

Short communication

Quantification of isosorbide 5-mononitrate in human plasma by liquid chromatography–tandem mass spectrometry using atmospheric pressure photoionization

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Abstract

Isosorbide 5-mononitrate (5-ISMN) is an organic nitrate widely used for its vasodilating properties in the treatment of angina pectoris. In the present study, an efficient, sensitive, robust method was developed for the determination and quantification of isosorbide 5-mononitrate, in human plasma, by liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS), using photospray ionization. Isosorbide 5-mononitrate was extracted from 0.5 mL human plasma by liquid–liquid extraction (LLE). The method had a chromatographic run of 2.0 min using a C₈ analytical column (100 mm × 2.1 mm i.d.) and the linear calibration curve over the range was linear from 20 to 2000 ng mL⁻¹ ($r^2 > 0.995$). The between-run precision, based on the relative standard deviation replicate quality controls, was 7.9% (60 ng mL⁻¹), 5.2% (300 ng mL⁻¹) and 7.0% (1800 ng mL⁻¹). The between-run accuracy was 94.9%, 94.1% and 88.8% for the above-mentioned concentrations, respectively. The method herein described was employed in a bioequivalence study of two tablet formulations of isosorbide 5-mononitrate 40 mg.

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1. Introduction

Isosorbide 5-mononitrate (5-ISMN) is an organic nitrate widely used for its vasodilating properties in the treatment of angina pectoris. The 5-ISMN has several advantages over the parent compound isosorbide dinitrate, such as a longer elimination half-life, no first-pass metabolism, and no active metabolites, which lead to a more predictable and reproducible clinical effect [1,2].

Several analytical methods mainly based on high resolution gas chromatography with an electron-capture detector (HRGC-ECD) [3–13], and high-performance liquid chromatography (HPLC) with detection by thermal energy analysis [14,15]

have been used to quantify isosorbide 5-mononitrate in human plasma.

The HRGC-ECD has been employed often, since it offers high sensitivity and selectivity. However, the previous data by HRGC show that this compound has a substantial decomposition when the injector temperature is higher than 150 °C. This injector temperature is low and promotes the condensation of non-volatile compounds caused contamination of the injector system and a rapid decrease of sensitivity; the injector liner needs to be replaced after only 60 injections [13].

Here we describe a specific, sensitive and high throughput liquid chromatography–tandem mass spectrometry (LC–MS/MS) method using the atmospheric pressure photospray ionization (APPI) in negative mode for quantification of isosorbide 5-mononitrate in human plasma. The procedure requires a simple liquid–liquid extraction (LLE) and was developed

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Table 1
Analysis conditions for 5-ISMN in APPI

Compound	Transition (<i>m/z</i>)	IS (V)	Temperature (°C)	DP (V)	FP (V)	CE (eV)	CXP (V)
Isosorbide 5-mononitrate	249.92/189.9	−1200	400	−80	−160	−26	−21

IS, ion transfer voltage; DP, declustering potential; FP, focusing potential; CE, collision energy; CXP, collision exit potential.

for pharmacokinetics studies. This method was applied to a bioequivalence study in healthy volunteers ($n = 24$).

2. Experimental

2.1. Chemicals and reagents

Isosorbide 5-mononitrate was obtained from Biosintetica (São Paulo, SP, Brazil). Acetonitrile (HPLC grade), acetone (HPLC grade), acetic acid (analysis grade) and ammonium acetate (analysis grade) were purchased from J.T. Baker (Phillipsburg, NJ, USA). Ultrapure water was obtained from a Gradient Millipore system (São Paulo, Brazil). Blank blood was collected from healthy, drug-free volunteers. Plasma was obtained by centrifugation of blood treated with anticoagulant sodium heparin. Pooled plasma was prepared and stored at approximately -70°C until needed.

2.2. Calibration standards and quality controls

The stock solution of isosorbide 5-mononitrate was prepared in acetonitrile/water 1:1 at concentration of 1 mg mL^{-1} . The calibration curve for isosorbide 5-mononitrate was prepared in blank human plasma at concentrations of 20, 50, 100, 200, 500, 1000 and 2000 ng mL^{-1} and performed in duplicate in each batch. Quality control samples were prepared in blank plasma at concentrations of 60, 300 and 1800 ng mL^{-1} .

