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DETERMINATION OF THE TWO MONONITRATE METABOLITES OF ISOSORBIDE DINITRATE IN HUMAN PLASMA AND URINE BY GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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SUMMARY

This paper describes a sensitive method for the determination of 2-isosorbide mononitrate and 5-isosorbide mononitrate as metabolites of isosorbide dinitrate at concentrations down to 2 ng/ml of 2-isosorbide mononitrate in both plasma and urine, and 5 ng/ml and 10 ng/ml of 5-isosorbide mononitrate in plasma and urine, respectively.

The two mononitrate metabolites are extracted at basic pH into ethyl acetate, which is then evaporated to dryness. The residue is dissolved in a basic aqueous solution, which is washed with heptane and then re-extracted into ethyl acetate. The metabolites are quantitated by gas chromatography, using a ^{63}Ni electron-capture detector. Conjugates of 2- and 5-isosorbide mononitrate are determined in urine after enzymatic hydrolysis.

INTRODUCTION

Isosorbide dinitrate (ISDN) is an organic nitrate vasodilator. Very low plasma levels are reached after oral administration to man, and ISDN is detectable below its limit of quantitation in the 12-24 h urine only [1]. A glutathione-dependent enzyme system, glutathione S-transferase, catalyses the denitration of ISDN during its passage through the liver to give two isosorbide mononitrates (ISMN): 2-ISMN and 5-ISMN [2].

In a study of the urinary excretion of ISDN in the rat, Rosseel and Bogaert [3] found (without quantitation) 5-ISMN to be the main metabolite, 2-ISMN being excreted in much smaller amounts, both mononitrates were largely excreted in conjugated form. Concentrations of ^{14}C-labelled ISDN, 2-ISMN, and 5-ISMN in blood were measured radiometrically after thin-layer chromatography [4]; the limit of accurate measurement of ISDN and both metabolites

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was 2 ng/ml, but 9 ml of blood were needed. Using this method to study the biotransformation of ISDN after oral administration of 5 mg of [^{14}C] ISDN to human subjects, Down et al. [5] showed that up to 13% was excreted as free and conjugated 5-ISMN and 1% as free 2-ISMN. Some gas chromatographic (GC) methods have been published [6—8], but they either lack sensitivity [6], or require a large sample volume [6, 7]. Rosseel and Bogaert [8] described a GC method with electron-capture detection (ECD) using a capillary column for the simultaneous determination of ISDN down to 0.5 ng/ml, and 2-ISMN and 5-ISMN down to, respectively, 2 ng/ml and 20 ng/ml only, from 2 ml of plasma.

In a recent paper, Geigenberger et al. [9] determined simultaneously ISDN, 2-ISMN and 5-ISMN in serum and not in urine by GC—ECD using benzene as extraction solvent.

A rapid and sensitive method of assaying unchanged ISDN in plasma and urine has recently been developed in our laboratories [1]. Using GC—ECD, this method can measure concentrations down to 0.5 ng/ml. The extraction conditions elaborated for ISDN, however, were not convenient for 2-ISMN and 5-ISMN, and the method therefore did not appear practicable for the simultaneous assay of ISDN and the two mononitrate metabolites in a single run due to their different polarity.

This present paper describes a procedure which permits the quantitative GC determination of free 2-ISMN and 5-ISMN in human plasma and urine. The conjugated metabolites can also be assayed in urine after enzymatic hydrolysis. The limits of quantitation are 2 ng of 2-ISMN per ml of plasma and urine, 5 ng of 5-ISMN per ml of plasma, and 10 ng of 5-ISMN per ml of urine.

EXPERIMENTAL

Chemicals and reagents

The two mononitrate metabolites (2-ISMN and 5-ISMN) of ISDN were supplied by Sanol (Monheim, F.R.G.). The solvents and reagents used are all of analytical grade: ethyl acetate (Nanograde 3427; Mallinckrodt, St. Louis, MO, U.S.A.), *n*-heptane (Uvasol 4366; Merck, Darmstadt, F.R.G.), sodium hydroxide (Titrisol Merck 9956), potassium carbonate (Merck 4928, used to alkalinize plasma or urine).

The pH 5 buffer is prepared with 14.8 ml of 0.2 *M* acetic acid solution and 35.2 ml of 0.2 *M* sodium acetate solution, which are diluted to 100 ml with water. β-Glucuronidase (Sigma, St. Louis, MO, U.S.A.) is bacterial β-glucuronidase containing about 61.5 units/g.

Calibration solutions

The methanolic solutions of the two metabolites contain 2—500 ng of 2-ISMN and 5-ISMN per 25 μl.

