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Short communication

Solid-phase extraction of isosorbide dinitrate and two of its metabolites from plasma for gas chromatographic analysis

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Abstract

A rapid, accurate and selective method for the determination of isosorbide dinitrate and its 2- and 5-isosorbide mononitrate metabolites in 1.0 ml of human plasma has been developed. Before chromatographic quantitation by gas–liquid chromatography with electron-capture detection, the compounds are subjected to solid-phase extraction, using ENVI 18 cartridges (Supelco). The intra-day and inter-day coefficients of variation are less than 10%, except the inter-day coefficient of variation for the assay of 5-isosorbide dinitrate which is less than 15%. Limits of quantitation are 10, 10 and 20 ng/ml for isosorbide dinitrate, 2-isosorbide mononitrate and 5-isosorbide mononitrate, respectively. Recoveries are in excess of 90% for isosorbide dinitrate and 70% for its two metabolites.

1. Introduction

Isosorbide dinitrate (ISDN) is an organic nitrate widely used for its vasodilating properties in the treatment of angina pectoris. ISDN is metabolized in liver by partial denitratation. It is biotransformed to two active metabolites with are regioisomers: 5-isosorbide mononitrate (5-ISMN) and 2-isosorbide mononitrate (2-ISMN). The 5-ISMN/2-ISMN ratio ranges from 3/1 to 7/1. 5-ISMN which is the most important metabolite is responsible for the long acting activity of ISDN. Because of its better biodisposability than that of ISDN after oral administration, it has recently been used in therapeutics [1].

Available methods for determination of ISDN and its active metabolites in plasma include gas–liquid chromatography with electron-capture detection [2–10] or high-performance liquid chromatography with detection by thermal energy analysis [11,12]. To analyse ISDN and its two metabolites, we used a gas chromatographic method based on the studies of Santoni et al. [10] and Michel et al. [6].

The feature of this method is the way in which the compounds are extracted from plasma. Solid-phase extraction is used, unlike the other methods which employed liquid–liquid extraction [1–9] or Extrelut extraction [10]. Solid-phase ex-

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traction has various advantages compared with liquid–liquid extraction, particulary convenience and rapidity [13,14].

2. Experimental

2.1. Materials

The solid-phase extraction was performed using the Vac-Elut system (Analytichem International, Harbor City, CA, USA) and ENVI 18 500-mg cartridges (Analytichem International).

The CPG system consisted of a Perkin Elmer gas chromatograph (Norwalk, CT, USA) equipped with a constant-current ^{63}Ni electron-capture detector and a fused-silica capillary column (25 m × 0.32 mm I.D.) coated with BP 1 of 0.5 μm film thickness (Perkin Elmer). The carrier gas was helium and the make-up gas was nitrogen. Peak areas were measured with a Model D 2500 integrating printer–plotter (Merck, Darmstadt, Germany).

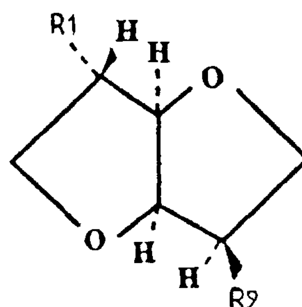
The analytical standards used were ISDN (I) (Théraplix, Paris, France), 2- and 5-ISMN (II and III) (Ethypharm, Houdan, France) and nitroglycerine (TNG) (IV) (Merck, Darmstadt, Germany). Their structures are given in Fig. 1. Reagents used were methanol (>99% purity) (Carlo Erba, Milan, Italy) and sterile water (Bruneau, Boulogne-Billancourt, France).

2.2. Sample processing

Standards of compounds I, II, III and IV were prepared at 1 μg/ml in water. Dilutions of these stock solutions were used to prepare the appropriate working standard solutions (mixed standard solutions). After shaking, 1-μl aliquots were injected onto the chromatograph.

The chromatographic conditions were as follows: injector temperature, 150°C; detector temperature, 230°C; oven temperature, programmed from 110 to 140°C at 15°C/min.

The extraction procedure consisted of the following four steps. Samples were prepared in haemolysis tubes (for single use), according to their type: (i) preparation of samples used for



Compound	R_1	R_2
Isosorbide dinitrate	ONO_2	ONO_2
5-Isosorbide mononitrate	OH	ONO_2
2-Isosorbide mononitrate	ONO_2	OH

Fig. 1. Structure of I, II and III.

calibration: 1 ml of plasma (Lyotrol, BioMerieux, Lyon, France) to which 100 μl of standard solutions were added; (ii) preparation of control blank: 1 ml of plasma to which 100 μl of methanol were added; (iii) preparation of the sample itself: 1 ml of patient's plasma to which 100 μl of internal standard solution were added.