2.3. Sample preparation

Aliquots (0.50 mL) of human plasma were employed for liquid–liquid extraction without the use of internal standard. The tubes were vortex mixed for 20 s and allowed to stand at room temperature for 2 min. Four milliliter of diethyl ether–hexane (80:20, v/v) were added and the samples were vortex mixed for 40 s, the upper layer transferred to clean tubes and the solvent evaporated under N_2 (40°C). The dry residue was re-dissolved with $200\text{ }\mu\text{L}$ of acetonitrile:water (50:50, v/v). The samples were transferred into glass microvials, capped and placed in an autosampler.

2.4. Liquid chromatography and mass spectrometry conditions

A HPLC system (LC10AD, Shimadzu, Japan) consisting of a pump and an autosampler was used for all analysis. The chromatographic system consisted on a C_8 Genesis analytical column ($100\text{ mm} \times 2.1\text{ mm i.d.}$, $4\text{ }\mu\text{m}$ particle size), and the mobile phase was a mixture of acetonitrile/water/acetone (80:15:5, v/v/v, 20 mM of ammonium acetate and 0.1% of acetic

acid) at a flow rate of $350\text{ }\mu\text{L min}^{-1}$. The total run time was set for 2.0 min. The column was operated at room temperature and presented a void time of 0.8 min. The temperature of the autosampler was maintained at 8.0°C and was set up to make $40\text{ }\mu\text{L}$ sample injection. Mass spectrometry was performed in a Sciex API 3000 triple stage quadrupole mass spectrometry (Applied Biosystems, Foster City, CA, USA) equipped with an APPI source operating in negative mode. The source block temperature was set at 400°C and the photoionization capillary voltage at 1.2 kV; nitrogen was used as the collision gas. The ions monitored in multiple reaction monitoring (MRM) and the conditions are described in Table 1. Data were acquired by Analyst software (1.4, Applied Biosystems) and calibrations curves for the analyte were constructed using the isosorbide 5-mononitrate peak-area via a weighted ($1/x^2$) least-squares linear regression. Peak areas were then interpolated from the calibration curve to provide concentrations of isosorbide 5-mononitrate.

2.5. Specificity/selectivity

Each blank sample from five different pools of plasmas, including a pool of lipemic and another of haemolyzed, were tested for interference using the proposed extraction procedure and analytical conditions.

2.6. Recovery

Preliminary experiments were conducted to evaluate the recovery with the extraction method described above. The percentage recovery was calculated as the ratio of the peak area for extracted blank plasma spiked at each standard concentration (60, 300 and 1800 ng mL^{-1}) relative to the peak area of the equivalent blank plasma samples spiked after the extraction.

2.7. Stability

Quality control samples prepared to test stability (60, 300 and 1800 ng mL^{-1}) were subjected to short-term (6 h) room temperature, three freeze-thaw cycles and 24 h autosampler (8°C) stability tests. For long term test (196 days) two quality control samples were prepared at 120 and 1200 ng mL^{-1} . Subsequently the isosorbide 5-mononitrate concentrations were measured in comparison with freshly prepared samples.

2.8. Precision and accuracy

To assess the precision and accuracy of the developed analytical method, three distinct concentrations in the range of expected concentrations were evaluated using eight determinations per concentration.

The precision and accuracy were assessed according to a within-day basis (intra-batch), which defines those parameters during a single analytical run, and a between-day basis (inter-batch), which measures the variability between days, involving different analysts and reagents.

2.9. Bioequivalence study

The method was applied to evaluate the bioequivalence of two 40 mg tablet formulations of 5-ISMN in healthy volunteers: isosorbide 5-mononitrate (test formulation from Laboratórios Biosintética, Brazil; lot no. DNP008/01, expiration date Apr/2002) and Monocordil® (standard reference formulation from Baldacci S/A, Brazil; lot no. BB110, expiration date Feb/2002).