Equipment

All the glassware (flasks, glass tubes) is pretreated to prevent adsorption. It is immersed in toluene containing hexamethyldisilazane, trimethylchlorosilane and pyridine (1%, v/v, each) for 15 min and rinsed with methanol. The treat-

ment is repeated every month. Between treatments, the glassware is cleaned as usual and rinsed with methanol.

A Hewlett-Packard Model 5713 A gas chromatograph equipped with a Hewlett-Packard electron-capture detector (Model 18 713 A) is used. The thermoregulators of the detector and the injection port have been modified to permit continuous adjustment of the temperature. The peak areas are given by a Hewlett-Packard computing integrator (Model 3388 A). The column is operated at 123°C with argon—methane (90:10) at a flow-rate of 75 ml/min; the injector temperature is 162°C and the detector is set at 205°C. The glass column (1 m × 2 mm I.D.) is washed [10], and packed with 3% QF-1 on Gas-Chrom Q (80—100 mesh) (Supelco, Bellefonte, PA, U.S.A.). The filled column is flushed with the carrier gas at a flow-rate of 75 ml/min and heated to 150°C at a rate of 1°C/min. The column temperature is held overnight at 150°C and increased to 210°C for conditioning by injection of 200 μl of Silyl 8 (Pierce, Rockford, IL, U.S.A.) in fractions of 10 μl. After this treatment, the column is held at 210°C for 45 min and it is ready for use at 123°C.

Enzymatic hydrolysis of 2-ISMN and 5-ISMN conjugates in urine

One millilitre of urine, 1 ml of acetate buffer (pH 5) and approximately 10 mg of β-glucuronidase are heated for 16 h at 37°C. The extraction procedure is then performed as described below for the free mononitrate metabolites.

Extraction of free 2-ISMN and 5-ISMN from plasma and urine

One millilitre of plasma or urine is introduced into a 5-ml glass centrifuge tube with approximately 100 mg of potassium carbonate and shaken on a Vortex mixer for 1 min; then 3 ml of ethyl acetate are added. The tube is stoppered and shaken mechanically for 10 min at 250 rpm in an Infors shaker, then centrifuged for 3 min at 2500 *g*.

An aliquot of the ethyl acetate phase is transferred to another 5-ml tube and taken to dryness under a stream of nitrogen at room temperature. Evaporation must be performed very carefully and stopped just as the tube reaches dryness.

One millilitre of 2 *M* sodium hydroxide and 2 ml of heptane are added to the dry residue, and the tube is then shaken mechanically for 10 min at 250 rpm and centrifuged. The heptane phase is separated and discarded. Then 1 ml of ethyl acetate is added to the sodium hydroxide phase, shaken for 10 min at 250 rpm and centrifuged. An aliquot of the ethyl acetate phase is transferred into another tube and carefully taken to dryness, as mentioned above; 150 μl ethyl acetate are added to the dry residue and the tube is shaken on a Vortex mixer.

Gas chromatography

A 3-μl portion of the ethyl acetate solution is injected into the gas chromatograph. The content of 2-ISMN and 5-ISMN is calculated from the peak areas by reference to the two respective calibration curves. These curves are obtained by extraction of plasma or urine spiked with increasing amounts of 2-ISMN and 5-ISMN (from 2 to 500 ng/ml). Standard samples for the calibration curves are extracted and plotted every day.

Study in man

A healthy male subject, who had been advised to take no drugs during the 14 days preceding the experiment and none besides ISDN throughout the duration of the study, received 5 mg of ISDN as one Isoket® tablet. Blood samples were collected before and 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 8, 12 and 24 h after the administration of the drug, and centrifuged. Plasma was removed and stored at −20° C until analysis. Urine was collected during the following time intervals: 0—4, 4—8, 8—12, 12—24, 24—48, 48—72, 72—96 and 96—104 h. The volume was measured and an aliquot was stored at −20° C.

RESULTS AND DISCUSSION

Extraction procedure

No internal standard was used. Attempts to use 1,2-dinitrobenzene and 2,3-dinitrotoluene were unsuccessful: the extraction conditions for 2-ISMN and 5-ISMN are different from those required for these two compounds. The chromatographic conditions mentioned above do not permit the use of isomannide mononitrate, which has the same retention time as 5-ISMN.

Hydrolysis of conjugated 2-ISMN and 5-ISMN in urine

Preliminary experiments were carried out to determine the best conditions for the hydrolysis of the 2-ISMN and 5-ISMN conjugates in human urine. Stability tests showed that 2-ISMN and 5-ISMN are stable in urine at 37° C for 48 h.

