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Short communication

Gas chromatographic–mass spectrometric determination of isosorbide 5-mononitrate in human plasma

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Abstract

A highly specific and precise method using gas chromatography–mass spectrometry was developed for the measurement of isosorbide 5-mononitrate in plasma using isomannide mononitrate as internal standard. With regard to the numerous analytical problems encountered when organic mononitrates were determined in plasma, such as thermal instability and adsorption, compounds were silylated before gas chromatography. In order to increase the specificity of the assay, two specific ions of the isosorbide 5-monitrate were simultaneously recorded. The accuracy of the assay was tested day to day with quality specimens spiked blind to the analyst.

1. Introduction

Organic mononitrates as long-acting nitrates are more suitable for prophylaxis of angina pectoris than short-acting nitrates such as glyceryl trinitrate. These mononitrates are well absorbed [1] and slow-release formulations were developed especially for isosorbide 5-mononitrate (5-ISMN). Previous work on the determination of 5-ISMN in plasma, as reviewed by Tzeng and Fung [2], emphasized the high GC thermal instability of organic mononitrates and the irreversible adsorption on the GC inlet systems. Our preliminary assays with non-derivatized mononitrates, exhibited an substantial decomposition of 5-ISMN when the injector temperature was higher than 150°C. Yet, at low temperatures, condensation of non-volatile compounds caused considerable contamination of the injector glass-linear and a rapid decrease of sensitivity. Thus, derivatization of mononitrates before GC seemed to be more suitable. Although gas chromatography with electron-capture detection is a sensitive and popular method, its reliability appears to be insufficient [3]. Besides, this method cannot be applied to silylated derivatives. Some authors [4] proposed a derivatization with trifluoroacetic anhydride. When acetylation procedures were assayed, we observed the formation of two peaks for 5-ISMN

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and thermal degradation occured at an injection port temperature higher than 120°C. Therefore, we developed a capillary gas chromatographic method coupled with mass spectrometry (GC–MS) after silylation of isosorbide 5-mononitrate. This method is an improvement of that reported by Zuccaro *et al.* [5], which did not use an internal standard. As it was proved that no interconversion occured in vivo nor during analysis [2], isomannide mononitrate (IMMN), a positional isomer of 5-ISMN, could be used as internal standard.

In the present report we describe an improved, specific and validated GC–MS assay for 5-ISMN determination in plasma which proved to be reliable for pharmacokinetic studies.

2. Experimental

2.1. Chemical and reagents

All reagents and solvents were of analytical-reagent grade. Ethyl acetate was obtained from SDS (Peypin, France) and ethanol was from Carlo Erba (Milan, Italy). BSTFA and TMCS were purchased from Pierce (Rockford, IL, USA).

5-ISMN, stabilized in lactose (10% w/w) was a gift of Ciba-Geigy laboratories (Rueil Malmaison, France). IMMN, used as internal standard, was synthesized from isomannide dinitrate according to the method reported by Jackson and Hayward [6]. Stock solutions at 1 mg/ml were prepared in ethyl acetate and stored at 4°C. Working solutions were daily prepared before analysis by dilution in ethanol.

2.2. Gas chromatography–mass spectrometry (GC–MS)

Determinations were carried out on a GC–MS HP system (Hewlett-Packard, Palo Alto, CA, USA) consisting of an HP 5890 Series II gas chromatograph connected to a HP 5971 A mass selective detector. GC was performed on an OV 1701 fused-silica capillary column (25 × 0.20 mm I.D., 0.1 μm film thickness, Chrompack, Les Ulis, France) protected by a deactivated fused-silica precolumn (1 m × 0.22 mm I.D., SGE, Villeneuve-Saint-Georges, France). Helium was used as the carrier gas (inlet pressure 69 kPa). The injector and the transfer line temperatures were set at 200 and 280°C respectively. The oven temperature was initially set at 60°C for 0.5 min, then programmed at 35°/min to 120°C and at 10°/min to 140°C and finally at 35°/min to 280°C. The MS system was operated in the electron-impact mode with an electron energy of 70 eV and an ion source temperature of 180°C.

2.3. Sample preparation

Aliquots (1 ml) of plasma samples were transferred to 10-ml polypropylene tubes, spiked with internal standard (100 μl of a 1 μg/ml solution of IMMN) and stabilized with ascorbic acid (0.2 ml of a 0.2 g/l solution). After dilution with 1 ml of distilled water, mononitrates were immediately extracted with 4.5 ml of ethyl acetate using a reciprocal shaker at a low speed for 30 min. After centrifugation for 10 min at 1600 g, the organic layer was transferred to a 5-ml polypropylene tube and evaporated at room temperature to a volume of ca. 0.2 ml using a Speed Vac concentrator. The residue was transferred to a 1.5-ml Eppendorf tube and so were the ethyl acetate volumes (2 × 0.2 ml) used to rinse the extraction tubes. The pooled organic layers were evaporated to complete dryness using the Speed Vac concentrator.