Twenty-four healthy volunteers of both sexes were selected for the study. The study followed a single dose, two-way randomized crossover design with a two-week washout period between doses. Blood samples were collected at 0, 10 min, 20 min, 30 min, 40 min, 1 h, 1 h 30 min, 2 h, 2 h 30 min, 3 h, 3 h 30 min, 4 h, 5 h, 6 h, 7 h, 8 h, 10 h, 12 h, 14 h, 16 h, 18 h and 24 h post-dosing. The bioequivalence of the two formulations was assessed according to US-FDA guidelines.

3. Results and discussion

3.1. Method development

Atmospheric pressure photospray ionization mode is virtually a universally adopted procedure for efficiently identifying various classes of substances, including apolar substances such as the polycyclic aromatic compounds. This ionization mode has been shown to be a powerful technique in analyzing different drugs in biological matrixes, including polar compounds such as chloramphenicol [16]. This procedure opens a new window to compounds that can be analyzed by LC–MS/MS, as for example in the present case of isosorbide 5-mononitrate. One of the main advantages of this ionization system is that the molecule ionization is soft and produces minimal fragmentations and a predominant molecular ion signal [17]. For isosorbide 5-mononitrate, the formation of the ion was observed in negative mode corresponding to an adduct with the acetate anion $[M + \text{acetate}]^-$ of m/z (249.9, whose abundance is ten times higher than the deprotonated molecular ion $[M - H]^-$ of m/z 189.9. The maximum abundance of the ion m/z 249.9 was obtained with the minimum (in module) ion transfer voltage (IS)–1200 V, and the increase (in module) of the IS reduced the abundance of this acetate adduct (data not shown). The formation of the ion $[M + \text{acetate}]^-$ was not observed in electron spray ionization (ESI) due to the higher voltage used (4000–5500 V) in this ionization mode. In atmospheric pressure chemical ionization (APCI), the ion of m/z 249.9 can be observed, but in very low abundance (Fig. 1C). In the analysis of isosorbide 5-mononitrate by HRGC–MS using electron impact, independently on whether the molecule is derivatized or not, the high fragmentation and low abundance of the molecular ion [18] provides no advantages in terms of