Samples of the same urine fraction, containing the conjugates of 2-ISMN and 5-ISMN, from a volunteer treated with ISDN were subjected to enzymatic hydrolysis. The reaction was studied in aliquots of this urine incubated with 10 mg of β-glucuronidase and acetate buffer (pH 5) for 1, 2, 3, 4, 16 and 24 h at 37° C.

Fig. 1 shows that 5-ISMN is less easily hydrolyzed than 2-ISMN; the maximum yield of 5-ISMN is obtained after incubation for 16 h whereas that of 2-ISMN is reached after 4 h.

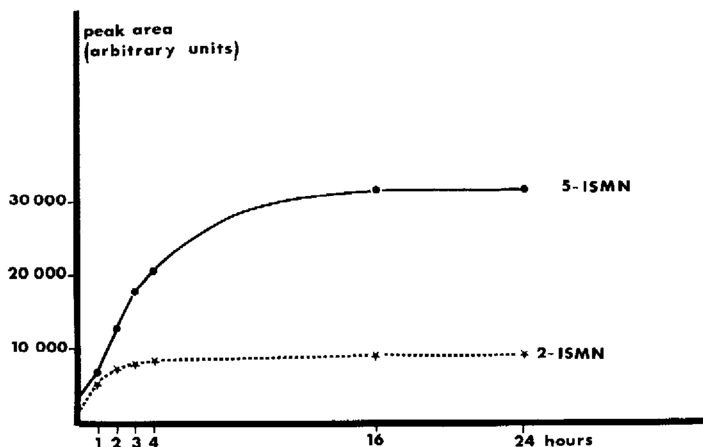

Fig. 1. Effect of incubation time on hydrolysis of 2-ISMN and 5-ISMN conjugates.

Identical results were found after a further 24-h incubation during which fresh enzyme (10 mg) was added five times.

Incubation with β-glucuronidase—arylsulphatase did not modify the hydrolysis. It may be concluded that the conjugates of 2-ISMN and 5-ISMN are glucuronides.

Within-day precision

The within-day precision of the method was checked by determining six plasma and urine samples spiked with different concentrations of 2-ISMN and

TABLE I

WITHIN-DAY PRECISION OF THE ASSAY AND RECOVERY OF 2-ISOSORBIDE MONONITRATE IN SPIKED PLASMA AND URINE SAMPLES

Amount added (ng/ml)	In plasma			In urine		
	Mean amount found (ng/ml) (n = 6)	Standard deviation (±)	Recovery (%)	Mean amount found (ng/ml) (n = 6)	Standard deviation (±)	Recovery (%)
2	1.97	0.09	98.5	2.0	0.40	100.0
5	4.8	0.79	96.0	5.4	0.59	108.0
10	9.2	0.73	92.0	9.8	0.43	98.0
50	51	5.8	102.0	51	2.2	102.0
100	99	7.2	99.0	97	4.5	97.0
200	201	7 0	100.5	—	—	—
500	497	41.4	99.4	466	26.1	93.2
Mean			98.2			99.7
C.V. (%)			3.4			5.0

TABLE II

WITHIN-DAY PRECISION AND RECOVERY OF 5-ISOSORBIDE MONONITRATE IN SPIKED PLASMA AND URINE SAMPLES

Amount added (ng/ml)	In plasma			In urine		
	Mean amount found (ng/ml) (n = 6)	Standard deviation (±)	Recovery (%)	Mean amount found (ng/ml) (n = 6)	Standard deviation (±)	Recovery (%)
5	4.7	0.3	94.0	—	—	—
10	11.2	0.8	112.0	9.6	2.0	96.0
50	55	4 2	110.0	59	2.2	118.0
100	98	7.2	98.0	110	5.3	110.0
200	188	9.5	94.0	—	—	—
500	470	26.7	94.0	526	51.7	105.2
Mean			100.3			107.3
C.V. (%)			8.4			8.6

5-ISMN. The results obtained with the procedure described are given in Tables I and II.

Day-to-day precision

The day-to-day precision was checked in plasma and urine by determining two concentrations (10 and 100 ng/ml) of 2-ISMN and 5-ISMN, in duplicate, every day for one week. The results obtained with the procedure described are given in Tables III and IV.