Derivatization was carried out by adding 50 μl of BSTFA and 10 μl of TMCS to the residue (vortex mixed, 1 min). The reaction was immediate and 1 μl was injected for GC–MS analysis using an autosampler injector.

2.4. Stability study

In order to determine the stability of plasma samples kept frozen at −20°C, a study was carried out with plasma samples spiked at 3 concentration levels (25, 75, and 350 ng/ml) and analysed after storage at −20°C for 2, 4, 7, 14

and 30 days. Three samples were analysed for each assayed concentration at each storage period.

2.5. Quality controls

Specimens covering the range from blank to the highest anticipated concentration were prepared independently by a technician who did not participate in the study. The quality control samples were stored frozen at −20°C until analysis. Three samples were analysed blind and concomitantly with the plasma samples of the pharmacokinetic study.

2.6. Preliminary pharmacokinetic study

Six healthy male volunteers participated in the study. Following informed consent, each subject received a 60-mg oral dose of 5-ISMN in a slow-release form. Blood samples (10 ml) were collected in heparinized tubes at 0, 15, 30, 45 min and 1, 1.5, 2, 4, 5, 6, 8, 12, 15, 24 and 34 h following oral administration of the drug. Plasma was immediately separated by centrifugation and stored at −20°C in 2 different 5-ml polypropylene tubes until analysis.

A last control was carried out at the end of the study by analysing some plasma samples of the second lot kept frozen for two months.

3. Results and discussion

3.1. Mass spectra and specificity

Mass spectra of silylated derivatives of 5-ISMN and IMMN are reported in Fig. 1. Derivatization as described here leads to bis-(trimethylsilyl) derivatives of 5-ISMN and IMMN, not only on the hydroxyl group but also by substitution of the nitro-group. As it was shown [2] that no interconversion between mononitrates occurs *in vivo* nor during GC analysis, IMMN could be used as internal standard.

In order to determine the specificity of the assay, ions at m/z 101 and at m/z 290 were simultaneously recorded for both 5-ISMN and IMMN and the ratios 101 (5-ISMN)/101 (IMMN) and 290 (5-ISMN)/290 (IMMN) were compared. The proportion between these ratios has to be unchanged in standard solutions, calibration points and assay points. An interfering compound occured in the determination of the ion at m/z 101 of the internal standard. This only occured in plasma samples of the pharmacokinetic study and was possibly caused by a metabolite of 5-ISMN.

Finally, quantitation was carried out by monitoring of the 290 (5-ISMN)/290 (IMMN) ratio. Ion chromatograms are presented in Fig. 2. The 101 (5-ISMN)/290 (IMMN) ratio was also routinely calculated as a specificity control. The proportion of the area ratios 290 (5-ISMN)/290 (IMMN) and 101 (5-ISMN)/290 (IMMN) has to remain circa 0.1 as the relative abundance of the ion at m/z 290 is ca. 10% of that of the ion at m/z 101 in the mass spectra of 5-ISMN.

3.2. Validation of the assay

Extraction recovery

The extraction recoveries were $75.7 \pm 7.3\%$ ($n = 4$), $76.3 \pm 1.1\%$ ($n = 2$) and $75.9 \pm 5.8\%$ (n = 6) for plasma samples spiked with 5-ISMN at 25, 200 and 500 ng/ml, respectively. The extraction recovery for IMMN was $81.6 \pm 1.0\%$ ($n = 2$) at 100 ng/ml.

Linearity and reproducibility

Ten-point calibration graphs were obtained by plotting the peak-area ratios of ions at m/z 290 of 5-ISMN and IMMN vs. the concentration of 5-ISMN. Over the concentration range studied, i.e. 0–1000 ng/ml, the linearity was satisfactory as shown by the equation of the mean plots ($n = 10$), $y = 0.00335x + 0.00143$ ($r = 0.999$). The slopes of the calibration graphs were reproducible all through the study: 0.00331 ± 0.00028 (mean ± S.D.) with a coefficient of variation of 8.4%.