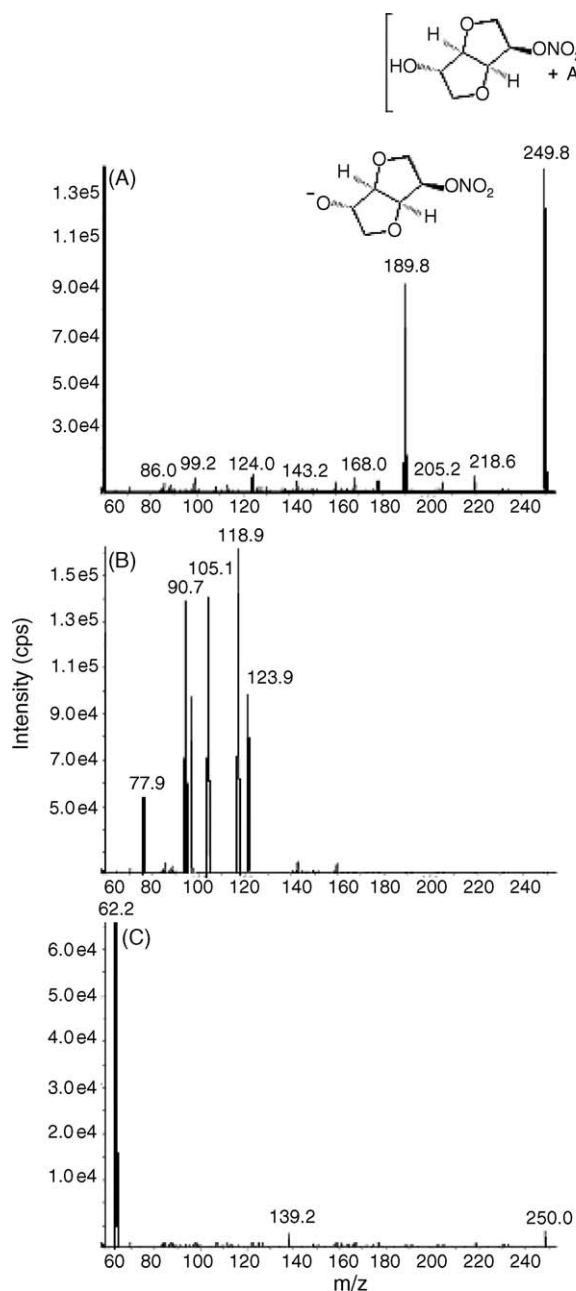


Fig. 1. (A) Full-Scan mass spectrum of the isosorbide 5-mononitrate in photo-spray ionization, ion source voltage = -1200 V. (B) Full-scan mass spectrum of isosorbide 5-mononitrate in turbo ionspray source, ion source voltage = -4500 V (equipment API 4000). (C) Full-scan mass spectrum of isosorbide 5-mononitrate in APCI source, corona discharge needle = $5 \mu\text{A}$ (equipment API 4000).

selectivity and sensitivity over the use of the electron-capture detector.

The main transition observed by the dissociation of the adduct ion $[M + \text{acetate}]^-$ is the deprotonated molecular ion $[M - H]^-$ (Fig. 1A). The transition m/z 249.9 \rightarrow 189.9 had the highest intensity and the lowest detectable interference caused by human blank plasma, and therefore was selected for the present method.

Different molecules with chemically unrelated structures with isosorbide 5-mononitrate have been previously used in HRGC and HRGC–MS analysis as an internal standard such

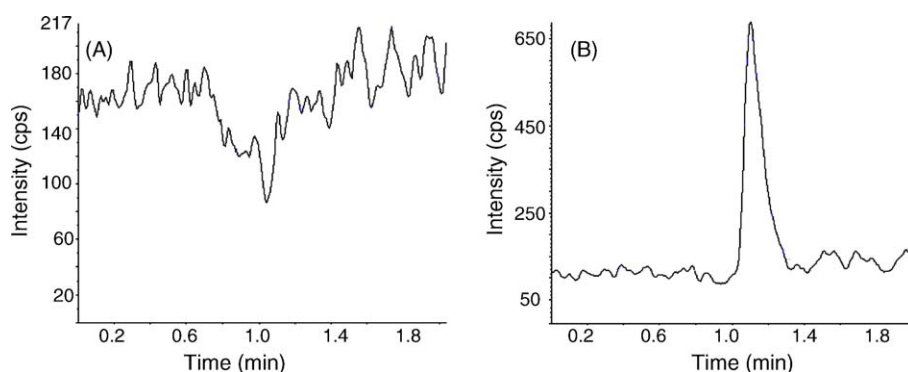


Fig. 2. (A) MRM chromatogram of blank pooled human plasma for the isosorbide 5-mononitrate and (B) MRM chromatogram of the isosorbide 5-mononitrate spiked in human plasma at a final concentration of 20 ng mL⁻¹.

as, nitroglycerine and 9-fluorenone. During the development of this method, different substances were evaluated as possible internal standards such as chloramphenicol, pentanitate and *p*-nitrobenzyl alcohol. However, they did not show similar retention times and/or suitable response factor in photospray ionization. Thus, we decided not to use an internal standard in this methodology.

The limit of quantification (LOQ) was validated for 20 ng mL⁻¹ and the run time of the 2.0 min allowed for a high throughput. The mass chromatograms of a blank and LOQ samples are shown in Fig. 2.

