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Note

Determination of isosorbide dinitrate and its metabolites in plasma by gas chromatography on a capillary column with electron-capture detection

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Isosorbide dinitrate (ISDN) is used for the treatment of various cardiovascular diseases. After oral administration of this drug, low plasma levels are observed [1]. Its metabolites, isosorbide 5-mononitrate (5-ISMN) and isosorbide 2-mononitrate (2-ISMN), both of which contribute to the pharmacological activity of ISDN [2–4], are present in much higher concentrations in plasma.

The gas chromatographic determination of ISDN and its metabolites on packed or capillary columns with splitless injection has been described [5–7]. Isomannide dinitrate has also been reported as an internal standard [8,9].

We describe here a gas chromatographic method with electron-capture detection for the quantitation of ISDN and its metabolites. This method was adapted from those described by Lutz et al. [7], but the capillary column was a fused-silica SE-30 bonded phase with a 0.5-μm film and the injection system was an all-glass falling-needle injector without a dead volume [10,11]. Two internal standards were used for the simultaneous quantitation of ISDN and

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its metabolites, 4-nitrobenzyl alcohol (NBA) and trinitroglycerine (TNG) and one external standard, 1,3-dinitrobenzene (DNB).

EXPERIMENTAL

Chemicals and reagents

ISDN, 2-ISMN and 5-ISMN (Ethypharm, Houdan, France), TNG (Fournier, Dijon, France), DNB (Merck, Darmstadt, F.R.G.) and NBA (Ega-Chemie, Steinheim, F.R.G.) were used without further purification. Dichloromethane (Merck) and ethyl acetate (Carlo Erba, Milan, Italy) were of the grade for pesticide determination.

Gas chromatography

A Packard 427 gas chromatograph (Packard, Delft, The Netherlands) was 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 SE-30 of 0.5-μm film thickness (Spiral, Dijon, France). The all-glass falling needle injector system (Spiral) was without a dead volume. The conditions were as follows: injector temperature, 160°C; detector temperature, 200°C; oven temperature, programmed from 140 to 250°C at 2°C/min. The carrier gas was helium at 0.7 bar and the make-up gas was nitrogen at a flow-rate of 30 ml/min. Peak areas were measured with a model 3390 A integrating printer–plotter (Hewlett-Packard, Avondale, PA, U.S.A.).

Sample preparation

Plasma samples were stored at −20°C. After thawing at 4°C, 1 ml of plasma was added to 100 ng of NBA and 25 ng of TNG (standard solutions were prepared at 1 mg/ml in ethanol and working solutions at 10 and 1 ng/ml in ethyl acetate) in a silanized 15-ml screw-capped tube. The mixture was incubated for 30 min at 37°C. After addition of 8 ml of dichloromethane, the mixture was shaken gently for 30 min on a rotating shaker and centrifuged at 1000 *g* for 10 min. The aqueous layer was discarded and the dichloromethane phase was transferred into a 10-ml silanized tube. The solution was evaporated to dryness under a stream of nitrogen at 0°C in an ice–water bath and 62.5 ng of DNB (external standard) in 250 μl of ethyl acetate were added. After shaking, 1 μl was injected into the chromatograph. The needle was dropped in the injector just before complete solvent evaporation.

Human study

The method was used to determine the plasma concentrations of ISDN and its metabolites after oral administration of ISDN to healthy subjects. A single-dose pharmacokinetics study was made on six healthy subjects. Three doses of ISDN were applied, 40, 80 and 120 mg. To avoid degradation and losses of

organic nitrates, blood samples were drawn in Vacutainer tubes (ref. A3204SV72) and immediately centrifuged at 4°C (at 1000 *g* for 10 min) to remove cells. The supernatant plasma was transferred into a special siliconed Vacutainer tube (ref. A3204SV73) at −20°C until taken for analysis. Thirteen samples were taken: before administration (time 0) and after administration of ISDN at times 0.5, 2, 3, 4, 5, 6, 8, 10, 12, 15, 18 and 24 h.