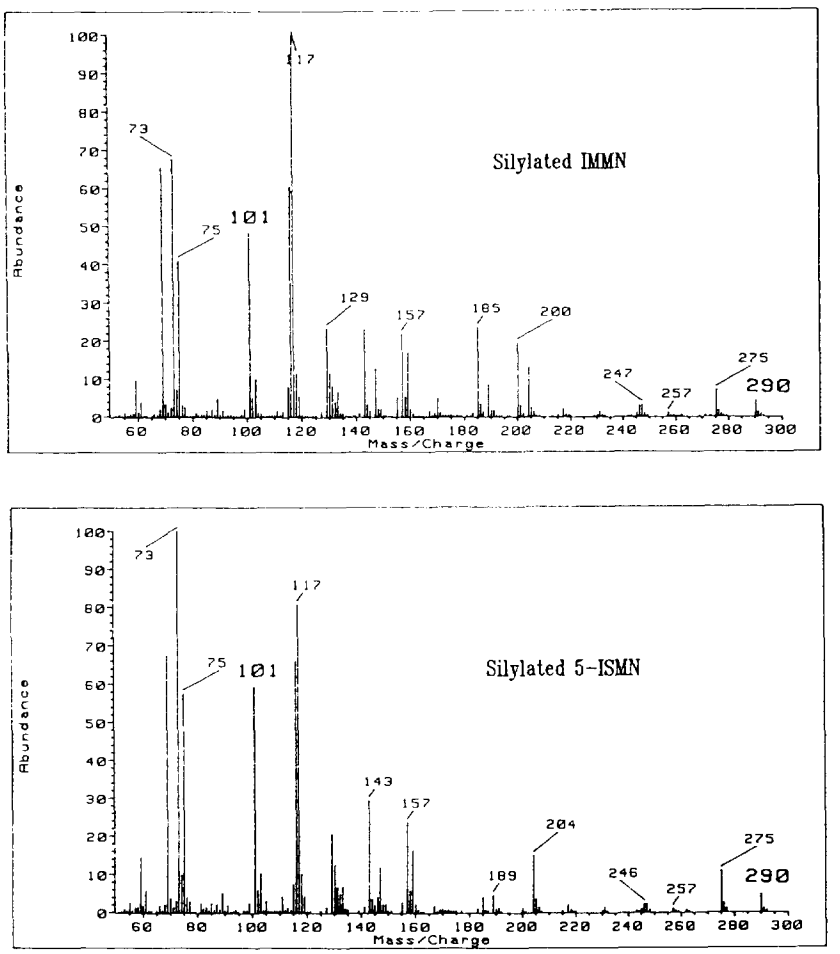

Fig. 1. Electron-impact mass spectra of silylated derivatives of isosorbide-5-mononitrate and isomannide mononitrate.

Precision and accuracy

The within-day precision and accuracy were determined by analysing replicate plasma samples ($n = 10$) spiked with 5-ISMN at 25 and 750 ng/ml. The calculated concentrations of 5-ISMN (mean ± S.D.) were 23.0 ± 3.6 and 734.5 ± 45.9 ng/ml with coefficients of variation of 15.1 and 6.2% and mean errors (in %) of 15.9 and 2.1 respectively. The inter-day precision and accuracy were determined on different days ($n = 10$) over a period of two months at four concentration levels (10, 25, 100 and 750 ng/ml) by the coefficient of variation and the mean error (Table 1).

Limit of quantification

The background, although quite close to zero (Fig. 2) appears to be different from one assay to another. In order to determine an accurate limit of quantitation which is particularly essential in pharmacokinetic analysis, the statistical approach as proposed by Girault et al. [7] was used. The mean calculated value ±S.D. for ten blank plasma samples was 0.67 ± 1.06 ng/ml. The limit of detection, defined as the concentration yielding a significantly higher signal than blank specimens ($p < 0.05$) was 6.2 ng/ml. However, the coefficient of variation for ten replicates spiked at this concentration was higher

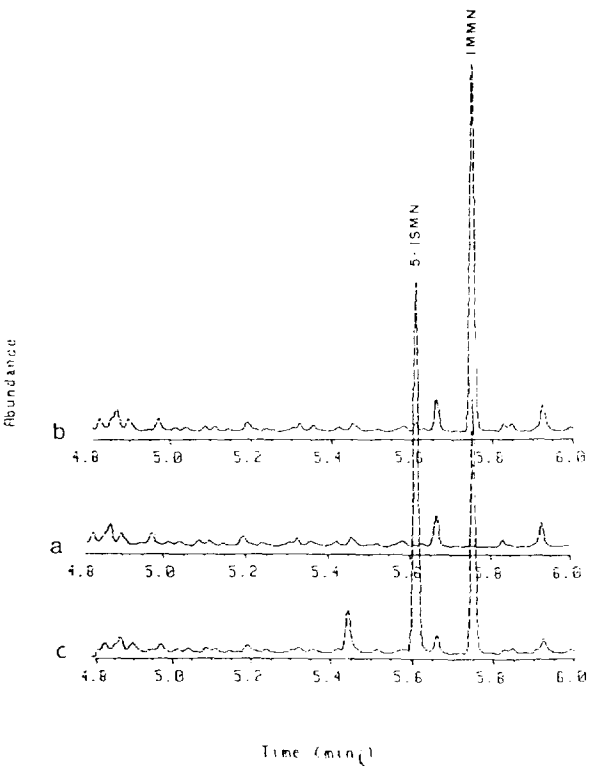

Fig. 2. Selected-ion monitoring (SIM) signals of a blank plasma sample (a) and plasma samples spiked with IMMN as internal standard and with 5-ISMN at the limit of quantitation (b) and at 1000 ng/ml (c).

than 30%. So, the limit of quantitation was in fact 10 ng/ml with regard to the precision (coefficient of variation 18.3%) and the accuracy (mean percentage error +12.5%) for ten replicates. Ion chromatograms of a blank plasma sample and a plasma sample spiked at 10 ng/ml are shown in Fig. 2.