Methanol (2 ml) and then water (2 ml) were run through the cartridges. This was followed by passage of the plasma sample through the conditioned cartridges. Water (3 ml) was run through the cartridges and these were then left to dry for 1 or 2 min. The cartridges were eluted with 1 ml of methanol, and the eluate was directly injected onto the CPG.

The specificity of the method is validated by the retention times of the compounds.

Calibration samples were prepared using drug-free plasma. Aliquots (1 ml) were spiked by addition of ISDN, 2-ISMN and 5-ISMN working standard solutions to produce concentration ranges of 10–100 ng/ml, 25–200 ng/ml and 50–300 ng/ml, respectively. TNG (25 ng/ml) was added according to the routine extraction procedure.

Calibration curves were obtained by plotting the peak-area ratios (the peak areas of I, II, III

to the peak area of the internal standard, IV) as a function of the concentration of I, II, III per ml of plasma. The concentrations of I, II or III in a patient sample were calculated by interpolation from the standard curve (equation of linear regression: $y = ax + b$).

Accuracy and precision of the method for I, II and III are evaluated with the intra- and inter-day assay variabilities. The intra-day and inter-day variabilities are determined for the three compounds in series consisting of ten spiked plasma samples with respect to a standard calibration curve.

Limit of detection is evaluated from ten solutions of compounds in methanol and the limit of quantitation from ten extracted plasma samples.

Recoveries of ISDN and its metabolites were determined by comparison of the peak-area ratios obtained from processed plasma samples (the peak areas of I, II and III to the peak area of the internal standard, IV) with the peak-area ratios of directly injected standards. Plasma samples with known ISDN, 2-ISMN and 5-ISMN concentrations of 10 and 100 ng/ml, 25 and 200 ng/ml and 50 and 300 ng/ml, respectively, as well as standard solutions in methanol were used. TNG (25 ng/ml) was added to the extracted plasma before injection.

3. Results and discussion

3.1. Calibration

The characteristics of the calibration graphs established with plasma are given in Table 1. The results demonstrate that the calibration curves are linear in the ranges 10–100 ng/ml (ISDN), 25–200 ng/ml (2-ISMN) and 50–300 ng/ml (5-ISMN).

3.2. Limit of detection and limit of quantitation

The limits of detection are 5, 1 and 5 ng/ml, and the limits of quantitation are 10, 10 and 20 ng/ml for ISDN, 2-ISMN and 5-ISMN, respectively, with repeatability better than 10% at these concentrations.

3.3. Accuracy and precision

The results of precision and accuracy of the method for I, II and III are given in Tables 2 and 3. The results show the good precision of the method for the simultaneous determination of ISDN and its two metabolites. The intra-day and inter-day coefficients of variation for the assay are less than 10% except the inter-day coefficient of variation for the assay of 5-isosorbide dinitrate which is less than 15%.

The accuracy of the method is satisfactory ($< 10\%$) for all compounds tested.

3.4. Recoveries

The extraction recoveries of ISDN, 2-ISMN and 5-ISMN are shown in Table 4. Recoveries are higher than 90% for ISDN, and higher than 70% for its two metabolites.

3.5. Duration of assay

One of the goals of the present study was to reduce as much as possible the time needed for

Table 1
Calibration graphs for ISDN, 2-ISMN and 5-ISMN in spiked blank plasma ($n = 10$)

Compound	Lower concentration (ng/ml)	Higher concentration (ng/ml)	Equation of linear regression	Coefficient of correlation (r) (mean ± S.D.)
ISDN	10	100	$y = 0.0066x - 0.0133$	0.9985 ± 0.0008
5-ISMN	50	300	$y = 0.0019x + 0.0068$	0.9977 ± 0.0007
2-ISMN	25	200	$y = 0.0080x + 0.0602$	0.9987 ± 0.0008

Table 2
Validation of the method: inter-day assay variability and accuracy

Compound	Concentration added (ng/ml)	Concentration found[a] (ng/ml)	Coefficient of variation (%)
ISDN	10	10.8 ± 1.0	9.6
	100	100.9 ± 0.5	0.5
2-ISMN	25	25.8 ± 2.7	10.2
	200	199.7 ± 2.9	1.4
5-ISMN	50	53.7 ± 8.5	15.8
	300	302.5 ± 4.7	1.6

[a] Mean ± standard deviation; $n = 10$.