3.2. Assay performance

The optimized method was validated by assessment of recovery, linearity, quantification limit, precision and accuracy. Coefficients of variation and relative errors of less than 15% were considered acceptable, except for the quantification limit (LOQ), whose values were extended to 20%, as recommended by Shah et al. [19] and Bressole et al. [20] for the analysis of biological samples for pharmacokinetics studies. Three calibration curves, prepared independently from different master solutions, were evaluated into the pre-study validation. Although the method showed a linear calibration curve response (20–2000 ng mL⁻¹), the analytical methodology was fully validated with a linear equation $y = 322 \pm 25x - 998 \pm 302$ and $r^2 > 0.998$. The recovery of isosorbide 5-mononitrate calculated from the peak area ratios of extracted human plasma previously spiked at 60, 300 and 1800 ng mL⁻¹ ($n = 15$ for each concentration) were 61.7%, 59.8% and 60.6%, respectively. Between- and within-run accuracy and precision for the quality controls are summarized in Table 2.

The ion suppression evaluated as proposed by Bonfiglio et al. [21] showed no matrix effect.

The stability tests performed indicated no significant degradation following freeze-and-thaw test and short term room temperature test (data not shown). The autosampler stability test (samples maintained at 24 h-autosampler at 8 °C), showed variations of 2.2%, -2.1% and -0.8% for samples of 60, 300 and 1800 ng mL⁻¹ ($n = 5$ for each concentration), respectively. For the long term stability test of 196 days, the concentrations of 120 and 1200 ng mL⁻¹ ($n = 5$ for each concentration) were used;

Table 2

Accuracy and precision of the method for quantifying 5-ISMN based on an analysis of the quality controls

Parameter	Nominal concentration (ng mL ⁻¹)			
	20	60	300	1800
Intra-batch				
Mean found ($n = 8$) (ng mL ⁻¹)	19.8	56.2	281	1580
Precision (%)	99.2	93.6	93.7	87.6
Accuracy (%)	9.1	7.9	5.2	7.0
Mean found ($n = 3$) (ng mL ⁻¹)	19.5	56.9	282	1600
Inter-batch				
Precision (%)	97.5	94.9	94.1	88.8
Accuracy (%)	12.0	9.9	6.7	8.9

the variations were 4.9% and 0.1%, respectively, compared to freshly spiked samples.

3.3. Bioequivalence

The mean peak plasma concentration (C_{max}) for isosorbide 5-mononitrate after oral administration to volunteers is shown in Fig. 3. The geometric mean and respective 90% confidence intervals (CI) for the isosorbide 5-mononitrate/Monocordil® percent ratios were 98.38% (94.60%–102.31%) for AUC_{last},

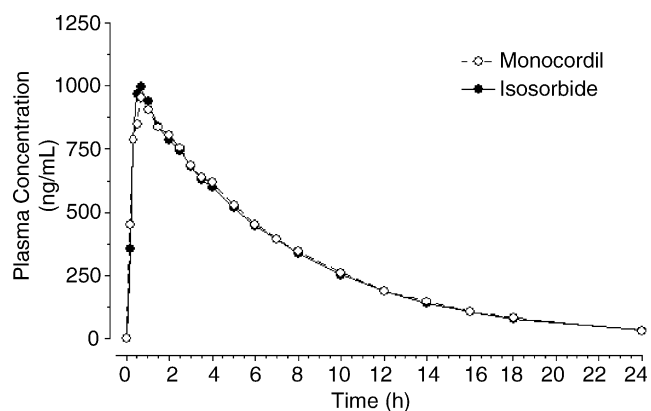


Fig. 3. Mean concentration data vs. time. Monocordil is the standard reference formulation and isosorbide is the formulation received from Biosintetica Laboratory.

98.96% (95.12%–102.95%) for AUC_{0-inf} and 105.41% (95.93%–115.82%) for C_{max} . The 90% CI were therefore within the 80%–125% interval defined by the US Food and Drug Administration [22].

4. Conclusions

This study reports for the first time on a novel alternative method for measuring isosorbide 5-mononitrate using LC–MS/MS. This method offers advantages over those previously reported, in terms of requiring only a simple liquid–liquid extraction without clean-up procedures and a faster run time (2.0 min). The LOQ of 20 ng mL^{-1} is appropriate for pharmacokinetic studies (the estimate $LOQ/C_{max} < 3\%$) and could be further improved by sample concentration if required. The assay performance results indicate that the method is sufficiently precise and accurate for the routine determination of the isosorbide 5-mononitrate in human plasma.

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