Stability study

Results of the stability study, expressed as the mean calculated concentrations (±S.D.) of 5-ISMN determined in plasma after storage for different periods of time at −20°C are presented in Table 2. The results show that the stability of 5-ISMN in plasma kept frozen at −20°C is quite satisfying for a 1-month period.

Quality controls

The analysis of quality controls ($n = 18$) showed that the calculated concentrations correlated well with the theoretical values ($r = 0.997$). The mean difference between the theoretical and calculated values was 5.55%.

3.3. Assay application

After oral administration of a 60-mg dose of 5-ISMN to six healthy volunteers, pharmacokinetic analysis indicated that the C_{max} value (mean ± S.D.) was 625.8 ± 140.0 ng/ml and most of the drug was absorbed within 5.83 ± 1.07 h. The mean half-life was 6.74 ± 0.49 h and the total area under the 5-ISMN plasma concentration–time curve was 8494.6 ± 1433.3 ng h/ml.

A final control was carried out by analysing plasma samples (at C_{max}) kept frozen for two

Table 1
Day-to-day precision and accuracy of 5-isosorbide monitrate in plasma samples

Spiked concentration (ng/ml)	Calculated concentration (mean ± S.D., $n = 10$) (ng/ml)	Coefficient of variation (%)	Mean error (%)
10	10.6 ± 2.1	19.8	6.0
25	26.3 ± 4.4	16.8	1.3
100	98.3 ± 10.6	10.8	−1.6
750	749.7 ± 38.1	5.1	−0.3

($n = 10$, over two months).

Table 2
Precision and accuracy of the assay of 5-isosorbide mononitrate in plasma samples and stability at −20°C

Amount added (ng/ml)	Days at −20°C	Amount found (mean ± S.D., $n = 3$) (ng/ml)	Precision (C.V., %)	Accuracy (mean error, %)
25	0	25.3 ± 4.6	18.2	1.2
	2	26.2 ± 1.2	4.6	4.8
	4	22.4 ± 1.2	5.3	−10.4
	7	24.2 ± 0.8	3.3	−3.2
	14	20.0 ± 1.3	6.5	−20.0
	30	22.7 ± 2.3	10.3	−9.2
75	0	70.2 ± 1.7	2.4	−6.4
	2	83.8 ± 4.0	4.8	11.7
	4	73.5 ± 5.9	8.0	−2.0
	7	73.4 ± 3.9	5.3	−2.1
	14	71.2 ± 2.9	4.1	−5.1
	30	78.6 ± 1.7	2.2	+4.8
350	0	347.9 ± 28.8	8.3	3.3
	2	370.0 ± 12.6	3.4	5.7
	4	340.2 ± 12.2	3.6	−2.8
	7	355.0 ± 34.9	9.8	1.4
	14	336.9 ± 16.2	4.8	−3.7
	30	347.9 ± 33.3	9.6	−0.6

Three replicates per concentration and per day were determined.

months at −20°C. Results are reported in Table 3 and exhibit the reliability of the method.

The mean C_{max} and AUC values obtained in this study are slightly higher than those reported previously for slow release 5-ISMN formulations. Thus, the data of Lemmer et al. [8] showed C_{max} and AUC values of 509 ± 97 ng/ml and 6730 ± 1190 ng ml/h, respectively, after an oral dose of 60 mg. Previously published methods for GC analysis of 5-ISMN in plasma are numerous. Most of them used electron-capture detection; however, these methods, although quite sensitive, require careful handling to ensure reproducibility. Problems of stability during GC analysis may possibly explain in part the slight differences observed in the 5-ISMN levels.

Table 3
Comparison of the concentrations of 5-ISMN determined in plasma during the first assay and at the end of the study

Subject	Concentration at T_{max} (ng/ml)		Deviation (assay 1 − assay 2) (%)
	Assay 1	Assay 2	
1	668.4	643.8	−3.7
2	890.4	878.0	−1.4
3	658.4	713.6	8.4
4	480.1	510.5	5.3
5	664.7	721.1	8.5
6	483.1	510.0	5.6

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