RESULTS AND DISCUSSION

GC system characteristics

The use of an SE-30 bonded phase with a 0.5-μm film gave good chromatographic responses (symmetrical peaks for the three reference compounds and the two internal standards). High-resolution chromatography avoided interferences from coextracted plasma compounds and allowed several internal and external standards for quantitative analysis to be chosen. Optimization of the nature and flow-rate of the carrier gas led to good efficiency and rapid chromatographic analysis, which are necessary for achieving high sensitivity with an electron-capture detector. The use of nitrogen as the make-up gas in the detector cell led to better sensitivity in comparison with argon–methane (95:5) (a factor of three increase in the ISDN response).

The solvent evaporator injector made it possible to concentrate the plasma extract on the needle and to prevent solvent contamination in the detector cell. To obtain a reproducible response for these volatile compounds, it was necessary to inject just before total evaporation of the diluting solvent occurred.

The chromatographic system was stable for routine plasma analysis.

Extraction and internal standards

Table I gives the absolute extraction recoveries obtained with dichloromethane, which is a sufficiently polar solvent to be used successfully for the simultaneous determination of ISDN and its more polar metabolites. Extrac-

TABLE I

EXTRACTION RECOVERIES OF ISDN, 2-ISMN, 5-ISMN AND INTERNAL STANDARDS TNG AND NBA

Compound	Concentration (ng ml^{-1})	Extraction recovery (mean ± S.D., $n=6$) (%)
ISDN	25	91.8 ± 4.5
2-ISMN	50	94.1 ± 4.6
5-ISMN	50	91.7 ± 6.6
TNG	100	96.7 ± 4.5
NBA	25	87.5 ± 4.8

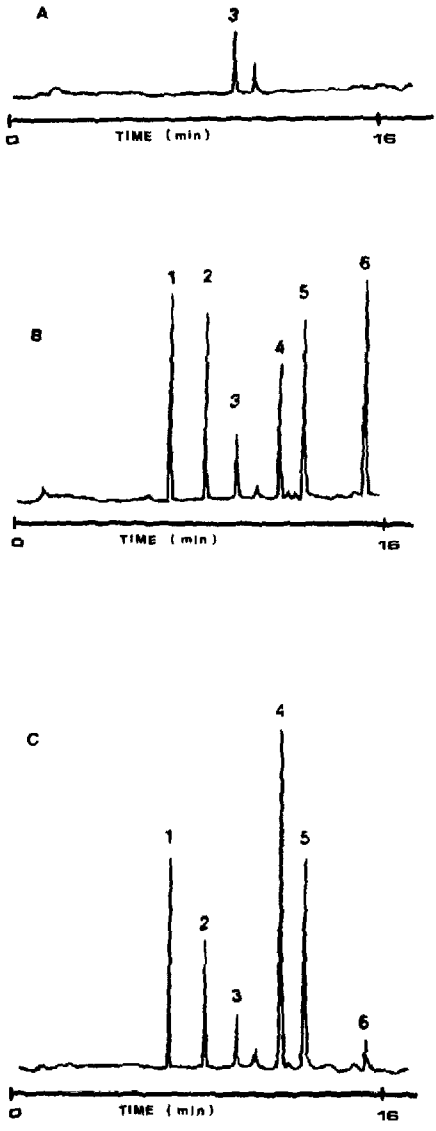

Fig. 1. Chromatograms of plasma extracts. (A) Blank plasma; (B) blank plasma spiked with 100 *ng/ml* ISDN, 2-ISMN and 5-ISMN; (C) plasma from a healthy subject to which ISDN (8 ng/ml), 2-ISMN (76 ng/ml) and 5-ISMN (660 ng/ml) were added. Peaks: 1 = TNG (internal standard, 25 ng/ml); 2 = 2-ISMN; 3 = DNB (external standard, 62.5 ng/ml); 4 = 5-ISMN; 5 = NBA (internal standard, 100 ng/ml); *6* = ISDN.

