The inter-day accuracy was between 97.2 and 102.7% with a precision of 5.4–9.9%. The results are presented in Table 1. A chromatogram of ISMN at the

Table 1. The intra- and inter-batch precision and accuracy of the method for the determination of ISMN in human plasma

Concentration (ng mL^{-1})	Conc. found (mean ± SD, ng mL^{-1})	Mean accuracy (%)	Precision (%RSD)
Intra-day			
151.7	160.1 ± 13.8	105.5	8.6
908.3	862.1 ± 78.9	94.2	9.2
1816.7	1899.6 ± 131.3	104.6	6.9
Inter-day			
151.7	155.8 ± 15.4	102.7	9.9
908.3	883.2 ± 64.5	97.2	7.3
1816.7	1854.7 ± 100.2	102.1	5.4

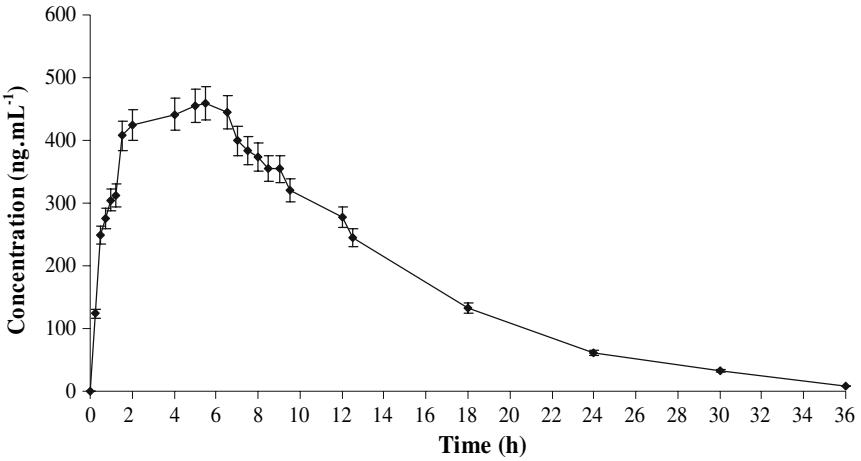


Fig. 2. Time course of the plasma ISMN concentration in healthy subjects after a single 60 mg (SR) oral dose. Each point represents the mean ± SD ($n = 21$)

Table 2. Pharmacokinetic parameters of ISMN in 21 healthy subjects after a single 60 mg sustained release oral dose

Parameters	Mean ± SD
C_{max} (ng mL^{-1})	512.2 ± 256.1
T_{max} (h)	3.9 ± 2.1
$AUC_{36\ h}$ (h ng mL^{-1})	6604.2 ± 3468.4
$t_{1/2}$ (h)	6.5 ± 2.9

LQC concentration with an internal standard in shown in Fig. 1.

The accuracy and precision obtained on LQC samples prepared using EDTA plasma ranged from 98.6 to 102.0% with precision of 2.3 and 99.6% to 101.3% with a precision of 1.0% on HQC sample indicating that there was no anticoagulant effect and the biostudy samples could be collected using EDTA as an anticoagulant.

ISMN was found to be stable in the plasma matrix for 30 days at −20 °C (LTS) with percent differences of 4.6 and 2.7% for LQC and HQC, respectively, for 8 h at ambient temperature with percent difference of 8.0 and −4.5% for LQC and HQC, respectively, for 24 h in the autosampler with percent difference of 12.4 and −1.0% for LQC and HQC, respectively. The dried extract was stable for 24 h at 2–8 °C with percent difference of 2.6 and −1.8% for LQC and HQC, respectively and ISMN in plasma was stable for three freeze–thaw cycles with percent difference of 9.2 and −13.6% for LQC and HQC, respectively. The stock solution of ISMN was found to be stable for 6 days at 2–8 °C with %RSD of 1.2%.

Application to Pharmacokinetic Study

Figure 2 shows the time course of the ISMN plasma concentration of 21 healthy human subjects who received 60 mg sustained release ISMN tablets under fasting conditions. The study was conducted strictly in accordance with guidelines laid down by the International Conference on Harmonisation and USFDA [19]. The pharmacokinetic data are tabulated in Table 2.

Conclusion

The developed and validated LC method proved to be cost effective, simple, rapid, selective and convenient for the determination of ISMN in human plasma. The method has the advantages of good selectivity, simple sample processing procedure, high throughput and wide linear range. It has been successfully applied to the pharmacokinetics of ISMN in healthy volunteers after single oral administration of 60 mg sustained release formulation.

References

1. O'Neil Maryadele J (ed) (2001) The Merck index, 13th edn, monograph no. 5246, p 935 and monograph no. 4571, p 812. Merck Research Laboratories, Division of Merck, Whitehouse Station
2. Chasseaud LF (1987) Cardiology 74:6. doi:10.1159/000174255
3. Major RM, Taylor T, Chasseaud LF, Darragh A, Lambe RF (1984) Clin Pharmacol Ther 35:653
4. Pastera J, Vyslouzil L, Kvetina J (2004) J Chromatogr B Analyt Technol Biomed Life Sci 800:271–274. doi:10.1016/j.jchromb.2003.10.069
5. Gremeau I, Sautou V, Pinon V, Rivault F, Chopineau J (1995) J Chromatogr B Analyt Technol Biomed Life Sci 665:399–403. doi:10.1016/0378-4347(94)00543-E
6. Michel G, Fay L, Prost M (1989) J Chromatogr A 493:188–195
7. Santoni Y, Rolland PH, Cano J-P (1984) J Chromatogr A 306:165–172
8. Morrison RA, Fung H-L (1984) J Chromatogr A 308:153–164. doi:10.1016/S0021-9673(01)87542-7

9. Maddock J, Lewis PA, Woodward A, Massey PR, Kennedy S (1983) J Chromatogr A 272:129–136
10. Marzo A, Treffner E (1985) J Chromatogr A 345:390–395. doi:10.1016/0378-4347(85)80177-8
11. Pennings JM, De Haas JM (1995) J Chromatogr B Analyt Technol Biomed Life Sci 675:332–336
12. Sioufi A, Pommier F (1984) J Chromatogr A 305:95–103
13. Pommier F, Gauducheau N, Pineau V, Sioufi A, Godbillon J (1996) J Chromatogr B Analyt Technol Biomed Life Sci 678:354–359. doi:10.1016/0378-4347(95)00570-6
14. Jain DS, Subbaiah G, Sanyal M, Shrivastav PS, Pal U, Ghataliya S, Kakad A, Bhatt J, Munjal V, Patel H, Shah S (2006) Rapid Commun Mass Spectrom 20(19): 2921–2931. doi:10.1002/rcm.2684
15. Silva LC, Oliveira LSOB, Mendes GD, Garcia G, Pereira ADS, Nucci GD (2006) J Chromatogr B Analyt Technol Biomed Life Sci 832:302–306. doi:10.1016/j.jchromb.2005.12.027
16. Sun X, Li X, Cai S, Qin F, Lu X, Li F (2007) J Chromatogr B 846:323–328
17. Lauro-Marty C, Lartigue-Mattei C, Chabard JL, Beyssac E, Aiache JM, Nadesclaire M (1995) J Chromatogr B Analyt Technol Biomed Life Sci 663:153–159. doi:10.1016/0378-4347(94)00436-9
18. Guidance for Industry, Bioanalytical Method Validation, Center for Drug Evaluation and Research (CDER), May 2001
19. Guidance for Industry, E6 Good Clinical Practice: Consolidated Guidance, Center for Drug Evaluation and Research (CDER), April 1996