TABLE III

DAY-TO-DAY PRECISION AND RECOVERY OF 2-ISOSORBIDE MONONITRATE IN SPIKED PLASMA AND URINE SAMPLES

Amount added (ng/ml)	In plasma			In urine		
	Mean amount found (ng/ml) (n = 8)	Standard deviation (±)	Recovery (%)	Mean amount found (ng/ml) (n = 8)	Standard deviation (±)	Recovery (%)
10	10.6	0.7	106	10.0	0.5	100
100	105	7.8	105	97	4.6	97

TABLE IV

DAY-TO-DAY PRECISION AND RECOVERY OF 5-ISOSORBIDE MONONITRATE IN SPIKED PLASMA AND URINE SAMPLES

Amount added (ng/ml)	In plasma			In urine		
	Mean amount found (ng/ml) (n = 8)	Standard deviation (±)	Recovery (%)	Mean amount found (ng/ml) (n = 8)	Standard deviation (±)	Recovery (%)
10	9.6	1.1	96	10.5	1.3	105
100	104	9.9	104	99	9 6	99

Analysis of control samples

Four plasma samples spiked with concentrations of 2-ISMN and 5-ISMN unknown to the analyst were prepared in duplicate. The accuracy demonstrated by calculating the recovery for each concentration is 102.1 ± 5.6% and 94.0 ± 5.7%, respectively, for 2-ISMN and 5-ISMN.

Plasma and urine interference

Fig. 2 shows the chromatograms of an extract of human plasma (1 ml) and of the same extract spiked with 10 ng of 2-ISMN and 5-ISMN. No interference from normal plasma components was recorded. 2-ISMN and 5-ISMN are well separated from the normal components of the urine extract.

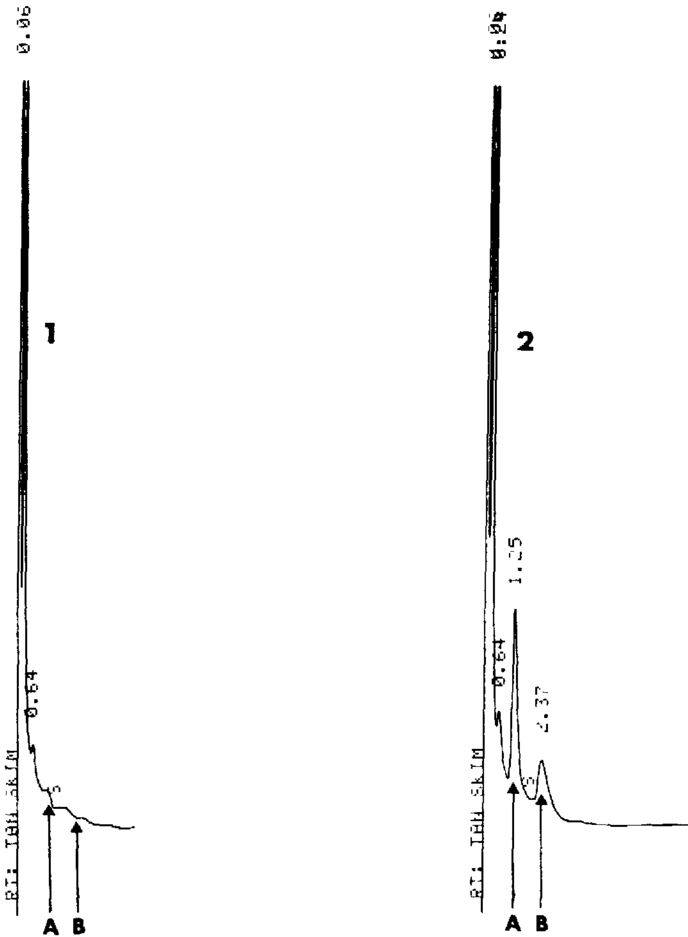

Fig. 2. Examples of chromatograms: (1) human plasma blank (1 ml plasma); and (2) the same plasma spiked with 10 ng of 2-ISMN (A) and 5-ISMN (B).

Storage stability of 2-ISMN and 5-ISMN in human plasma

No decrease in the 2-ISMN and 5-ISMN content (10 ng/ml and 100 ng/ml) was observed in plasma samples when stored frozen for 1, 8 and 15 days, 1, 3, 6 and 9 months, and 1 year at −20° C.

Application

The present method was used to determine the plasma concentrations and the urinary excretion of the mononitrate metabolites (2-ISMN and 5-ISMN) after oral administration of ISDN to a healthy subject. The corresponding plasma concentrations of the parent drug (ISDN) have also been determined [1]. Fig. 3 shows the plasma concentration curves of 2-ISMN and 5-ISMN and Fig. 4 the urinary excretion of the free and conjugated mononitrates after a single dose of 5 mg of ISDN in the form of one Isoket tablet. In the 0—104 h urine, 14% of the administered dose was recovered as mononitrate metabolites. Only 0.3% corresponds to free 2-ISMN and 2.2% to free 5-ISMN.