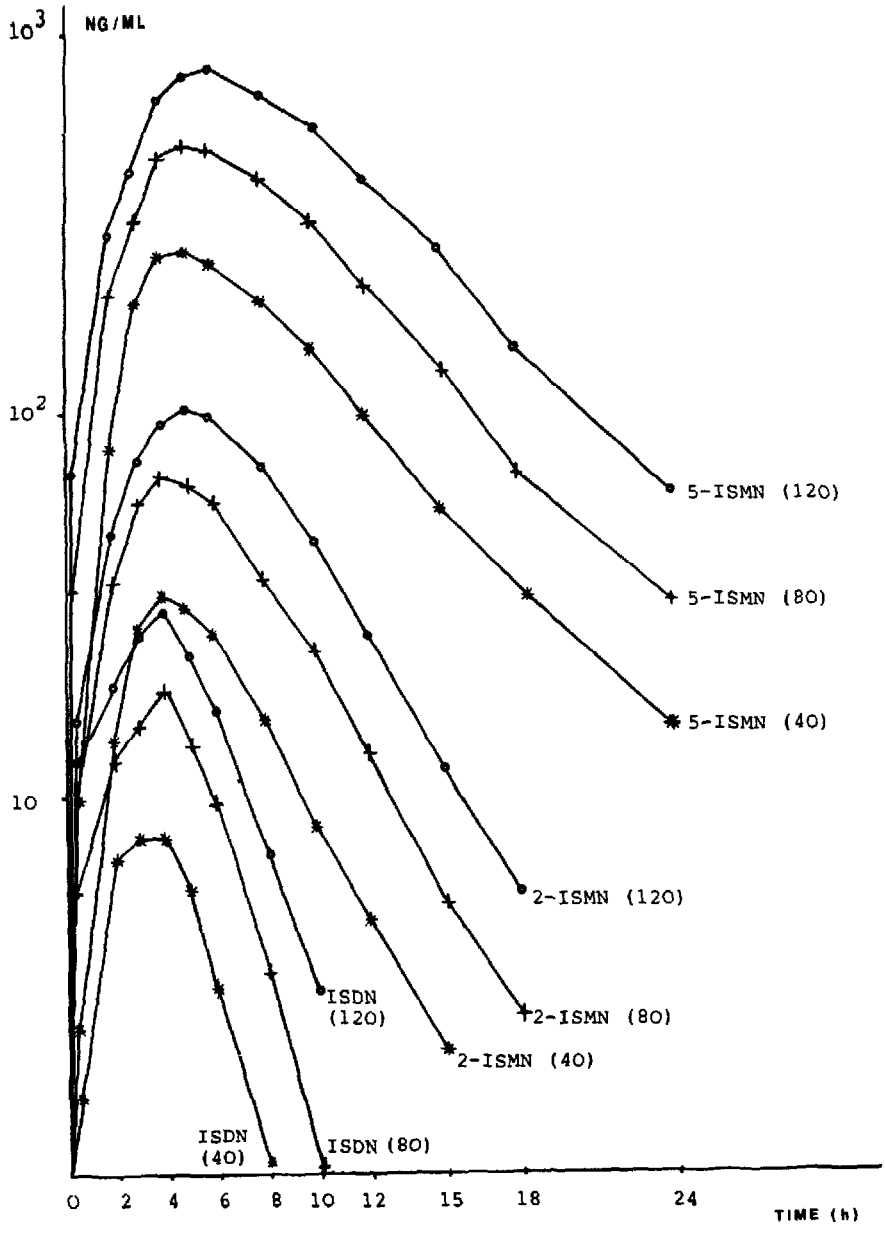

Fig. 2. Curves of mean plasma levels (six subjects) versus time for ISDN, 2-ISMN and 5-ISMN for single doses of (*) 40, (+) 80 and (○) 120 mg of ISDN.

TABLE II

CALIBRATION GRAPHS FOR ISDN, 2-ISMN AND 5-ISMN IN SPIKED BLANK PLASMA ($n=6$)

Compound added	Lower amount (ng ml^{-1})	Higher amount (ng ml^{-1})	Internal standard (ng ml^{-1})	Equation of linear regression $y=ax+b$	Correlation coefficient (r)
ISDN	1	250	TNG (25)	$y=0.0236x-0.0088$	0.9999
			NBA (100)	$y=0.0156x+0.0035$	0.9999
2-ISMN	5	1000	NBA (100)	$y=0.0207x-0.0079$	0.9997
5-ISMN	5	1000	NBA (100)	$y=0.0143x+0.0222$	0.9999

TABLE III

ACCURACY AND PRECISION OF DETERMINATION OF ISDN, 2-ISMN AND 5-ISMN IN PLASMA ($n=3$)

Compound added	Amount added (ng ml^{-1})	Amount found (ng ml^{-1})	Recovery (%)	Coefficient of variation (%)
ISDN	1	1.15	115	4.3
	5	5.15	103	2.1
	10	9.95	99.5	1.0
	25	24.8	99.2	2.9
2-ISMN	5	4.60	92.0	1.7
	10	10.0	100	1.6
	50	48.7	97.4	3.2
	100	105.1	105.1	4.3
5-ISMN	5	4.7	94	2.0
	25	22.8	91.2	3.7
	100	96.0	96	3.7
	250	242.8	97.1	5.9

tion recoveries were measured by spiking 1 ml of blank sample with known amounts of the compounds.