Table 3
Validation of the method: intra-day assay variability and accuracy

Compound	Concentration added (ng/ml)	Concentration found[a] (ng/ml)	Coefficient of variation (%)
ISDN	10	11.3 ± 0.8	6.7
	100	105.2 ± 4.4	4.2
2-ISMN	25	27.3 ± 1.3	4.9
	200	201.9 ± 9.8	4.8
5-ISMN	50	50.2 ± 4.9	9.7
	300	308.7 ± 14.1	4.6

[a] Mean ± standard deviation; $n = 10$.

Table 4
Extraction recoveries of ISDN, 2-ISMN AND 5-ISMN (n = 5)

Compound	Concentration (ng/ml)	Extraction recoveries (%)[a]
ISDN	10	96.0 ± 3.6
	100	91.0 ± 1.3
2-ISMN	25	76.4 ± 5.0
	200	83.9 ± 1.2
5-ISMN	50	70.4 ± 5.1
	300	99.4 ± 8.0

[a] Mean ± standard deviation; $n = 5$.

the assay of ISDN and its metabolites. The methods previously used to simultaneously extract ISDN and its metabolites are quite time-consuming: Extrelut extraction needs more than 35 min [10], liquid–liquid extractions techniques need more than 30 min [6,8]. In the present study solid-phase extraction presents a rapid alternative. The characteristics of the described assay make it possible to dispense with the time-consuming purification procedures prior to the extraction without giving interfering peaks in the chromatogram, a phenomenon frequently encountered in liquid–liquid extraction. The choice of the cartridge and the high purity of the

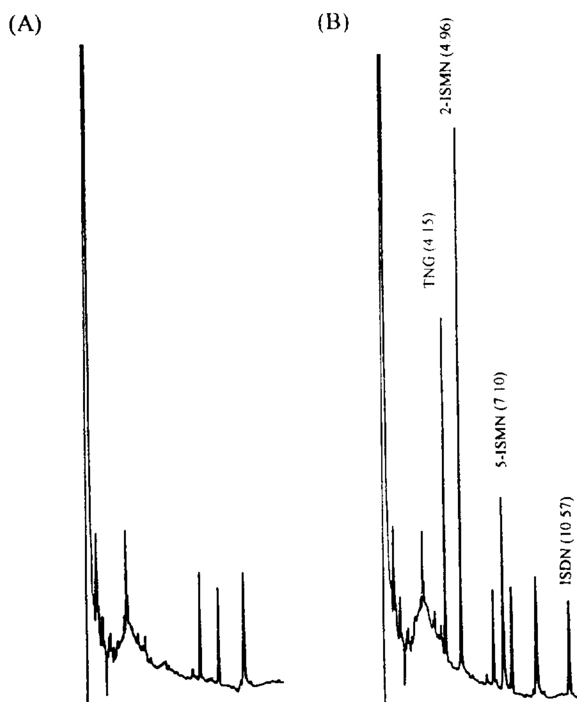


Fig. 2. (A) Chromatogram of a blank plasma extract. (B) Chromatogram of a plasma extract containing 25 ng/ml ISDN, 50 ng/ml 5-ISMN, 75 ng/ml 2-ISMN and 25 ng/ml internal standard (TNG).

solvents (water and methanol) used for the extraction are the most essential characteristics of our method. An other advantage is the use of methanol as the elution solvent which renders subsequent evaporation unnecessary, except when the eluate has to be concentrated. The eluate can thus be directly injected on the CPG. Extraction of ISDN and its metabolites takes only 14 min. As the chromatographic analysis of the compounds needs 11 min, the total time needed for processing is 25 min. The method is therefore particulary well suited for routine analysis of large quantities of samples.

Fig. 2 shows chromatograms obtained with this method. Retention times of ISDN, 5-ISMN and 2-ISMN are 10.5, 7.1 and 4.9 min, respectively.

4. Conclusions

Several papers have described CPG or HPLC methods for the analysis of ISDN and its metabolites. However these methods used liquid–liquid or Extrelut extraction. This paper describes a new method for the extraction of ISDN and its two metabolites: solid-phase extraction which was preferred because of its rapidity, its convenience, the low volume of solvents required, the small amount of plasma required and the good recoveries.

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