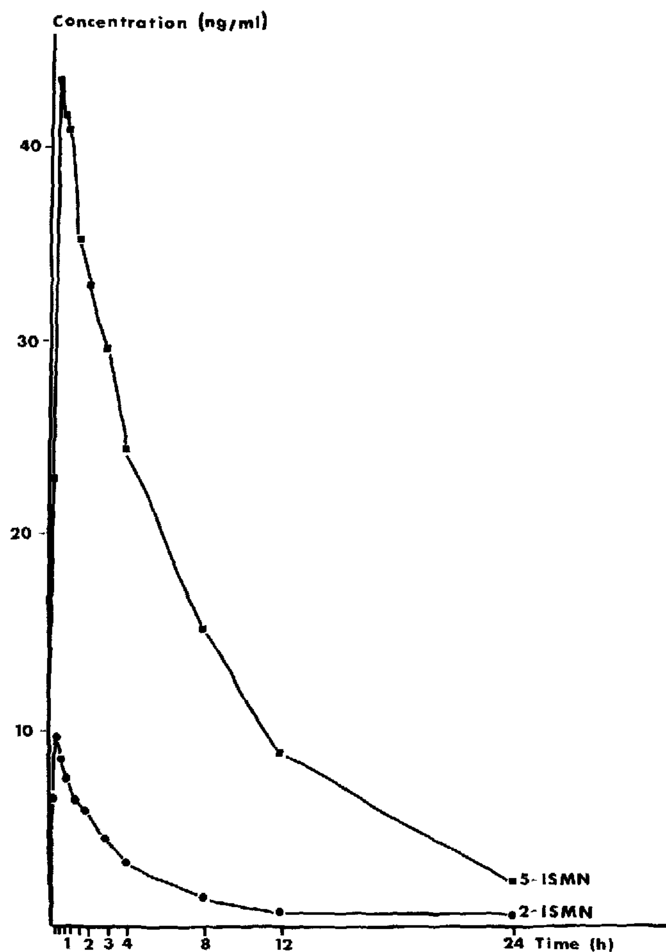

Fig. 3. 2- and 5-isosorbide mononitrate plasma concentrations in a healthy subject after oral administration of 5 mg of ISDN as one Isoket tablet. (•), 2-ISMN; (▪), 5-ISMN.

Enzymatic hydrolysis suggests that about 11.1% is excreted as 5-ISMN glucuronide whereas only 0.4% of the dose is excreted as 2-ISMN glucuronide.

CONCLUSION

The proposed gas chromatographic technique permits the quantitative assay of 2-ISMN and 5-ISMN in human plasma and urine and can be applied for the determination of free and conjugated 2-ISMN and 5-ISMN as metabolites of ISDN.

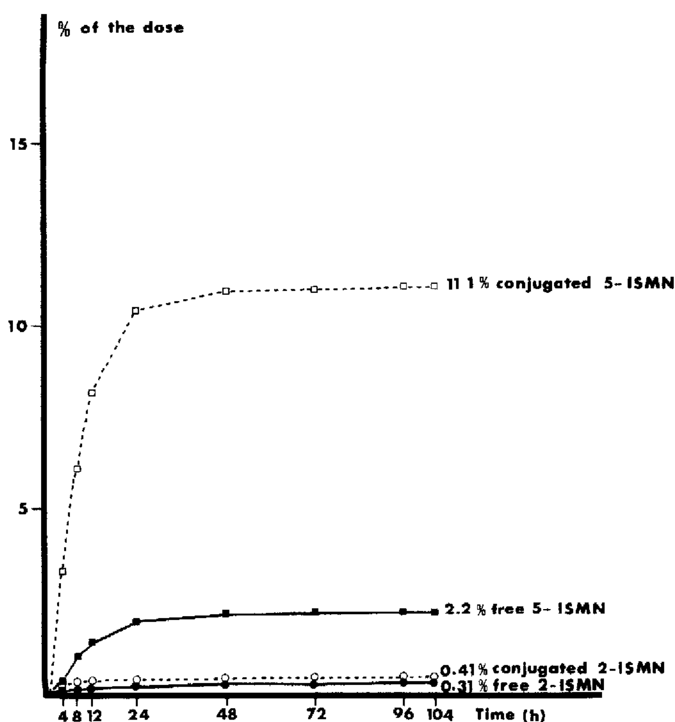

Fig. 4. Cumulative urinary excretion in a healthy subject after oral administration of 5 mg of ISDN as one Isoket tablet. (●), Free 2-ISMN; (○), conjugated 2-ISMN; (■), free 5-ISMN; (□), conjugated 5-ISMN.

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