Dichloromethane led to 'blank plasma' chromatograms without interferences at the retention times of the compounds of interest (Fig. 1). Two internal standards were used for quantitation, TNG for ISDN and NBA for 2-ISMN and 5-ISMN. Hence it was possible to adjust two amounts of internal standards for good quantitation of the three compounds: 25 ng of TNG to calculate low levels of ISDN and 100 ng of NBA to calculate high levels of 2-ISMN and 5-ISMN. This was the only means of simultaneously determining the accuracy and precision.

The peak shapes for the sample molecules and internal standards, indicated by the peak of areas and heights given by the integrator, are similar (TNG

with ISDN and NBA with 2-ISMN and 5-ISMN); the use of DNB as an external standard just before chromatographic analysis permitted control of each plasma extraction yield.

Calibration graphs

Calibration graphs were constructed by spiking drug-free plasma with different amounts of ISDN, 2-ISMN and 5-ISMN. The graphs were linear in the ranges 1–250 ng/ml (ISDN) and 5–1000 ng/ml (2-ISMN and 5-ISMN). Table II gives the characteristics of the calibration graphs established with plasma. For each compound these calibration graphs had the same characteristics with plasma and pure solutions and demonstrated the selectivity of the electron-capture detector under the described optimized chromatographic conditions. The detection limits for ISDN, 2-ISMN and 5-ISMN are 0.5, 1 and 1 ng/ml, respectively. These results are similar to those obtained by Lütz et al. [7].

Accuracy and precision

The accuracy was checked by three assays on four identical plasma samples spiked with various amounts of ISDN, 2-ISMN and 5-ISMN. The results, presented in Table III, show the good accuracy of the method for the simultaneous determination of ISDN and its two metabolites. For determinations on three days the low coefficient of variation for ISDN in particular is due to the use of silanized tubes for sampling and extraction. Also, the chromatographic system allowed the use of internal standards which had similar behaviour (physico-chemical and chromatographic) to ISDN and its metabolites.

Application

The procedure was applied successfully to the determination of ISDN and its two metabolites in human plasma. The graphs of plasma levels versus time for single doses of 40, 80 and 120 mg are presented in Fig. 2. The results are means obtained for six healthy volunteers.

REFERENCES

1 H. Bogaert, in P. Needleman (Editor), Organic Nitrates, Springer, Berlin, 1975, p. 25.
2 L.F. Chasseaud, E. Doyle, T. Taylor, A. Darragh and R.F. Lambe, Int. J. Clin. Pharmacol., 21 (1983) 514.
3 T. Taylor, L.F. Chasseau, R. Major and E. Doyle, Biopharm. Drug Dispos., 2 (1981) 255.
4 H.L. Strae and R. Galeazzi, Clin. Pharmacol. Ther., 38 (1985) 140.
5 R.A. Morrison and H.L. Rung, J. Chromatogr., 308 (1984) 153.
6 A. Sioufi and F. Pommier, J. Chromatogr., 305 (1984) 95.
7 D. Lütz, J. Rasper, W. Cielsdorf, J.A. Settlage and H. Jaeger, J. High Resolut. Chromatogr. Chromatogr. Commun., 7 (1984) 58.
8 A. Sioufi and F. Pommier, J. Chromatogr., 229 (1982) 347.
9 Y. Santoni, P.H. Rolland and J.P. Cano, J. Chromatogr., 306 (1984) 165.

10 J. de Graeve, F. Berthou and M. Prost, Méthodes Chromatographiques Couplées à la Spectrométrie de Masse, Masson, Paris, 1986, p. 61.
11 G. Lavoue, P. Chebroux, M. Prost and P. Rigaud, Les Nouvelles Dimensions de la Chromatographie en Phase Gazeuse, Labo-France, Paris, 1982, p. 105.